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Dynamic Antagonism between Phytochromes and PIF Family Basic Helix-Loop-Helix Factors Induces Selective Reciprocal Responses to Light and Shade in a Rapidly Responsive Transcriptional Network in *Arabidopsis* [C][W]

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Plants respond to shade-modulated light signals via phytochrome (phy)-induced adaptive changes, termed shade avoidance. To examine the roles of Phytochrome-Interacting basic helix-loop-helix Factors, PIF1, 3, 4, and 5, in relaying such signals to the transcriptional network, we compared the shade-responsive transcriptome profiles of wild-type and quadruple *pif* (*pifq*) mutants. We identify a subset of genes, enriched in transcription factor–encoding loci, that respond rapidly to shade, in a PIF-dependent manner, and contain promoter G-box motifs, known to bind PIFs. These genes are potential direct targets of phy-PIF signaling that regulate the primary downstream transcriptional circuitry. A second subset of PIF-dependent, early response genes, lacking G-box motifs, are enriched for auxin-responsive loci, and are thus potentially indirect targets of phy-PIF signaling, mediating the rapid cell expansion induced by shade. Comparing deetiolation- and shade-responsive transcriptomes identifies another subset of G-box–containing genes that reciprocally display rapid repression and induction in response to light and shade signals. These data define a core set of transcriptional and hormonal processes that appear to be dynamically poised to react rapidly to light-environment changes via perturbations in the mutually antagonistic actions of the phys and PIFs. Comparing the responsiveness of the *pifq* and triple *pif* mutants to light and shade confirms that the PIFs act with overlapping redundancy on seedling morphogenesis and transcriptional regulation but that each PIF contributes differentially to these responses.

INTRODUCTION

The phytochrome (phy) family of sensory photoreceptors (phyA to phyE in *Arabidopsis thaliana*) constantly monitor the environment for informational light signals and direct plant growth and developmental adaptations to the prevailing conditions throughout the life cycle (Rockwell et al., 2006; Schafer and Nagy, 2006; Quail, 2010). These phy-directed responses include the induction of seed germination, seedling deetiolation (the transition from skotomorphogenic to photomorphogenic development), shade avoidance, and floral induction (Franklin and Quail, 2010; Strasser et al., 2010). Light signal perception involves photoconversion of the phy molecule from its inactive Pr to its active Pfr conformer, which then initiates an intracellular transduction process that culminates in the altered expression of nuclear genes that control the overt photomorphogenic responses.

Current data indicate that the transduction process that initiates seedling deetiolation in dark-grown plants involves rapid translocation of the light-activated photoreceptor molecule from the cytoplasm to the nucleus (Nagatani, 2004), where it interacts physically with a subset of members of the basic helix-loop-helix (bHLH) transcription factor family, termed Phytochrome-Interacting Factors (PIFs), inducing transcriptional responses in target genes (Castillon et al., 2007; Jiao et al., 2007; Bae and Choi, 2008; Leivar and Quail, 2011). The striking *constitutively photomorphogenic*-like phenotype of quadruple *pif1 pif3 pif4 pif5* (*pifq*) mutant seedlings grown in complete darkness (Leivar et al., 2008a) has provided compelling evidence that these PIF family members function collectively, in a partially redundant or overlapping fashion, to constitutively promote skotomorphogenesis (repress photomorphogenesis) in young dark-grown seedlings (Leivar et al., 2008a; Shin et al., 2009). Additional evidence shows that photoactivated phy reverses this promotion/repression, upon

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initial exposure to light, by inducing rapid degradation of the PIF molecules, via the ubiquitin-proteasome system (Bauer et al., 2004; Park et al., 2004; Shen et al., 2005, 2008; Oh et al., 2006; Nozue et al., 2007; Shen et al., 2007; Lorrain et al., 2008). This process involves rapid, phy-induced phosphorylation of the interacting bHLH protein in the nucleus, as a prelude to ubiquitin-proteasome system–mediated proteolysis (Al-Sady et al., 2006, 2008; Shen et al., 2007, 2008; Lorrain et al., 2008).

In fully deetiolated, light-grown seedlings, although PIF levels have been strongly decreased, they are not reduced to zero. The evidence indicates instead that a lower constant level of the protein is established under prolonged, continuous red light (Rc) irradiation or under continuous white light (WLc), such as during the daylight period for seedlings grown under day/night cycles (Monte et al., 2004; Nozue et al., 2007). A considerable number of studies have shown that monogenic and various higher order *pif* mutant seedlings display light-hypersensitive phenotypes (shorter hypocotyls and larger cotyledons than the wild type) at the completion of deetiolation when grown in prolonged, Rc or WLc (several days) (Huq and Quail, 2002; Kim et al., 2003; Fujimori et al., 2004; Huq et al., 2004; Monte et al., 2004; Oh et al., 2004; Khanna et al., 2007; Leivar et al., 2008b; Lorrain et al., 2008). Despite interpretive complications raised by the discovery of a mutually negative feedback loop between the PIF proteins and the phyB photoreceptor (Khanna et al., 2007; Monte et al., 2007; Al-Sady et al., 2008; Leivar et al., 2008b, 2012), these data suggest that the PIF levels present continue intrinsically to promote skotomorphogenic-like growth and development at a strongly reduced level in the light (de Lucas et al., 2008; Lorrain et al., 2008; Leivar and Quail, 2011). Return of seedlings to darkness results in reaccumulation of higher levels of PIF protein, and the rate of this reaccumulation is strongly accelerated by a short, terminal pulse of far-red light (so-called end-of-day far-red [EOD-FR] treatment) before return to darkness (Monte et al., 2004; Shen et al., 2005; Nozue et al., 2007; Leivar et al., 2008a). These data indicate that photoactivated phy continues to function in the light, and early postirradiation darkness, to sustain the repression of PIF levels and that this repression is relieved rapidly upon step function removal of Pfr by the far-red (FR) pulse and further incubation in the absence of phy photoactivation (Monte et al., 2004; Shen et al., 2005).

A qualitatively similar, but quantitatively less robust, reduction in Pfr levels than for the end-of-day FR pulse treatments is induced in green plants growing in normal white light (WL) upon exposure to the FR-enriched light generated by vegetational shade (Child and Smith, 1987; Smith and Whitelam, 1997; Franklin, 2008). Light filtered through, or reflected from, neighboring vegetation is depleted in red (R), but not FR, photons to a greater or lesser extent, depending on the density and proximity of this vegetation. This results in a quantitatively variable reduction in the ratio of R-to-FR light (variably lower R:FR ratio) compared with open sunlight. This shade signal drives the phy photoequilibrium back toward the inactive Pr conformer, thus decreasing the levels of the active Pfr conformer in the cell, despite the maintenance of sustained irradiation. Plants react to this signal with a suite of growth and developmental responses, termed the shade avoidance syndrome (SAS) (or shade avoidance response), which include accelerated extension growth rates in hypocotyls, internodes (detectable within 5 to 10 min) and petioles, retarded expansion rates in cotyledons, and retarded chloroplast development (Child and Smith, 1987; Smith and Whitelam, 1997; Franklin, 2008). Experimentally, FR-enriched light is frequently provided by FR supplementation of otherwise unchanged irradiation with WLc. This protocol selectively alters the R:FR ratio without altering the photosynthetically active radiation available to the plant. Although not directly mimicking true vegetational shade (which also reduces R levels and, thus, PAR), this strategy allows assessment of the participation of the phy system in the response, in the absence of additional effects due to reduced photosynthesis (Smith and Whitelam, 1997; Franklin, 2008) and/or blue light signaling through cryptochrome 1 (Keller et al., 2011). Here, we use the term "simulated shade" (Smith and Whitelam, 1997) to refer to such FR supplementation of WLc (also called a low R:FR ratio in the literature; Salter et al., 2003; Franklin, 2008; Lorrain et al., 2008) unless otherwise indicated.

There is evidence that PIF4 and PIF5 function in the shade-induced response. The abundance of these proteins increases rapidly in WL-grown wild-type seedlings upon exposure to simulated vegetative shade, and *pif4*, *pif5*, and *pif4 pif5* double mutants exhibit a reduced acceleration of hypocotyl elongation in response to this signal compared with the wild type (Lorrain et al., 2008). Conversely, PIF4 and PIF5 overexpressors display close to constitutively long hypocotyls and petioles, with consequent reduction in residual capacity for shade-responsiveness. Together with the observation that the *pif4 pif5* mutations suppress the shade avoidance-like long-hypocotyl phenotype of the *phyB* mutant in WLc (Lorrain et al., 2008), these data indicate that these two PIFs act intrinsically to promote the SAS in fully green plants.

Transcriptome analysis of the deetiolation process in wild-type and *pifq* mutant seedlings has defined the transcriptional network regulated by the PIF family (Leivar et al., 2009) and has documented the pleiotropic function of these factors in implementing phy control of target gene expression during normal light-induced seedling development (Leivar et al., 2009; Lorrain et al., 2009; Shin et al., 2009). The data show that, of the alterations in gene expression induced in dark-grown seedlings in the genetically imposed absence of PIF1, 3, 4, and 5 in the *pifq* mutant, the large majority of changes are normally evoked by light in the wild type (via the phy system) during the transition to the fully deetiolated state in prolonged irradiation. The broad spectrum of responsive genes identified is consistent with the multifaceted biochemical, cellular, and morphogenic changes that encompass this switch in developmental direction from heterotrophic to autotrophic growth.

Comparison of the most rapidly light-responsive genes defined in the wild type with those altered by PIF absence in the *pifq* mutant in the dark has identified a subset (designated Class 7) that are potential direct targets of these bHLH factors (Leivar et al., 2009). The rapidly light-repressed genes in this class are particularly enriched in a variety of transcription factor–encoding genes, suggesting that the PIFs function to directly regulate and amplify the downstream transcriptional network that executes the phy-regulated, deetiolation developmental program. Consistent with this suggestion, PIFs 1, 3, 4, and 5 have all been shown to have inherent transcriptional activation activity (Huq et al., 2004; Al-Sady et al., 2008; de Lucas et al., 2008; Shen et al.,

2008; Hornitschek et al., 2009). Similarly, several studies using quantitative PCR (qPCR)-based chromatin immunoprecipitation (ChIP) assays of preselected gene promoters have provided initial evidence of in vivo binding of each of the PIFs to potential target genes in seeds or developing seedlings (Oh et al., 2007, 2009; Shin et al., 2007; de Lucas et al., 2008; Feng et al., 2008; Moon et al., 2008; Hornitschek et al., 2009; Toledo-Ortiz et al., 2010), although evidence of association with G-box or E-box motif-containing sequences was lacking in several cases.

The question of the potentially differential or overlapping contributions of the individual PIF family members to the overall molecular phenotype of the developing seedling has been addressed in a limited number of expression profiling studies with single and double *pif* mutants. Microarray analysis of dark-grown monogenic *pif3* mutant seedlings identified only 14 genes that were differentially expressed, in statistically significant and twofold (SSTF) fashion, between the mutant and the wild type (Monte et al., 2004; Leivar et al., 2009), although the increased number of statistically differentially expressed genes defined by lowering the fold change threshold from 2 to 1.5, in a recent reanalysis of these data, revealed additional targets of PIF3 regulation (Sentandreu et al., 2011). A similar study with *pif1* defined only two genes as statistically different in expression between the mutant and the wild type, and these differences were less than twofold (Moon et al., 2008). These findings are consistent with the weak or absent visible morphogenic phenotypes in these mutants in the dark (Huq et al., 2004; Leivar et al., 2008a, 2009; Shin et al., 2009; Stephenson et al., 2009; Sentandreu et al., 2011). Profiling of a *pif4 pif5* double mutant grown in darkness identified 113 genes that were misregulated in the mutant compared with the wild type (Lorrain et al., 2009). The degree of overlap of these genes with those misregulated in the *pifq* mutant suggests that PIF4 and PIF5 together, additively or redundantly, contribute significantly to the combined regulatory activities of the PIF1, 3, 4, 5 quartet (Sentandreu et al., 2011).

Two previous studies analyzed the rapid genome-wide transcriptional responses elicited by vegetative shade in light-grown wild-type seedlings using Affymetrix ATH1 microarrays and defined a subset of genes that respond rapidly (within 1 h) to the shade signal (Sessa et al., 2005; Carabelli et al., 2007; Tao et al., 2008). In addition, an initial examination of the potential role of the PIFs in regulating these responses has been reported (Hornitschek et al., 2009). Using qPCR analysis of two selected rapidly shade-responsive genes, *PHYTOCHROME INTERACTING FACTOR 3-LIKE1* (*PIL1*) and *XYLOGLUCAN ENDOTRANSGLYCOSYLASE7* (*XTR7*), as markers, these authors showed that *pif4*, *pif5*, and *pif4 pif5* double mutants displayed reduced shade-induced elevation of expression compared with the wild type. These results indicate that PIF4 and PIF5 act positively to promote these shade-responsive transcriptional changes. Consistent with this conclusion, ChIP analysis indicates that PIF5 binds in vivo to the G-box–containing regions of the promoters of the marker genes *PIL1*, *XTR7*, and *LONG HYPOCOTYL IN FAR-RED* (*HFR1*) (Hornitschek et al., 2009).

Here, we broadened the question of the functional roles of the PIF bHLH factors in shade avoidance to include PIF1 and PIF3, in addition to PIF4 and PIF5, by examining the shade-induced gene expression changes in the *pifq* mutant compared with the wild type, and expanded the expression analysis to be genome-wide using microarray profiling. In addition, we explored the question of whether the rapidly shade-induced, PIF-dependent transcriptional responses represent incipient reversal of those triggered in dark-grown seedlings at the inception of deetiolation by initial exposure to light. To begin to dissect the relative contributions of the individual PIF family members to the cumulative regulated transcription of individual target genes, we also assayed the relative expression levels of selected rapid response genes in the various triple *pif* mutant combinations compared with the *pifq* quadruple mutant.

RESULTS

Simulated Vegetative Shade Induces Rapid Increases in PIF3 Levels in Light-Grown Seedlings under Dichromatic Irradiation Conditions

Early experiments under dichromatic (Rc ± continuous far-red [FRc]) irradiation conditions showed that Rc light–grown seedlings exposed to supplemental FRc displayed rapid (within 5 to 10 min) and robust reaccumulation of the PIF3 protein, in the absence of changes in the corresponding transcript levels (see Supplemental Figure 1 and Supplemental Analysis 1 online). Therefore, these data indicate that PIF3 levels are regulated dynamically by photoactivated phy, in deetiolated seedlings, in response to simulated shade, at the protein rather than the gene expression level.

Multiple PIF Family Members Are Necessary for Normal Responsiveness of WL-Grown Seedlings to Simulated Vegetative Shade

Examination of WL-grown, fully green seedlings showed that they likewise reaccumulate endogenous PIF3 in response to simulated vegetative shade (WLc with supplemental FRc) (Figure 1A). Wild-type seedlings exposed to such simulated shade conditions display the long hypocotyls and petioles characteristic of the shade avoidance response, but this response is attenuated in the *pifq* mutant (Figures 1B and 1C). The extent of this reduction in responsiveness is significantly, albeit moderately, greater in the *pifq* mutant than in the *pif4 pif5* double mutant, indicating that PIF1 and/or PIF3 contribute to this response, in addition to that previously reported for PIF4 and PIF5 (Lorrain et al., 2008), but that the magnitude of this contribution is quantitatively less than that of PIF4 and PIF5.

A response pattern similar to shade avoidance is known to be observed in seedlings subjected to so-called EOD-FR treatments, where seedlings growing under diurnal light-dark cycles are administered a terminal FR light pulse at the end of each light period to remove Pfr for the duration of the subsequent dark period. Figure 1D shows that this treatment accelerates the growth rate in the wild type, as expected, but that this acceleration is progressively reduced in the *pif4 pif5* double and *pifq* mutant seedlings. This result indicates that, in this case, PIF1 and/or PIF3 function robustly together with PIF4 and/or PIF5 to promote hypocotyl elongation during the diurnal dark period in a manner that is accelerated by the absence of Pfr (Figure 1D, right panel).

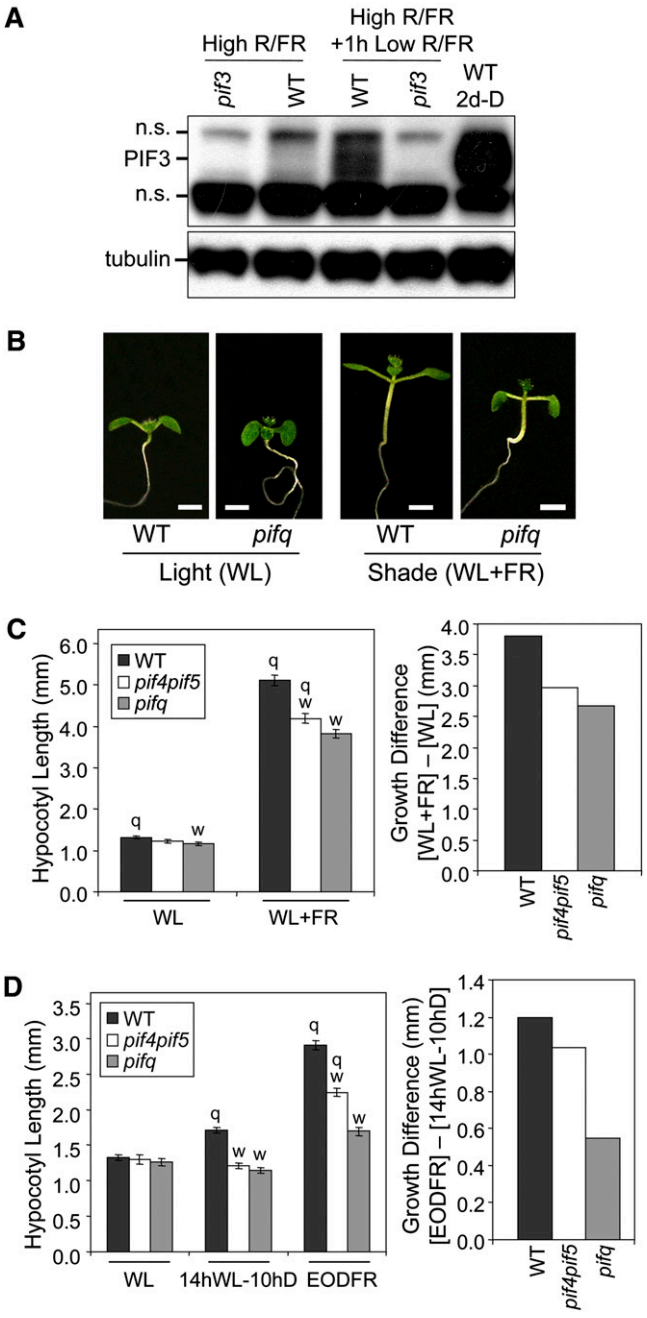

Figure 1. Multiple PIF Family Members Are Necessary for Normal Responsiveness to Simulated Vegetative Shade in WLc-Grown Seedlings.

(A) Endogenous PIF3 levels rise in response to FRc supplementation in WLc-grown seedlings. Two-day-old wild-type (WT) seedlings were grown in darkness (D) or WLc (20 μmol m^{-2} s^{-1}) (high R:FR ratio 6.48) from germination onward, and the latter were (high R/FR + 1 h low R/FR ratio 0.006) or were not (high R/FR) exposed to supplemental FRc for 1 h before immunoblot analysis of PIF3 protein levels. A *pif3* null mutant was included as a negative control, and tubulin was used as a loading control. n.s., nonspecific.

(B) Visible phenotypes of wild-type and *pifq* mutant seedlings grown in WLc (20 μmol m^{-2} s^{-1}) for 2 d from germination onward and then for an additional 5 d in WLc, either with (WL+FR, Low R/FR) or without (WL, High R/FR) supplemental FRc. Bars = 2mm.

(C) Quantification of hypocotyl lengths for wild-type, *pif4 pif5* double, and *pifq* quadruple mutant seedlings grown as in **(B)**. Left panel: Absolute hypocotyl lengths for light (WL)- and shade (WL+FR)-grown seedlings. Right panel: Differential shade responsiveness ([WL+FR] – [WL]) of wild-type, *pif4 pif5*, and *pifq* seedlings.

(D) Quantification of hypocotyl lengths for wild-type, *pif4 pif5*, and *pifq* mutant seedlings in EOD-FR conditions. Seedlings were grown in WL (20 μmol m^{-2} s^{-1}) for 2 d and then transferred for five additional days to constant WLc (WL) or to diurnal cycles of 14 h of WL followed by 10 h of darkness with (EOD-FR) or without (14 hWL-10 hD) a terminal saturating pulse of FR administered prior to the 10-h dark treatment. Left panel: Absolute hypocotyl lengths. Right panel: Differential EODFR responsiveness ([EODFR] – [14 hWL-10 hD]) of wild-type, *pif4 pif5*, and *pifq* seedlings.

(C) and **(D)** Left panels: Data represent mean values and SE (bars) from at least 20 seedlings. Statistically significant differences from the wild type (w) and/or the *pifq* mutant (q) values, defined by the Student's *t* test ($P \leq 0.05$), are indicated for each light treatment.

[See online article for color version of this figure.]

Definition of Rapid Shade-Induced Transcriptional Responses Genome-Wide

To examine the molecular phenotype of the shade avoidance response and to define genes most likely to be early, if not direct, targets of phy signaling through the PIF1, 3, 4, 5 quartet, we performed a transcriptome analysis of simulated shade–induced changes in expression in wild-type and quadruple *pifq* mutant seedlings. For this purpose, RNA was extracted after transfer of 2-d, WLc-grown seedlings (WL0) to supplemental FRc light for 1 (FR1), 3 (FR3), and 24 (FR24) h or retention in WLc for an additional 24 h (WL24). The primary data and all statistical parameters generated in this analysis for all 22,000 genes on the Affymetrix ATH1 microarray have been deposited at the Gene Expression Omnibus (GSE28297) and for all genes discussed here (defined in Supplemental Figure 2 online) are presented in Supplemental Data Set 1. This analysis identified a total of 169 rapidly shade-responsive genes that were induced (131 genes; see Supplemental Data Set 2 online) or repressed (38 genes; see Supplemental Data Set 3 online) within 1 h of FRc supplementation (FR1) in the wild-type in an SSTF manner compared with the WLc-grown control before FRc treatment (WL0) (SSTF@FR1 genes; see Supplemental Figure 2 online). A comparison of these data with those of two formally similar, but experimentally different, earlier studies (Carabelli et al., 2007; Tao et al., 2008) is presented in Supplemental Analysis 2, Supplemental Figure 3, and Supplemental Data Sets 2 to 8 online together with analysis of the shade-induced expression changes at FR3 in wild-type seedlings (SSTF@FR3; see Supplemental Figure 2 online).

Identification of PIF-Dependent Rapidly Shade-Responsive Genes

To assess the potential role of the PIF proteins in the rapid shade-induced expression changes observed in this study, we identified those genes that exhibited statistically significant differences in transcript abundance between wild-type and *pifq* seedlings following 1 h of FRc supplementation (PIF-dependent genes in

Supplemental Figures 2 and 4 online). The procedure used in this analysis (detailed in Supplemental Analysis 3, Supplemental Figures 2 to 5, and Supplemental Data Sets 9 to 17 online) defined a total of seven classes of genes (A to G), of which Classes A and B combined (Classes A+B) comprised those designated as displaying rapid (at FR1), statistically significant, PIF-dependent shade responsiveness (see Supplemental Figure 4 online). A total of 123 such Class A+B genes (103 induced, Supplemental Data Set 9 online; 20 repressed, Supplemental Data Set 10 online) were identified. This genome-wide pattern of rapidly shade-responsive gene expression provides strong evidence of the involvement of the PIF quartet in early shade signaling.

Functional Dichotomy between Genes with and without Promoter-Located G-Box Motifs

The magnitude of the contribution of the four PIFs to the shade responsiveness of each shade-induced gene was defined as the fold induction ratio (FIR) (Monte et al., 2004; Tepperman et al., 2004; see Methods). This parameter quantitatively measures the magnitude of the change in expression induced by the simulated shade treatment at FR1 in the wild type (wild-type fold induction [FI]) compared with the *pifq* mutant (*pifq* FI), expressed as a ratio (wild-type FI/*pifq* FI). A ratio of 1.0 signifies no detectable contribution of PIF1, 3, 4, and 5 to the shade-induced response, independently of whether the underlying response to shade is intrinsically large or small for that gene. Deviations from 1.0 provide a quantitative measure of the robustness of the PIF quartet contribution to the shade response.

The 103 shade-induced PIF-dependent genes at FR1 of Classes A and B (see Supplemental Figure 4B and Supplemental Data Set 9 online) were arrayed in rank order of FIR value at 1 h of supplemental FRc and further divided into subsets according to the range of those values, as depicted in Figure 2A. Genes at the left extreme of this array are those exhibiting the strongest dependence on the four PIFs for FRc-induced expression, whereas those toward the right exhibit the least PIF quartet dependence. We define those genes with FIR values >1.5 as being moderately to robustly dependent on the four PIFs for shade responsiveness (Table 1) and those with FIR values <1.5 as being marginally to minimally dependent on these PIFs for shade responsiveness (see Supplemental Data Set 18 online). Several genes previously well documented as rapidly shade-induced marker genes, including *ARABIDOPSIS THALIANA HOMEOBOX PROTEIN2* (*ATHB2*), *XTR7*, *HFR1*, *PHY RAPIDLY REGULATED1* (*PAR1*), and *INDOLE-3-ACETIC ACID INDUCIBLE29* (*IAA29*) (Roig-Villanova et al., 2006, 2007; Carabelli et al., 2007; Lorrain et al., 2008), are identified here as being robustly dependent on the PIF quartet for this rapid shade responsiveness (Table 1; see Supplemental Data Set 18 online).

The time-course curves for the mean expression of the four FIR subsets of genes graphically depict the gradation in dependence on the PIF quartet for rapid responsiveness to shade (Figure 2B). The data show the very weak early shade responsiveness in the *pifq* mutant of the 25 genes with a FIR >2 at 1 h of FRc compared with the wild type (Figure 2B1), and the declining differential in wild-type and *pifq* expression levels in shade-treated seedlings across the other gene subsets (Figure 2B, panels left [B1] to right [B4]). It is also notable that the percentage of genes that contain G-box motifs in their promoters within these subsets declines from left to right as follows: FIR >2, 80%; FIR 1.5 to 2, 69%; FIR 1.25 to 1.5, 41%; FIR 1.0 to 1.25, 50% (Figure 2B).

These PIF-dependent genes within each FIR subset were divided into G-box–containing and non-G-box–containing groups and assigned to functional categories. The percentage of the annotated genes falling into each category is displayed in Figure 2C. It is notable that the G-box–containing genes (Figure 2C, top) are enriched overall for genes encoding putative or established transcription-related factors (52%), compared with the non-G-box–containing group (Figure 2C, bottom) (24%). However, most strikingly, the G-box–containing, high-FIR genes are particularly enriched in such transcription factor–encoding genes: FIR >2 group, 67%, and FIR 1.5 to 2 group, 61% (Figure 2C, two top left panels) (Table 1; see Supplemental Data Set 18 online). Conversely, the non-G-box–containing group is preferentially enriched in hormone-related genes (38%) compared with the G-box–containing set (10%). Again, this enrichment is prominent for the higher FIR genes in the non-G-box–containing gene-set: FIR >2, 67%; FIR 1.5 to 2, 50%; and FIR 1.25 to 1.5, 55% compared with FIR 1 to 1.25, 0%.

Also very striking, auxin-responsive genes (including *SMALL AUXIN UP RNA* (*SAUR*) *1*, *7*, *9*, *15*, *23*, *25*, *28*, *62*, *63*, *64*, *65*, *66*, *67*, and *68*; Table 1; see Supplemental Data Set 18 online) dominate this hormone category, making up 88% of the total number of genes overall in this category and 92% of the non-G-box–containing subset. Because as noted above, the majority of hormone-related, shade-induced genes are non-G-box containing, it follows that the majority of these auxin-related genes are non-G-box containing. Furthermore, in our functional classification scheme, we chose to classify the *AUXIN/INDOLE-3-ACETIC ACID* (*AUX/IAA*) genes as transcription related, given the known function of their encoded proteins in regulating other genes in response to auxin. Five of these genes, *IAA2*, *IAA3*, *IAA4*, *IAA19*, and *IAA29*, are identified here as being G-box–containing, shade-induced genes, with four of the five displaying moderate to robust dependence on the PIF quartet for induction (Table 1; see Supplemental Data Set 18 online). This observation both enhances the apparent strong involvement of auxin in early phy-PIF–induced shade responses and suggests a possible dichotomy within the auxin-related gene set, whereby the regulatory *AUX/IAA* genes provide potential direct targets for the PIFs through their G-box motifs, and the *SAUR*/auxin-responsive group may be indirect targets, given that all but two lack G-box motifs in their promoters. It is also notable that an additional subset of seven rapidly shade-induced genes (albeit in a non-PIF–dependent manner) (Classes D and E in Supplemental Figure 4B online) are auxin related (*IAA1*, *IAA5*, *IAA34*, *SAUR10* [*At2G18010*], *SAUR46* [*At2G37030*], *GH3.3*, and *GH3.4*) (see Supplemental Data Set 19 online). These represent 41% of the total number of genes in this category.

Collectively, these data identify a subset of transcription factor–encoding genes as strong candidate direct targets of rapid phy-PIF–mediated shade signaling, including several *AUX/IAA* genes involved in transcriptional regulation in the auxin response pathway, and document similarly rapid, but perhaps indirect, induction of a suite of auxin-responsive genes (Table 1). These results suggest that the phy-PIF pathway may act directly

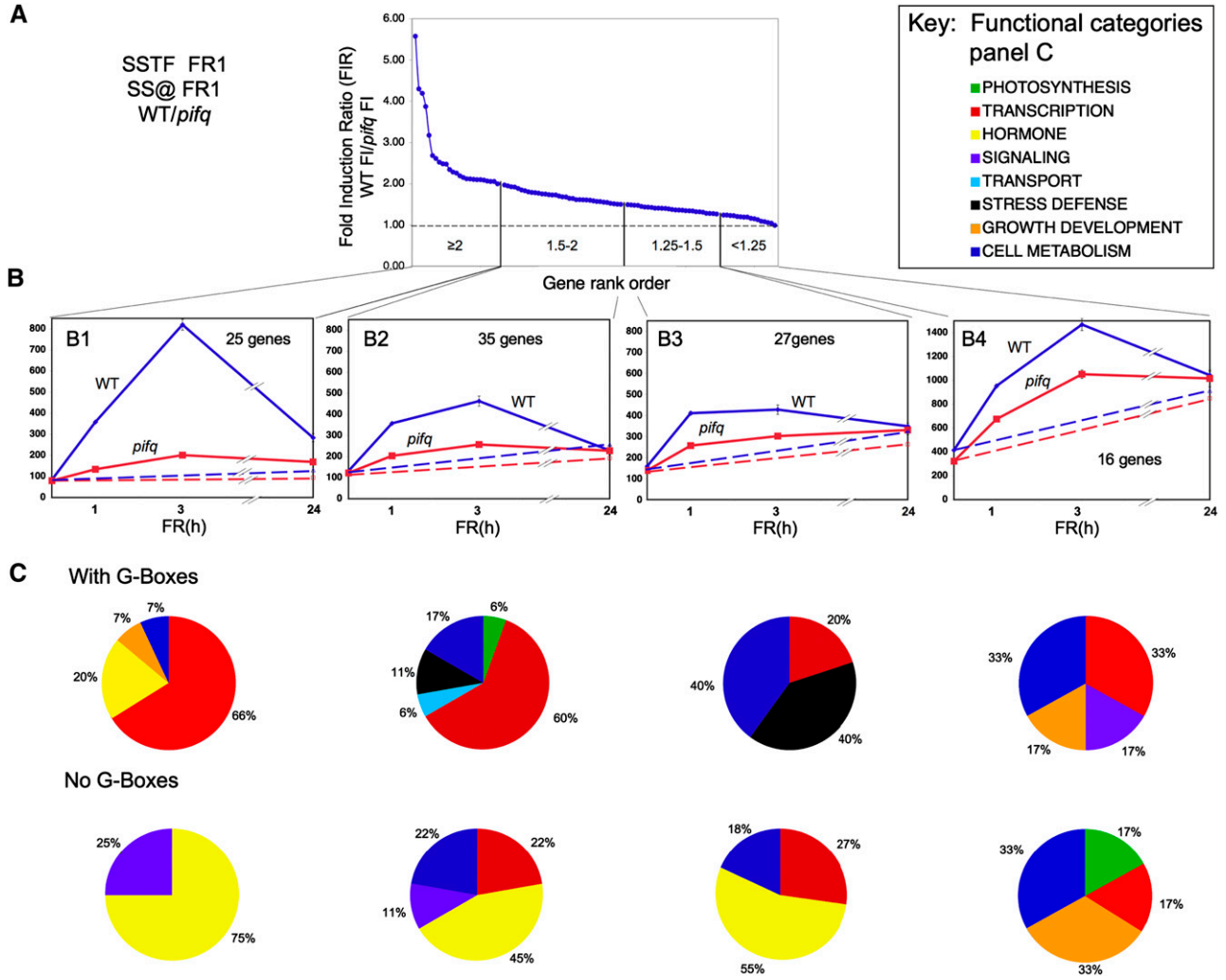

Figure 2. Transcription Factor–Encoding and Auxin-Responsive Genes Dominate the Rapid, PIF-Dependent, Shade-Induced Gene Set.

(A) Shade-induced genes arrayed in rank order of relative responsiveness to 1 h of FRc supplementation in the wild type (WT) compared with *pifq* mutant seedlings. The curve depicts the distribution of the 103 genes defined statistically both as SSTF induced by 1 h of FRc in the wild type and as significantly dependent on the PIF quartet for that FRc responsiveness, arrayed in order of descending FIR of the wild type/*pifq*. Vertical lines divide the array into bins (**[B1]** to **[B4]**) according to FIR value (range shown within each bin).
(B) Time course of mean expression of all genes in each bin over the 24-h shade exposure period. Blue, the wild type; red, *pifq*. Solid lines, shade exposed (WLc+FRc); dashed lines, not shade exposed (i.e., retained in WLc). Numbers of genes in each bin are indicated. Error bars indicate SE.
(C) Distribution of rapidly shade-induced genes among functional categories, expressed as a percentage of the total annotated genes within each bin, after division into groups with or without G-box motifs in their promoters. Functional category color code is shown in **(A)**, right. Genes with unknown function were eliminated from the analysis.

to induce or modulate auxin signaling as a major conduit by which rapid growth responses to shade (known to occur within minutes) are triggered.

Rapidly Shade-Elicited Transcriptional Responses Represent Selective Incipient Reversal of Rapidly Light-Triggered Transcriptional Responses Initiated during Deetiolation

To examine whether and to what extent the rapid, PIF-regulated changes in gene expression evoked by shade represent incipient reversal of the initial changes triggered upon first exposure of dark-grown seedlings to light at the onset of deetiolation, we performed a meta-analysis that merged our present shade microarray data with those of our previous deetiolation microarray study (Leivar et al., 2009). The data for this analysis are in Supplemental Data Set 20 online. This analysis provides a direct comparison between the genome-wide changes triggered by Rc light at the initiation of the deetiolation process and those induced by FRc light enrichment at the initiation of the shade avoidance process. Here, we focused on the rapidly (1 h) shade-induced genes.

Table 1. Genes Displaying Rapid Responsiveness to Shade in a Robustly to Moderately PIF-Dependent Manner (Classes A and B combined [see Supplemental Figure 4B online] with a FIR >1.5; Figure 2)

Locus	TAIR Annotation	Wild-Type FI[a]	*pifq* FI[b]	FR1 FIR[c]
Genes with G-Boxes in Promoter (44 Genes)				
Transcription				
AT4G32280	**IAA29**	**25.72**	**10.20**	**2.52**
AT4G16780	**ATHB-2**	**13.34**	**3.10**	**4.30**
AT1G02340	**HFR1**	**8.47**	**1.52**	**5.58**
AT2G42870	**PAR1**	**5.98**	**2.29**	**2.61**
AT3G15540	**IAA19**	**5.37**	**2.56**	**2.10**
AT5G39860	**PRE1**	**4.09**	**2.30**	**1.78**
AT4G01250	WRKY22	3.84	2.21	1.73
AT3G23030	**IAA2**	**3.71**	**2.37**	**1.57**
AT1G69570	**Dof-type Zn finger**	**3.34**	**1.52**	**2.19**
AT1G18400	**BEE1**	**2.74**	**1.71**	**1.60**
AT5G15160	bHLH 134	2.57	1.21	2.12
AT1G14920	**GAI**	**2.45**	**1.45**	**1.68**
AT1G01260	**bHLH 13**	**2.36**	**1.03**	**2.28**
AT1G69690	TCP15	2.33	1.45	1.61
AT3G57800	**bHLH 60**	**2.32**	**1.30**	**1.79**
AT5G43700	**IAA4**	**2.20**	**1.44**	**1.52**
AT5G28300	Trihelix DNA binding protein	2.15	1.03	2.09
AT2G46270	GBF3	2.14	1.27	1.68
AT5G53980	ATHB52	2.05	1.27	1.61
AT1G69010	**BIM2**	**2.04**	**0.99**	**2.07**
AT4G30410	Transcription factor	2.03	1.18	1.73
Hormone				
AT3G03830	**SAUR28**	**7.83**	**3.16**	**2.48**
AT4G13790	SAUR25	5.79	1.38	4.19
AT1G75450	**CKX5**	**2.68**	**1.08**	**2.48**
AT3G59900	ARGOS	2.30	1.22	1.88
Cellular metabolism				
AT5G54490	PBP1	3.06	1.77	1.73
AT4G25260	**Invertase/pectin methylesterase inhibitor**	**2.42**	**1.18**	**2.05**
AT1G04180	**FMO family protein**	**2.37**	**1.53**	**1.55**
AT3G54510	ERD protein-related	2.27	1.18	1.92
Growth/development				
AT4G14130	XTR7	3.59	1.59	2.26
Photosynthesis/chloroplast				
AT5G02020	SISR	2.14	1.42	1.50
Stress/defense				
AT4G28240	**Wound-responsive protein**	**2.25**	**1.43**	**1.57**
AT5G63650	SNRK2.5	2.07	1.18	1.75
Transport				
AT5G66110	Metal ion binding	2.08	1.15	1.80
Unknown				
AT5G12050	**Unknown protein**	**9.25**	**5.01**	**1.85**
AT3G29370	Unknown protein	5.48	2.55	2.15
AT5G02580	**Unknown protein**	**3.65**	**1.15**	**3.17**
AT5G50335	Unknown protein	3.25	1.55	2.10
AT3G55840	Unknown protein	3.15	1.72	1.83
AT5G66590	**Unknown protein**	**2.65**	**1.38**	**1.93**
AT1G21050	**Unknown protein**	**2.60**	**1.33**	**1.95**
AT3G29575	**Unknown protein**	**2.31**	**1.09**	**2.12**
AT5G57760	Unknown protein	2.31	1.12	2.05
AT3G50900	Unknown protein	2.05	1.27	1.61
Genes with No G-Boxes in Promoter (16 Genes)				
Transcription				
AT5G44260	**Zn finger (CCCH-type)**	**2.60**	**1.72**	**1.51**
AT3G60390	**HAT3**	**2.41**	**1.47**	**1.65**

(Continued)

Table 1. (continued).

Locus	TAIR Annotation	Wild-Type FI[a]	*pifq* FI[b]	FR1 FIR[c]
Hormone				
AT5G18060	**SAUR23**	**11.26**	**4.20**	**2.68**
AT1G29490	SAUR68	6.92	1.79	3.87
AT1G29440	**SAUR63**	**4.74**	**2.41**	**1.97**
AT4G38850	**SAUR15**	**4.20**	**2.10**	**2.00**
AT1G29460	**SAUR65**	**3.65**	**2.09**	**1.75**
AT2G21200	**SAUR7**	**3.24**	**2.04**	**1.58**
AT1G02400	GA2OX6	2.43	1.43	1.70
Cellular metabolism				
AT5G02540	**SDR**	**4.40**	**2.49**	**1.77**
AT4G27280	**Calcium binding EF hand**	**3.33**	**2.22**	**1.50**
Signaling				
AT5G14470	GHMP kinase-related	3.23	2.08	1.55
AT3G16800	**PP2C**	**2.32**	**0.99**	**2.34**
Unknown				
AT2G28400	Unknown protein	2.24	1.37	1.64
AT1G36940	Unknown protein	2.16	1.34	1.61
AT4G35720	Unknown protein	2.05	0.97	2.10

Genes in boldface type were previously identified as shade responsive (Carabelli et al., 2007; Tao et al., 2008). TAIR, The Arabidopsis Information Resource.

[a]FI in the wild type after exposure to 1 h of simulated shade signal.

[b]FI in *pifq* after exposure to 1 h of simulated shade signal.

[c]Wild-type FI/*pifq* FI.

Overall, 64% of early (1 h FRc), shade-induced genes (131 genes of the shade response Classes A, B, D, and E in Supplemental Figure 2B online) overlap with genes that are repressed by light exposure during deetiolation. Conversely, genes displaying rapid (within 1 h) repression in response to Rc light during deetiolation (deetiolation Classes 3, 6, and 7; Leivar et al., 2009) are strongly enriched in early shade-induced genes compared with classes where light-imposed repression was slow or absent during deetiolation (deetiolation Classes 1, 2, and 4; see Supplemental Figure 6 online). The data for the individual deetiolation class genes are summarized in Figure 3 and Supplemental Figures 7 to 10 online (see Supplemental Data Sets 21 to 35 online for associated gene lists). Section A of these figures contains a Venn diagram showing the extent of overlap between the dark-to-light repressed genes (deetiolation) and the 103 rapid, PIF-dependent light-to-shade induced genes at FR1 (Classes A and B; see Supplemental Figure 4B online) for each deetiolation gene class defined by Leivar et al. (2009). Section B displays the time courses of the mean expression level of all genes within each Venn sector, after dividing the genes between those with (three top panels) and without (three bottom panels) G-box motifs in their promoters. Within each of the six panels in Section B, the curves in the left subpanel show the light-repressed gene time courses (dark-to-light) derived from the data of Leivar et al. (2009), and those in the right subpanel (light-to-shade) show the shade-induced time courses derived from this study. Section C shows the functional category distribution of the genes within each respective panel in Section B of the figure.

We focus the initial analysis here on the Class 7 rapidly light-repressed (deetiolation) genes, as these appear to have an expression pattern in dark-grown and deetiolating seedlings that is consistent with being direct targets of phy-induced, PIF-mediated regulation (Figure 3). The middle Venn diagram sector (designated Meta-analysis Class M) represents those genes that display robust, rapid, reciprocal responsiveness to the onset of the light (Rc) and shade (supplemental FRc) signals, respectively (Figure 3A, Table 2; see Supplemental Data Set 21 online). This is visually apparent from direct comparison of the respective wild-type curves (Figure 3B, center panels, Class M). The data also show the robust dependence on the PIF proteins for the elevated levels of expression of these genes both in the dark and the shade. The left-hand Venn sector (designated Meta-analysis Class L) comprises genes that display robust, rapid repression of expression in the wild type by the initial light signal but subsequently markedly weaker and slower induction in the shade (Figure 3B, left panels) (see Supplemental Data Set 22 online). However, this minimal shade response does retain apparently substantial PIF dependence, most obviously at the FR3 time point. The right-hand Venn sector (designated Meta-analysis Class R) represents genes that lack robust, rapid light-imposed repression but display rapid and robust PIF-dependent shade induction (Figure 3B, right panels) (see Supplemental Data Set 23 online). The prominence of the transcription factor genes across both the light-repressed and shade-induced subsets is reinforced by this analysis (Figure 3C).

Overall, the data reveal dichotomies in the pattern of regulation within each of the PIF-dependent, rapidly light-repressed (Class 7; Leivar et al., 2009) and rapidly shade-induced (Classes A and B; see Supplemental Figure 4B online) gene sets. The subset common to both gene sets (Figure 3A, Class M), exhibiting dual, reciprocal light shade responsiveness, suggests that they are targets of reversible regulation by PIF-mediated phy signaling. The G-box–containing group within this subset appears to be the most likely to be direct targets of this signaling pathway (Table 2; see Supplemental Data Set 21 online). However, a larger subset

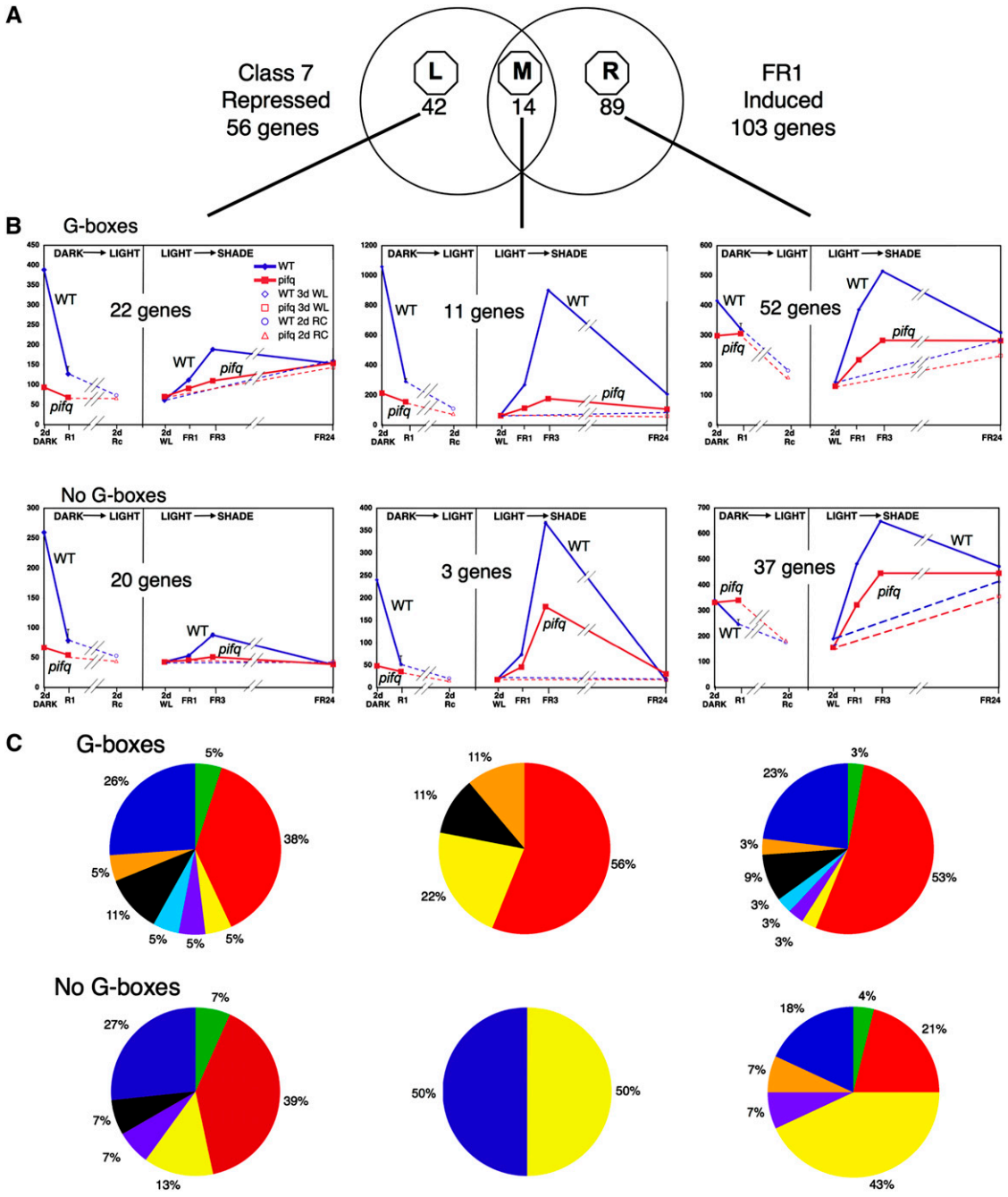

Figure 3. Merging Light-Repressed and Shade-Induced Transcriptome Data Identifies Genes Responding Rapidly and Reciprocally to Light and Shade Signals.

(A) Venn diagram depicting the overlap between the rapidly (R1 h) dark-to-light–repressed PIF-dependent (deetiolation Class 7) genes (Leivar et al., 2009) and the rapidly light-to-shade–induced PIF-dependent (FR1, Class A+B; see Supplemental Figure 4B online) genes (this study). Sectors are designated as left (L), middle (M), and right (R), and the number of genes in each is indicated.

(B) Time courses of the mean expression levels of all genes within each Venn sector, after dividing the genes between those with (top graphs) and without (bottom graphs), G-box motifs in their promoters. Curves in the left subpanel of each graph box show the light-repressed gene time courses (from Leivar et al., 2009), and those in the right subpanel the rapidly shade-induced genes identified here. Blue, the wild type; red, *pifq*. Solid lines: Light (Rc)- or shade (WLc+FRc)-exposed. Left subpanels: Break indicates noncontinuity of time course between 1 h R (R1) and 2 d R (Rc); right subpanels, dashed lines, not shade-exposed (i.e., retained in WLc). Error bars indicate SE. WT, the wild type.

(C) Functional category distribution of the genes within each respective graph box in **(B)**. Color code as in Figure 2.

Table 2. M Class Genes Displaying Rapid, Robust, Reciprocal Responsiveness to Both Light and Simulated Shade Signals (Figure 3)

Locus	TAIR Annotation	Light Repression in the Wild Type[a]	Shade Induction in the Wild Type[b]	FR1 FIR[c]
Genes with G-Boxes in Promoter (11 Genes)				
Transcription				
AT1G69690	TCP15	0.45	2.33	1.61
AT3G15540	**IAA19**	**0.36**	**5.37**	**2.10**
AT4G16780	**ATHB-2**	**0.11**	**13.34**	**4.30**
AT4G32280	**IAA29**	**0.11**	**25.72**	**2.52**
AT5G53980	ATHB52	0.10	2.05	1.61
Hormone				
AT1G75450	**CKX5**	**0.34**	**2.68**	**2.48**
AT4G13790	SAUR25	0.24	5.79	4.19
Growth/development				
AT4G14130	**XTR7**	**0.37**	**3.59**	**2.26**
Stress/defense				
AT5G63650	SNRK2.5	0.29	2.07	1.75
Unknown				
AT5G02580	Unknown	0.13	3.65	3.17
AT5G57760	Unknown	0.14	2.31	2.05
Genes with No G-Boxes in Promoter (Three Genes)				
Hormone				
AT4G36110	SAUR9	0.24	5.16	1.37
Cellular Metabolism				
AT5G02540	SDR	0.20	4.40	1.77
Unknown				
AT4G35720	Unknown	0.16	2.05	2.10

Genes in boldface type were previously identified as shade responsive (Carabelli et al., 2007). TAIR, The Arabidopsis Information Resource.
[a]Mean fold repression of expression in wild-type *Arabidopsis* seedlings exposed to 1 h Rc (R1) over the level in dark control seedlings (D1).
[b]Mean fold induction of expression in wild-type *Arabidopsis* seedlings exposed to 1 h of simulated shade (WL+FRc) over the level in WL control seedlings (WL0).
[c]FIR of shade induction in wild-type versus *pifq* seedlings after 1 h of simulated shade (FI/WT)/(FI/*pifq*).

of rapidly light-repressed genes does not exhibit rapid reciprocal PIF-dependent induction by shade (Figure 3, Class L), suggesting perhaps either an intrinsically low transcription rate coupled with a high turnover rate or a unidirectional change in the mode of transcriptional regulation of these genes during deetiolation (see Supplemental Data Set 22 online). Conversely, a large subset of rapidly shade-induced genes does not exhibit rapid reciprocal repression upon the initial dark-to-light transition of dark-grown seedlings (Figure 3, Class R). These genes thus appear to have newly acquired the capacity to respond to phy-PIF signaling during or after deetiolation (see Supplemental Data Set 23 online).

To examine the speed of the transcriptional responses elicited by the light and shade signals, selected genes displaying reciprocal responsiveness (Class M in Figure 3) were subjected to more detailed time-course analysis by qPCR over the first 60 min of exposure to each signal. The data are compared directly for each gene in Figure 4 and Supplemental Figure 11 online, normalized to the level of expression in 2-d dark-grown seedlings grown in parallel in each case. The data for the shade-induced expression increases were obtained in this study, whereas those for the light-imposed expression decreases were obtained in our previous study (Leivar et al., 2009) or in additional unpublished experiments. In addition to validating the microarray results (see Supplemental Figure 5 online), these data identify two subclasses of early-response genes. One subclass, which includes *PIL1*, *ATHB2*, *IAA29*, and *CYTOKININ OXIDASE5* (*CKX5*), displays rapid and robust, PIF-dependent shade induction to levels equivalent to, or exceeding, those of dark-grown seedlings within this 60-min period (Figure 4). This subclass comprises candidates for being direct targets of phy-PIF signaling and having key roles in central functions that initiate switching between skotomorphogenic and photomorphogenic developmental pathways. The other subclass, which includes *XTR7*, *SAUR25*, and *AT5G02580*, although displaying the twofold, statistically significant, PIF-dependent shade induction that defines this class (see insets), exhibits quantitatively less robust recovery in expression levels over the first 1 h of shade exposure compared with the levels originally present in dark-grown seedlings (Figure 4; see Supplemental Figure 11 online).

Morphogenic and Expression Analysis of *pif* Mutant Combinations Reveals Differential Contributions of Individual PIFs to Skotomorphogenesis and Shade Avoidance

Given the evidence that PIF1, PIF3, PIF4, and PIF5 (the PIF quartet) appear to contribute collectively to the maintenance of the etiolated state in dark-grown seedlings (Leivar et al., 2008a; Shin et al., 2009), that PIF1 appears to dominate the regulation of seed germination (Oh et al., 2004), and that PIF4 dominates the

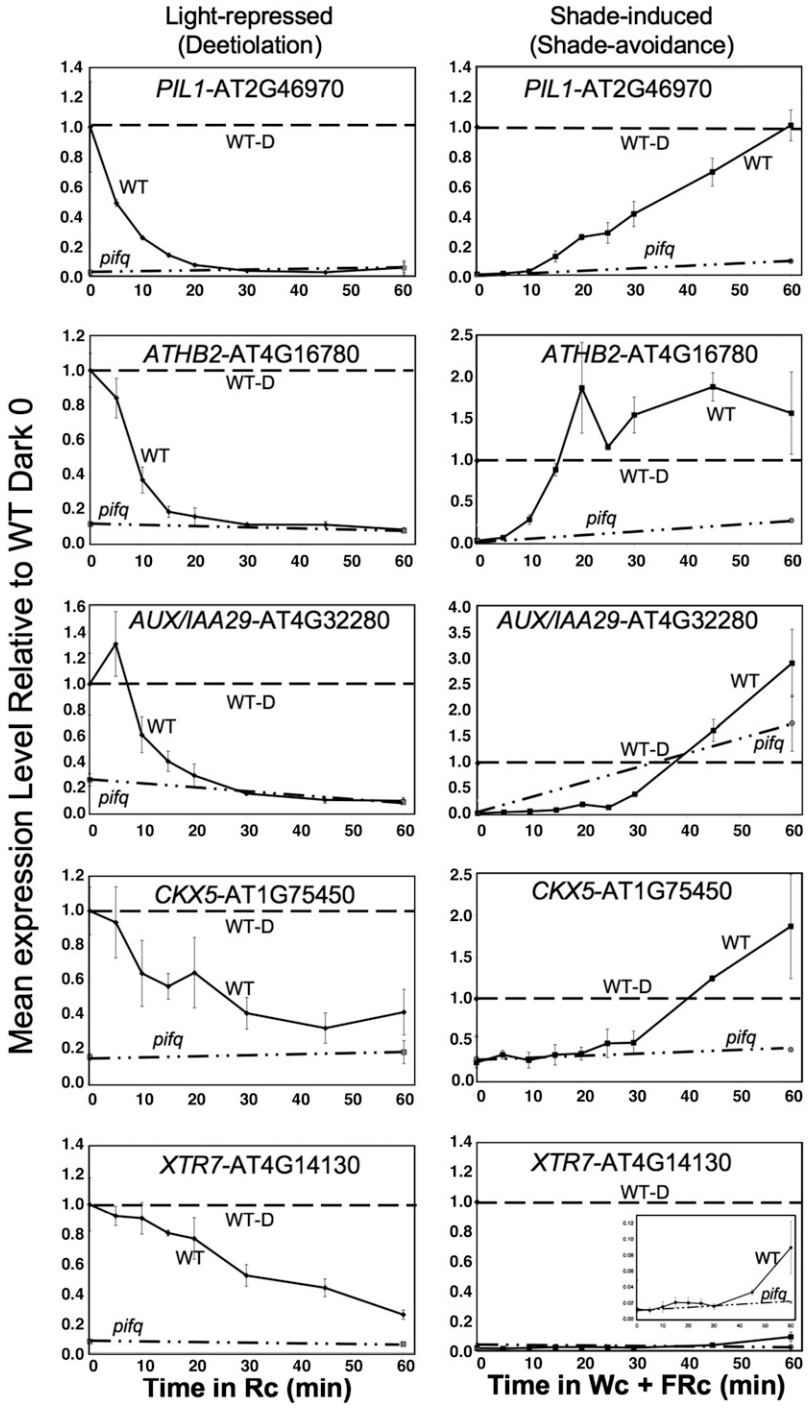

Figure 4. Selected G-Box–Containing, Class M Genes Display Rapid, Robust, PIF-Dependent, Reciprocal Transcriptional Responses to Light and Shade Signals.

Wild-type (WT) (solid curves) and *pifq* mutant (dot-dashed curves) seedlings were grown either for 2 d in the dark and then exposed to Rc (left panels) or for 2 d in WLc and then exposed to WLc+FRc (simulated shade) (right panels) for increasing periods from 0 to 60 min. Expression of the indicated genes was determined by qPCR, and *PROTEIN PHOSPHATASE2A* (*PP2A*) was used as a normalization control. Data are presented relative to the mean of 2-d dark-grown control seedlings (WT-D) in each case set at unity (horizontal dashed curve) and represent the mean and SE of three independent biological replicates. Deetiolation data for *PIL1*, *ATHB2*, and *IAA29* are from Leivar et al. (2009); the remainder is from this study.

hypocotyl elongation induced by warm temperature (Koini et al., 2009; Stavang et al., 2009), we wished to assess the individual contributions of these PIFs to the skotomorphogenic and shade avoidance morphological and molecular responses. To initiate this process, we analyzed *pif1*, *pif3*, *pif4*, and *pif5* single mutants (to assess the effect of the absence of each one of the PIFs in the presence of the others) and the *pif* triple mutant combinations *pif1 pif3 pif4*, *pif1 pif3 pif5*, *pif1 pif4 pif5*, and *pif3 pif4 pif5* (to assess the effect of the presence of each one of the PIF quartet separately in the absence of the other three by comparison to *pifq*). *pif1 pif3* and *pif4 pif5* double mutants were also included as controls for dark and shade phenotypes (Leivar et al., 2008a; Lorrain et al., 2008, 2009).

Phenotypic analysis of 2-d-old (Figures 5A and 5B) and 4-d-old (see Supplemental Figure 12A online) dark-grown seedlings suggest that PIF1 has the most prominent role in maintaining appressed cotyledons in etiolated seedlings but that it does this in a partially redundant manner with PIF3, PIF4, and PIF5. This conclusion comes from at least three observations: (1) Although the *pif1* single mutant shows only a minimal, nonstatistically significant cotyledon separation phenotype in the dark, higher order *pif* mutant combinations that include the *pif1* mutation show an increased degree of cotyledon separation compared with those that retain the wild-type *PIF1* locus. (2) The wild-type *PIF1* gene is the only *PIF* member able to fully complement the *pifq* cotyledon separation phenotype, since the *pif3 pif4 pif5* triple mutant behaves like the wild type. (3) *PIF3*, *PIF4*, and *PIF5* genes show more partial functions in maintaining appressed cotyledons in darkness in the absence of the other PIFs, as inferred from comparing *pif1 pif4 pif5*, *pif1 pif3 pif5*, and *pif1 pif3 pif4* triple mutants with *pifq*.

Overall, the hypocotyl elongation phenotype of young 2-d-old dark-grown seedlings broadly mirrors the cotyledon separation phenotype, in that elongation declines progressively with the absence of increasing numbers of PIF family members (cf. single, double, and triple mutants with *pifq*; Figures 5A and 5B, bottom). On the other hand, the contributions of each individual PIF to elongation in the absence of the other three (see the triple mutants) appear to be quantitatively more similar to each other than for the cotyledon phenotype in these seedlings (Figure 5B). In 7-d dark-grown seedlings, PIF1 emerges as the most active in complementing the *pifq* short-hypocotyl phenotype (cf. *pif3 pif4 pif5* to *pifq*), although relatively strong contributions are also observed for PIF3, PIF4, and PIF5 (cf. *pif1 pif4 pif5*, *pif1 pif3 pif5*, and *pif1 pif3 pif4* to *pifq*) (Figures 5C and 5D). Similar effects (although perhaps less pronounced) were also seen in 4-d-old dark-grown hypocotyls (see Supplemental Figure 12B online). These apparent quantitative differences between young and older seedlings might reflect developmental changes in PIF activity or perhaps the greater variability and smaller dynamic range of differences in hypocotyl length between the wild type and *pifq* in the 2-d-old than in the older seedlings.

Expression analysis of established PIF-dependent genes (*PIL1* and *ATHB2*) in the dark shows an expression profile pattern that is broadly consistent with the morphological phenotypes of the various *pif* mutant combinations tested above (Figure 6A). Among the *pif* single mutants, *pif1* shows the most robust *PIL1* misexpression in 2-d-old dark-grown seedlings (Figure 6A), and *PIF1* is also by far the most active gene among the *PIF* quartet in complementing the low-*PIL1* misexpression phenotype of the *pifq* mutant (cf. *pif3 pif4 pif5* to *pifq*). A similar pattern is observed for *ATHB2* expression (Figure 6A), although in this case the role of PIF1 in inducing *ATHB2* in the dark is quantitatively more similar to the other PIFs, and relatively strong contributions of PIF5 and, to a lesser extent, PIF4 are also observed (cf. *pif3 pif4 pif5*, *pif1 pif3 pif5*, and *pif1 pif3 pif4* to *pifq*). The data thus suggest that PIF1 is the most active player among the PIF quartet in promoting the expression of selected PIF-dependent genes during skotomorphogenesis, a function also exerted by the other PIF members in a partially redundant manner.

Phenotypic analysis of hypocotyl elongation responses to simulated vegetational shade of the various *pif* mutant combinations is presented in Figures 5C and 5E. The *pif1*, *pif3*, *pif4*, and *pif5* single and *pif1 pif3* double mutants show variable minor-to-absent hypocotyl phenotypes in response to long-term simulated shade, as documented in two independent experiments (Figure 5E, top; see Supplemental Figure 13 online). These data alone would thus suggest only marginal contributions of the individual PIF quartet members to the long-term hypocotyl elongation responses to simulated shade (i.e., in the presence of the other members of the PIF quartet). However, more consistently reproducible phenotypes are observed for *pif4 pif5*, *pif3 pif4*, and *pif3 pif5* double mutants compared with the wild type (Figure 5E, bottom; Lorrain et al., 2008; Leivar et al., 2012), suggesting that a certain degree of redundancy exists among these PIFs in regulating shade avoidance responses. The relatively smaller difference in hypocotyl shade responsiveness between the *pif4 pif5* and *pifq* mutants in this experiment than in Figure 1C falls short of statistical significance, reinforcing the conclusion that the PIF1-PIF3 pair contribute quantitatively very moderately to the collective activity of the quartet, in controlling this response, compared with PIF4 and PIF5 combined. However, combination of the *pif* mutations into higher order triple and quadruple *pif* mutants provides interesting insight into the relative activities of the individual PIFs (Figure 5E, bottom). In contrast with the triple *pif* mutant analysis in 7-d-old dark-grown seedlings (Figures 5C and 5D), *PIF1* does not dominate complementation of the *pifq* hypocotyl phenotype in response to long-term simulated shade, in the absence of the other three PIFs. Instead, *PIF5* is the only PIF quartet member that individually displays significant promotive activity in the absence of the other three (cf. *pif1 pif3 pif4* to *pifq* in Figure 5E, bottom).

Expression analysis of representative M-class genes, *PIL1* and *ATHB2*, in the various *pif* mutant combinations, in response to simulated shade, shows that *PIF5* is the most prominent contributor to the rapid induction of these genes in response to shade, with *PIF1* being the next strongest (Figure 6B). This prominent role of PIF5 is inferred from the robust reduction in shade-induced expression of *PIL1* and *ATHB2* in the *pif5* single mutant compared with the wild type, which is in contrast with the relatively more modest effect of *pif1* and the marginal-to-absent effects of *pif3* and *pif4* monogenic mutations (Figure 6B). Conversely, in good agreement with these data, the *PIF5* gene is the most active in complementing the loss-of-shade–induced *PIL1* gene expression phenotype of *pifq* (cf. *pif1 pif3 pif4* to *pifq*), followed by a relatively less pronounced effect of the *PIF1* gene (cf. *pif3 pif4 pif5* to *pifq*). A similar tendency is observed for *ATHB2*, albeit in a nonstatistically significant manner in this analysis (Figure 6B).

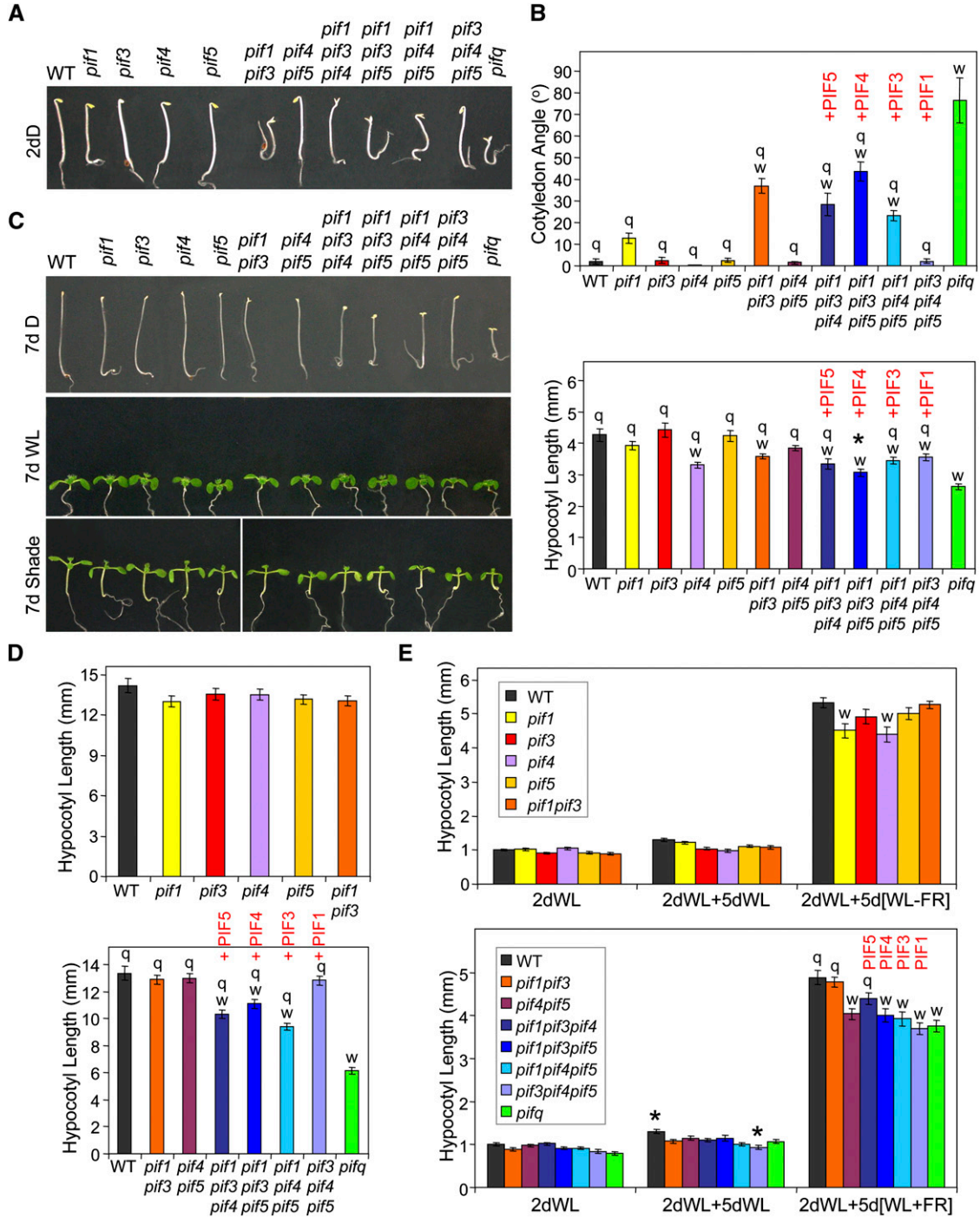

Figure 5. Phenotypic Analysis of *pif* Mutant Combinations Provides Evidence for Differential Contributions of Individual PIF Quartet Members to Skotomorphogenesis and Shade Avoidance.

Wild-type (WT) and *pif* mutants were analyzed phenotypically for cotyledon separation and/or for hypocotyl length in seedlings grown in darkness (D), in WLc, or in WL supplemented with FRc (shade, WL+FR). Light conditions were as in Figure 1A.

(A) Visible phenotypes of wild-type and *pif* mutant seedlings grown in the dark for 2 d (2dD).

(B) Quantification of cotyledon angle (top) and hypocotyl length (bottom) phenotypes of 2-d-old dark grown wild-type and *pif* mutant seedlings.

(C) Visible phenotypes of wild-type and *pif* mutant seedlings grown either in the dark for 7 d (7 d D), in WLc for 7 d (7 d WL), or in WL for 2 d and then in WL+FR for five additional days (7 d Shade).

(D) Quantification of hypocotyl length phenotype of 7-d-old dark-grown wild-type and *pif* mutant seedlings. *pif* single mutants are shown at the top,

DISCUSSION

Previously we defined the PIF-regulated transcriptional network that promotes skotomorphogenesis in dark-grown seedlings and that responds rapidly to PIF removal by phy-induced proteolysis at the inception of deetiolation upon first exposure to light (Leivar et al., 2009). Here, we identified a set of genes that respond rapidly to the shade signal, in a PIF-dependent manner, and uncovered a dichotomy within this set suggesting that transcription factor–encoding genes may dominate those that are direct PIF targets, whereas auxin-responsive genes may be indirect targets. In addition, we defined a further core subset of these genes that display diametrically opposed, rapid responsiveness to initial light and shade exposure. These data thus identify a subset of genes that apparently remain continuously under active, reversible, and potentially direct transcriptional regulation by phy-PIF signaling throughout seedling deetiolation and subsequent vegetative growth and development of the fully green plant. Moreover, we provided morphogenic and marker gene expression evidence that individual members of the PIF quartet contribute differentially to the collective action of these factors in exerting this regulation during skotomorphogenesis and shade avoidance, suggesting that they may provide selective channeling of the phy-mediated light signal to defined sectors of the transcriptional network.

Overall, the shade-induced expression profiles in wild-type seedlings observed here overlap significantly with those in previous similar studies (Devlin et al., 2003; Carabelli et al., 2007; Tao et al., 2008) (see Supplemental Figure 3 online). In particular, a considerable fraction of the genes responding within 1 h to the shade stimulus in our study (Table1; see Supplemental Data Set 18 online) have also been documented previously as rapidly shade responsive (Steindler et al., 1999; Salter et al., 2003; Roig-Villanova et al., 2006, 2007; Carabelli et al., 2007; Lorrain et al., 2008; Tao et al., 2008; Kozuka et al., 2010). This study expands this list of rapid-response genes and identifies those that require one or more members of the PIF quartet (PIF1, 3, 4, and/or 5) for normal, full responsiveness to the signal (Figure 2; see Supplemental Figure 4 online). We show that endogenous PIF3 levels increase rapidly in response to shade-induced reduction in phy Pfr levels (Figure 1; see Supplemental Figure 1 online), similarly to that reported for PIF4 and PIF5 proteins overexpressed ectopically in transgenic lines (Lorrain et al., 2008) and consistent with the involvement of these PIFs in the stimulation of enhanced axis elongation and other responses that make up the SAS (Smith and Whitelam, 1997; Franklin, 2008; Lorrain et al., 2008) (Figure 1C). Shade-induced genes (see Supplemental Figure 4B online) dominate the rapidly responsive class of loci (see Supplemental Figure 4A online) and have thus been the focus of our study.

Our analysis identifies those genes that are most robustly dependent on the PIFs for shade induction (FIR >1.5) (Figures 2B1 and 2B2, Table 1). Several of these genes have been identified previously as being rapidly shade responsive in wild-type seedlings, including *ATHB2*, *XTR7*, *HFR1*, *PAR1*, and *IAA29* (Steindler et al., 1999; Roig-Villanova et al., 2006, 2007; Carabelli et al., 2007; Lorrain et al., 2008; Hornitschek et al., 2009) (Table 1; see Supplemental Data Set 18 online). Our data show that genes with G-box–containing promoters are enriched among the robustly PIF-dependent loci (69 to 81% of genes with FIR >1.5 after 1 h of simulated shade) (Table 1; see Supplemental Data Set 18 online). Because PIF proteins have been shown to bind sequence specifically to the core G-box motif (Martínez-García et al., 2000; Huq and Quail, 2002; Huq et al., 2004; Shin et al., 2007; de Lucas et al., 2008; Leivar et al., 2008b; Moon et al., 2008; Hornitschek et al., 2009; Oh et al., 2009), this strong positive correlation is consistent with the possibility that those genes exhibiting robust PIF-regulated expression may be direct targets of transcriptional regulation by promoter-bound PIF proteins. The available ChIP analysis of targeted G-box–containing promoters, such as *XTR7*, *HFR1*,and *PIL1*, supports this suggestion (Shin et al., 2007; de Lucas et al., 2008; Moon et al., 2008; Hornitschek et al., 2009; Oh et al., 2009; Toledo-Ortiz et al., 2010). Moreover, comparison of the G-box–containing and non-G-box–containing genes within this set (Figures 2B1 and 2B2) reveals an interesting functional dichotomy. The G-box–containing subset is enriched for transcription factor–encoding genes (61 to 67%), suggesting a function in the primary transcriptional network that regulates downstream genes in the phy signaling cascade (Figure 2C, top). Conversely, the non-G-box–containing subset are enriched for hormone-related genes (50 to 67%), especially auxin-responsive genes (Figure 2C, bottom), reinforcing the established notion that auxin is strongly involved in the rapid growth responses (≤10 min) observed upon shade exposure (Smith and Whitelam, 1997; Steindler et al., 1999; Tanaka et al., 2002; Vandenbussche et al., 2003; Carabelli et al., 2007; Roig-Villanova et al., 2007; Franklin, 2008; Tao et al., 2008; Keuskamp et al., 2010; Kozuka et al., 2010; Cole et al., 2011; Grebe, 2011). However, closer inspection of the auxin-responsive gene set suggests a further dichotomy and level of complexity. There appears to be a possible dual track of rapid PIF regulation

Figure 5. (continued).

whereas *pif4 pif5* double, *pif* triple, and *pifq* mutants are shown at the bottom. Wild-type and *pif1 pif3* mutants were included in both experiments. **(E)** Quantification of hypocotyl length phenotype of wild-type and *pif* mutant seedlings grown in WLc from germination onward (2dWL) or for 2 days in WLc and then for an additional 5 d either in WLc (2dWL+5dWL) or in WL with supplemental FRc (2dWL+5d[WL+FR]). *pif* single mutants are shown at the top, whereas *pif4 pif5* double, *pif* triple, and *pifq* mutants are shown at the bottom. Wild-type and *pif1 pif3* mutants were included in both experiments. Graphical data in **(B)**, **(D)**, and **(E)** represent mean values and SE (bars) from at least 20 seedlings. Statistically significant differences from the wild type (w) and/or the *pifq* mutant (q) values, defined by Tukey's multiple comparison test ($P \leq 0.05$), are indicated for each treatment. An asterisk indicates statistically significant differences from the *pifq* mutant by Student's *t* test ($P \leq 0.05$), shown only for values that were not defined as "q" by the Tukey's test. PIF1, PIF3, PIF4, and PIF5 red labels on top of the triple *pif* mutant graphs are intended to highlight the *PIF* gene that is present as a wild-type copy in the corresponding triple mutant.

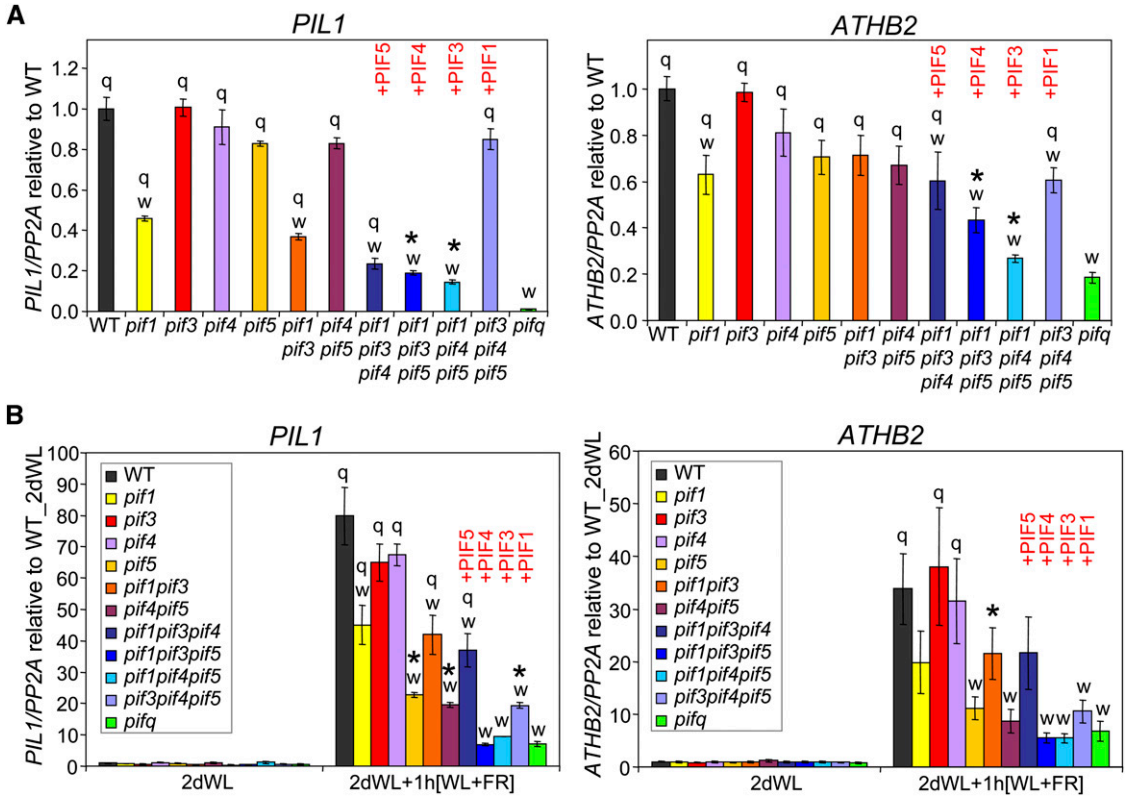

Figure 6. PIF1 and PIF5 Differentially Dominate Promotion of Expression of Selected M Class Genes during Skotomorphogenesis and Initiation of Shade Avoidance.

(A) *ATHB2* and *PIL1* gene expression in 2-d-old dark-grown seedlings. Data are presented relative to the wild-type (WT) mean, which was set at unity.
(B) *ATHB2* and *PIL1* gene expression in seedlings grown for 2 d in WLc (2dWL) or seedlings grown for 2 d in WL and then treated with WL supplemented with FRc for 1 h (2dWL+1 h[WL+FR]). Light conditions were as in Figure 1A. Data are presented relative to the mean of WT-WL set at unity.
ATHB2 and *PIL1* gene expression were measured by qPCR in wild-type and *pif* mutant seedlings grown under the indicated conditions, and *PP2A* was used as a normalization control as described (Shin et al., 2007). Data represent the mean and SE of at least three independent biological replicates. Statistically significant differences from the wild type (w) and/or the *pifq* mutant (q) values, defined by Tukey's multiple comparison test ($P \leq 0.05$), are indicated for each treatment. An asterisk indicates statistically significant differences from the *pifq* mutant by Student's *t* test ($P \leq 0.05$), shown only for values that were not defined as "q" by the Tukey's Test. PIF1, PIF3, PIF4, and PIF5 red labels on top of the triple *pif* mutant graphs are intended to highlight the *PIF* gene that is present as a wild-type copy in the corresponding triple mutant.

of the auxin signaling pathway: (1) A potentially indirect track stimulating bioactive auxin activity (perhaps via synthesis, transport, or activation of inactive conjugates) or signaling, with resultant induction of several *SAUR* genes with no G-boxes in their promoters (Figure 2C, bottom, Table 1; see Supplemental Data Set 18 online); and (2) a potentially direct track involving direct transcriptional activation of *AUX/IAA* genes by promoter-bound PIF proteins, represented by the G-box–containing genes *IAA2*, *IAA4*, *IAA19*, and *IAA29* (Figure 2C, top, Table 1; see Supplemental Data Set 18 online). As the *AUX/IAA* genes are negative regulators of auxin signaling (reviewed in Mockaitis and Estelle, 2008), it is possible that PIF promotion of their expression functions to precede and/or augment rapid auxin-induced accumulation of the transcripts of these genes to prevent an overresponse to the increased auxin and/or to generate a high capacity for rapid synthesis of the negative regulatory AUX/IAA proteins upon any subsequent reduction in auxin activity. Collectively, these data suggest that initiation or modulation of auxin signaling is at least one of the major circuits that the PIFs target initially in response to the shade signal, consistent with the role of this hormone in implementing rapid shade-induced growth responses (Tao et al., 2008; Cole et al., 2011). PIF modulation of auxin signaling might also operate in other conditions where growth is induced, as proposed for diurnal conditions (Nozue et al., 2011) and as recently demonstrated for PIF4 in response to high temperature (Koini et al., 2009; Franklin et al., 2011). Intriguingly, however, whereas Franklin et al. (2011) observe PIF4-dependent, high-temperature-induced increased expression of the auxin biosynthetic genes *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 and CYTOCHROME P450 CYP79B2*, we do not detect significant shade responsiveness of these genes. This observation might suggest that the phys and high-temperature signal through the PIFs to the auxin system via different pathways.

It is notable that bHLH factor genes (*PIL1*, *HFR1*, *PAR1*, *BES1-INTERACTING MYC-LIKE PROTEIN2* [*BIM2*], *BR ENHANCED EXPRESSION1* [*BEE1*], *AT5G15160*, *AT1G01260*, and *AT3G57800*) dominate the G-box–containing, robustly PIF-dependent, transcription factor–encoding gene set (Figure 2C, top, Table 1; see Supplemental Data Set 18 online). This suggests that these PIF-related factors may have evolved as variants from the same gene family recruited to function in the primary transcriptional network regulated by the PIFs in response to shade. The presence of homeodomain- (*ATHB2* and *ATHB52*) and a *TEOSINTE BRANCHED1/CYCLOIDEA* (*TCP*)-encoding (*TCP/PCF15* [*TCP15*]) gene in this group suggests early divergence of downstream targets in the transcriptional cascade. It is notable that several of these transcription factors have already been shown to have regulatory roles in the SAS, including *ATHB2* (Steindler et al., 1999), *PIL1* (Salter et al., 2003; Roig-Villanova et al., 2006), *HFR1* (Sessa et al., 2005; Hornitschek et al., 2009), and *PAR1* (Roig-Villanova et al., 2007). This is consistent with the PIFs acting to induce a transcriptional regulatory network that implements or fine-tunes the response to the shade signal. As *BIM2* and *BEE1* have been implicated in the brassinosteroid signaling pathway (Friedrichsen et al., 2002; Yin et al., 2005), these genes are potential regulatory nodes for PIF and brassinosteroid crosstalk (Sorin et al., 2009; Kozuka et al., 2010; Keller et al., 2011; Keuskamp et al., 2011) in response to shade. The presence of *CKX5* (a cytokinin oxidase–encoding gene) and *GA2OX6* (a gibberellin-2 oxidase) (Figure 2C, top; see Supplemental Data Set 18 online) suggests that cytokinin and gibberellin levels (two other proposed shade-regulatory hormones; Carabelli et al., 2007; Djakovic-Petrovic et al., 2007; Stamm and Kumar, 2010), in addition to auxin levels, may be early targets of PIF-mediated shade regulation. It is notable that GIBBERELLIC ACID INSENSITIVE (GAI), a repressor of the gibberellin pathway proposed to constrain shade avoidance responses (Djakovic-Petrovic et al., 2007), is induced by the shade signal in a robustly PIF-dependent manner. Since DELLA proteins, including GAI, have been proposed to constrain growth by inhibiting the PIFs (de Lucas et al., 2008; Feng et al., 2008), the data suggest that the PIFs induce a complex negative-feedback regulatory loop, possibly to modulate the response to the shade signal. Direct PIF regulation of cell wall metabolism, possibly related to the rapid growth responses to shade, is also suggested by the presence of *XTR7/XTH15* (a xyloglucan transglycosylase–encoding gene; Rose et al., 2002) in this group. This is consistent with the observation that *xth15* knockout mutants show reduced shade-induced growth responses (Sasidharan et al., 2010). Collectively, the data focus attention on a subset of genes that provide potential targets for future investigations aimed at securing more detailed insight into central cellular processes involved in the initiation of the shade avoidance response.

Certain facets of the SAS, such as accelerated hypocotyl and internode cell elongation rates and retarded chloroplastogenesis (Franklin, 2008), represent changes in growth and development in the direction of those that preexist in the etiolated seedling before light exposure. Our comparison of the transcriptome changes induced by light and shade at the inception of deetiolation and shade avoidance, respectively, has provided evidence (1) of a core subset of genes that respond rapidly and reciprocally to the production of, and reduction in, high Pfr levels upon initial light and shade exposure, respectively; (2) that require the PIF quartet for normal, maximal expression levels in both dark-grown and shade-exposed seedlings; and (3) whose promoters contain G-box motifs capable of direct recruitment of the PIF factors (Figure 3, Class M genes, and Table 2). The data suggest that these genes are direct targets of sustained, continuous modulation by the phy-PIF signaling system, whereby the PIFs constitutively promote their expression in an ongoing manner, and photoactivated phy quantitatively abrogates this activity by inducing controlled PIF proteolysis in response to impinging light signals. Therefore, these genes seem likely to comprise a central, primary transcriptional network module responsible for rapidly redirecting adaptive responses to the fluctuating light environment as the plant progresses through the life cycle. Consistent with this view, 13 out 14 of these class M genes (Table 2) have been shown in a recently published study to be upregulated during the growing phase at night in seedlings growing under diurnal conditions (Nozue et al., 2011), and among those, 10 of them require PIF4 and/or PIF5 for their expression.

Our comparison of the skotomorphogenic and shade-induced, visible and molecular phenotypes of monogenic, triple, and quadruple *pif* mutants in dark-grown and shade-exposed seedlings provides evidence that the individual PIF1, 3, 4, and 5 proteins contribute, qualitatively and quantitatively to different extents, to the collective activity of this PIF quartet in regulating growth and development. The data show that PIF1 dominates promotion of cotyledon appression in dark-grown seedlings, with PIF3, 4, and 5 contributing to a lesser extent (Figure 5B, top). These data are consistent with previous reports (Leivar et al., 2008a; Shin et al., 2009), although in one case the analysis was incomplete since it only involved a selected subset of *pif* mutant combinations (Leivar et al., 2008a) and in the other the appression data were not quantified by these authors (Shin et al., 2009). A similar, although less dramatic, pattern is observed for the hypocotyl length phenotype, where PIF1 again appears to dominate, especially in 7-d dark-grown seedlings (Figure 5D). Conversely, none of the PIF quartet appears to dominate promotion of shade-induced hypocotyl elongation, although if anything, PIF5 appears to show a more significant contribution in the absence of the other three PIF members (Figure 5E). By comparison, PIF4 has been shown to strikingly dominate high-temperature-induced elongation growth (Koini et al., 2009; Stavang et al., 2009; Franklin et al., 2011), PIF1 and PIF3 have been reported to dominate promotion of agravitropism in dark-grown seedlings (Shin et al., 2009), PIF1 dominates regulation of seed germination (Oh et al., 2006), and PIF3 plays a prominent role in promoting seedling growth under diurnal light-dark conditions (Soy et al., 2012) in conjunction with PIF4 and PIF5 (Nozue et al., 2007).

Marker gene expression analysis reveals a more complex picture, whereby the pattern of individual PIF involvement is different between skotomorphogenesis and shade avoidance. Whereas PIF1 dominates promotion of *PIL1* expression in dark-grown seedlings (with PIF3, 4, and 5 contributing less, but at comparable levels to each other), PIF5 dominates shade-induced promotion of *PIL1* expression (with PIF1, 3, and 4

contributing less) (Figure 6). *ATHB2* displays a similar general pattern of differential regulation in response to darkness and shade, except that PIF5 appears to have a significantly more prominent role in promoting this gene's expression in dark-grown seedlings in the absence of the other three PIF members, where its activity is comparable to that of PIF1. These data suggest that the different PIF family members may function to differentially distribute phy-regulated signaling across different sectors of the transcriptional network. In addition, the residual responsiveness of the *pifq* mutant to shade here, as well as to light during deetiolation (Leivar et al., 2008a, 2009), is suggestive of residual factors other than PIF1, 3, 4, and 5, that, like these PIFs, act continuously to promote facets of skotomorphogenic-like gene expression and development. Such factors might include additional PIF family members, such as PIF6, PIF7, and/or PIF8 (Leivar and Quail, 2011), or other potential regulators. Finally, emerging evidence of the central position of the PIF family at the nexus of multiple convergent signaling pathways (Leivar and Quail, 2011) indicates that further detailed definition of the functional roles of these factors in various cellular processes will be of considerable interest.

METHODS

Plant Materials, Seedling Growth, and Measurements

Except for *pif1 pif4 pif5* and *pif4 pif5*, all the *pif* mutant combinations used in this study were described elsewhere (Leivar et al., 2008a) and were generated using *pif1-1* (Huq et al., 2004), *pif3-3* (Monte et al., 2004), *pif4-2* (Leivar et al., 2008b), and *pif5-3* (Khanna et al., 2007) single mutants. *pif1 pif4 pif5* and *pif4 pif5* were obtained by crossing *pif1 pif3*, *pif4-2*, and *pif5-3* as described (Leivar et al., 2008a).

Seeds were sterilized and plated in germination medium without Suc as described (Leivar et al., 2009) and were then stratified for 5 d at 4°C in darkness. For phenotypic analysis under simulated shade conditions, seeds were transferred to WLc (19 μmol m^{-2} s^{-1}, R/FR ratio of 6.48) for 2 d at 21°C and then grown for five additional days under the same WL fluence rate with (WL-FR, R/FR ratio of 0.006) or without (WL) supplemental FRc.

For dark-grown seedlings, seeds were treated with a 3-h WL pulse to synchronize germination, followed by a 5-min FR pulse to avoid pseudo-dark effects as reported (Leivar et al., 2008a, 2009). Alternatively, a 5-min R pulse followed by a 3-h dark incubation and a terminal 5-min FR pulse was used (Leivar et al., 2008a, 2009). Seeds were then grown in the dark at 21°C for the indicated time.

Hypocotyl length and cotyledon opening were measured from at least 20 seedlings as described (Monte et al., 2003; Leivar et al., 2008a). Data were analyzed using Tukey's multiple comparison test (GraphPad Prism software) and/or Student's *t* test as indicated.

For the experiments performed under dichromatic R+FR simulated-shade conditions, seeds were induced to germinate directly in Rc (10 μmol m^{-2} s^{-1}), and seedlings were grown for 3 d in Rc. Seedlings were then maintained in the same fluence rate of Rc supplemented with FRc (low R/FR ratio of 0.1 or 0.02 as indicated) for the specified time.

Protein Extraction and Immunoblot and β-Glucuronidase Assays

Protein extracts of *Arabidopsis thaliana* seedlings and immunodetection of PIF3 in R+FR and WL+FR were done as described (Al-Sady et al., 2006; Leivar et al., 2008b). Briefly, protein extracts of *Arabidopsis* seedlings were prepared (Al-Sady et al., 2006; Leivar et al., 2008b), and total protein was quantified using a Protein DC kit (Bio-Rad). β-Mercaptoethanol was added to the samples before loading, and then equal amounts of protein were subjected to polyacrylamide gel electrophoresis. Immunodetection of PIF3 was performed using affinity-purified anti-PIF3 antisera (Al-Sady et al., 2006), and a mouse monoclonal antibody against α-tubulin (Sigma-Aldrich) was used as a loading control. Anti-rabbit-horseradish peroxidase and anti-mouse-horseradish peroxidase were used as secondary antibodies (Promega), and ECL or ECL plus chemiluminescence kits (Amersham) were used for detection. Alternatively, alkaline phosphatase–coupled anti-rabbit Antiserum (Promega) was used as a secondary antibody for detection of PIF3 (Al-Sady et al., 2006). β-Glucuronidase (GUS) activity assays with 35S:GUS:PIF3 transgenics were performed as described (Monte et al., 2004).

Gene Expression Analysis by qPCR and RNA Gel Blots

For qPCR experiments, ~250 seedlings were grown for 2 d in either darkness or WLc as indicated above. Two-day-old WLc-grown seedlings were then exposed to WL+FR as described above, and samples were harvested at the indicated times. Quantitative RT-PCR analysis was performed as described (Leivar et al., 2009). Briefly, an RNeasy Plus Plant Mini kit (Qiagen) was first used to extract RNA. Then, 1 μg of total RNA was treated with DNase I (Invitrogen) to further eliminate genomic DNA, and first-strand cDNA synthesis was done using the Super-Script First Strand cDNA synthesis for RT-PCR kit (Invitrogen) and oligo(dT20) as a primer. cDNA was treated with RNase H, and samples were then diluted 1:30 with water. Ten microliters of the diluted samples were used for real-time PCR (MyIQ single-color real-time PCR detection system; Bio-Rad). Eva-green (Biotium) was used for detection, and 0.1% Tween 20, 0.1 mg/mL BSA, and 5% DMSO were added to the PCR mix as described (Khanna et al., 2007). Each qPCR assay was repeated at least two times, and the mean expression values from these technical replicates were used for further calculations. Gene expression was measured from at least three biological replicates, and *PP2A* (*AT1G13320*) was used as a normalization control as described (Shin et al., 2007; Leivar et al., 2009). Normalized gene expression was represented relative to the wild type set as a unity. Primer sequences for qPCR of *PP2A* (*AT1G13320*), *PIL1* (*AT2G46970*), *HAT4/ATHB2* (*AT4G16780*), *AUX/IAA29* (*AT4G32280*), *SAUR25* (*AT4G13790*), and Unknown (*AT5G02580*) were described elsewhere (Leivar et al., 2009). Primer sequences for *CKX5* (*AT1G75450*) were as follows: CKX5_F (5′-ACCGTCCACCCTTCCGAC-3′) and CKX5_R (5′-CAGGTGACTTCAGCATACCGAA-3′). Primer sequences for *XTR7* (*AT4G14130*) were as follows: PLR145_F (5′-CGGCTTGCACAG-CCTCTT-3′) and PLR146_R (5′-TCGGTTGCCACTTGCAATT-3′). Data were analyzed using Tukey's Multiple Comparison Test (GraphPad Prism software) and/or a Student's *t* test as indicated.

For RNA gel blot analysis, total RNA extraction, loading, and hybridization conditions were as described (Monte et al., 2003). Specific probe for PIF3 was prepared as described (Monte et al., 2004).

Microarray-Based Expression Profiling: RNA Isolation, cRNA Synthesis, and Hybridizations

About 1500 *Arabidopsis* wild-type and *pifq* mutant (Leivar et al., 2008a) seeds were plated separately for each sample on germination medium without Suc at room temperature. During this procedure, the seeds were routinely exposed to WL for a total of 1.5 h after imbibition. Seeds were then stratified for 5 d at 4°C in darkness and then grown in WL (19 μmol m^{-2} s^{-1}, R/FR ratio of 6.48) for 2 d at 21°C (WL0 samples). Two-day-old WL-grown seedlings were then maintained in the same fluence rate of WL supplemented with FR light (WL-FR, R/FR ratio of 0.006) for 1 (FR1), 3 (FR3), or 24 (FR24) hours before harvesting. Control seedlings were also maintained in parallel in the same fluence rate of WL for 24 h (WL24) before harvesting. Three different biological replicates of each treatment were grown separately and extracted, processed, and analyzed

independently. Total RNA isolation, cRNA synthesis, and microarray hybridizations were performed as described (Monte et al., 2004) with minor variations (Leivar et al., 2009). Briefly, total RNA was prepared as described (Chang et al., 1993) followed by further purification using RNeasy columns (Qiagen). RNA target preparation was performed following the GeneChip 3′ IVT express kit (Affymetrix). ATH1 microarrays (Affymetrix) were used for gene expression detection. Hybridization and washes were performed as described by Affymetrix in the Functional Genomics Laboratory facility at the University of California at Berkeley (http://qb3.berkeley.edu/qb3/fgl/).

Microarray Data Analysis

Microarray data analysis was performed as described (Leivar et al., 2009) with minor modifications. Determination of statistical differences between genotypes and/or light treatments was made using the software analysis package AFFY (which is available as part of the Bioconductor project; www.bioconductor.org). To identify statistically significant, rapidly shade-responsive genes in the wild type, a moderated *t* test (Smyth, 2004) available as software analysis PLM and LIMMA AFFY packages as part of the Bioconductor project, was applied to the wild-type data, comparing gene expression at 2 d in WLc (WL0) with that after 1 h of WL+FRc irradiation (FR1). Log scale gene expression values were calculated using a Robust Multiarray Analysis. Fold-change values between various genotypes and treatments were calculated using the mean expression value of the replicate samples. Shade-responsive genes were defined as those that varied twofold from the WL0 time point with a P value (adjusted for false discovery rate) <0.05 SSTF genes (Hu et al., 2009). Similar analyses were done at the 3- and 24-h WL+FR (FR3 and FR24) time points. Genes that were differentially expressed with statistical significance in the *pifq* mutant compared with the wild type (P value <0.05 versus the wild-type, adjusted for false discovery rate) were identified at each time point (FR1, FR3, and FR24). If the ratio of gene expression of the wild type/*pifq* at WL0 was above 1.6 or below 0.625, they were defined as a separate class of genes, already different at WL0 before shade treatment in the *pifq* mutant (see Supplemental Figure 4, Supplemental Data Set 12, and Supplemental Data Set 15 online) and excluded from further analysis.

To provide a quantitative estimate of the robustness of the responsiveness of each gene to the shade treatment, we calculated a mean FI value for induced genes, separately for the wild type and *pifq* mutant. FI is defined as the mean of the triplicate expression values in 1-h WL+FRc-irradiated seedlings (designated FR1) divided by the mean of the triplicate expression values in the 2-d WLc (WL0)–treated seedlings:

$$\mathrm{FI} = \mathrm{FR1}/\mathrm{WL0}.$$

To provide a quantitative measure of the robustness of the contribution of the PIF quartet to the shade-induced response of each gene, we calculated a FIR value for induced genes as described (Monte et al., 2004). FIR is defined as the ratio of the FI values for the wild type and *pifq* mutant:

$$\mathrm{FIR} = (\mathrm{FI\ for\ WT})/(\mathrm{FI\ for}\ \textit{pifq}).$$

To simplify the functional classification analysis, genes were assigned to single functional categories based on the gene descriptions provided by Affymetrix and TIGR404 (http://compbio.dfci.harvard.edu/tgi/) and the classification provided by Munich Information Center for Protein Sequences (http://www.helmholtz-muenchen.de/en/mips/projects/funcat/index.html) as described (Leivar et al., 2009). Gene products targeted to the chloroplast were assigned to the "photosynthesis/chloroplast" category, whereas those with predicted or established transcription or DNA binding activity were assigned to the "transcription" category. Occurrence of G-boxes in promoter regions, defined as 3 kb upstream of the transcription start site, was determined using PATMATCH (http://www.Arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) as described (Leivar et al., 2009).

Meta-Analysis

CEL files from this study and those from our previous analysis of seedling deetiolation in the *pifq* mutant (Leivar et al., 2009) were combined in a single Robust Multiarray Analysis. Values derived from this analysis were used for graphic representations shown in Figure 3 and Supplemental Figures 7 to 10 online.

Accession Numbers

The microarray data reported in this article have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through Gene Expression Omnibus Series accession number GSE28297. Sequence data can be found in the Arabidopsis Genome Initiative database under accession numbers *AT2G20180* (*PIF1*/*PIL5*), *AT1G09530* (*PIF3*), *AT2G43010* (*PIF4*), and *AT3G59060* (*PIF5*/*PIL6*). Supplemental Data Sets 1 to 35 are deposited in the DRYAD repository: http://dx.doi.org/10.5061/dryad.gd031k26.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Reduction of Steady State Photoactivated-phy (Pfr) Levels in Continuous Light Induces Rapid Increases in PIF3 Abundance in *Arabidopsis* Seedlings.

Supplemental Figure 2. Genome-Wide Patterns of Rapid and Delayed Shade-Responsive Gene Expression in Wild-Type and *pifq* Mutant Seedlings.

Supplemental Figure 3. Limited Overlap of Rapidly Shade-Responsive Genes Identified Here with Those in Previous Transcriptome Analyses.

Supplemental Figure 4. Genome-Wide Patterns of Rapidly Shade-Responsive Gene Expression Establish PIF Involvement in Early Shade Signaling.

Supplemental Figure 5. qPCR Validation of Microarray Data.

Supplemental Figure 6. Genes Displaying Rapid Light-Triggered Repression during Deetiolation Are Enriched in Early Shade-Induced Genes.

Supplemental Figure 7. Merging of Deetiolation: Class 3 Light-Repressed and Rapidly Shade-Induced Transcriptome Data.

Supplemental Figure 8. Merging of Deetiolation: Class 6 Light-Repressed and Rapidly Shade-Induced Transcriptome Data.

Supplemental Figure 9. Merging of Deetiolation: Class 1 Light-Repressed and Rapidly Shade-Induced Transcriptome Data.

Supplemental Figure 10. Merging of Deetiolation: Class 4 Light-Repressed and Rapidly Shade-Induced Transcriptome Data.

Supplemental Figure 11. A Subset of G-Box–containing Class-M Genes Display SSTF but Quantitatively Minimal Shade-Induced Responsiveness Relative to Initial Expression Levels in Dark-Grown Seedlings.

Supplemental Figure 12. Phenotypic Analysis of 4-d-Old Dark-Grown Wild-Type and *pif* Mutant Seedlings.

Supplemental Figure 13. Quantification of the Hypocotyl Elongation Phenotype of Additional *pif* Single Mutants Grown under Simulated Shade.

Supplemental Analysis 1. Analysis of PIF3 Levels under Dichromatic Irradiation Conditions.

Supplemental Analysis 2. Comparison of Shade-Responsive Genes at FR1 with Previously Reported Studies and Definition of Shade-Responsive Genes at FR3.

Supplemental Analysis 3. Details of Identification of PIF-Dependent Rapidly Shade-Responsive Genes.

The following Supplemental Data Sets are deposited in the DRYAD repository: http://dx.doi.org/10.5061/dryad.gd031k26.

Supplemental Data Set 1. Expression Data and Primary Analysis for All Genes Identified in Supplemental Figure 2 Online (1381 Genes).

Supplemental Data Set 2. Expression Data and Primary Analysis for All Rapidly Induced Shade-Responsive Genes at FR1 (131 Genes).

Supplemental Data Set 3. Expression Data and Primary Analysis for All Rapidly Repressed Shade-Responsive Genes at FR1 (38 Genes).

Supplemental Data Set 4. Genes Induced Twofold or More in This Study and Either or Both Earlier Studies (Carabelli et al., 2007; Tao et al., 2008) in Response to 1 h (FR1) (131 Genes).

Supplemental Data Set 5. Expression Data and Primary Analysis for All FR3 Shade-Responsive Genes (878 Genes).

Supplemental Data Set 6. Genes Induced at FR1 1.75- to Twofold That Are SSTF Induced at FR3 (55 Genes).

Supplemental Data Set 7. Genes Repressed at FR1 1.75- to Twofold That Are SSTF Repressed at FR3 (60 Genes).

Supplemental Data Set 8. Genes Induced or Repressed 1.75- to Twofold in This Study That Overlap with Those Reported by Carabelli et al. (2007) (Six Genes).

Supplemental Data Set 9. SSTF-FR1–Induced Genes Exhibiting a Statistically Significant Difference in Expression between Wild-Type and *pifq* Seedlings at FR1 (103 Genes).

Supplemental Data Set 10. SSTF-FR1–Repressed Genes Exhibiting a Statistically Significant Difference in Expression between Wild-Type and *pifq* Seedlings at FR1 (20 Genes).

Supplemental Data Set 11. FR1 Shade-Responsive Genes Exhibiting No Statistical Differences between the Wild Type and *pifq* (26 Genes).

Supplemental Data Set 12. FR1 Shade-Responsive Genes with a Preexisting Difference between Genotypes at WL0 (20 Genes).

Supplemental Data Set 13. SSTF Shade-Responsive Genes at FR3 Exhibiting a Statistically Significant Difference in Expression between Wild-Type and *pifq* Seedlings at FR3 (579 Genes).

Supplemental Data Set 14. SSTF Shade-Responsive Genes at FR3 Displaying No Statistical Difference between Wild-Type and *pifq* Seedlings (249 Genes).

Supplemental Data Set 15. FR3 Shade-Responsive Genes with a Preexisting Difference between Wild-Type and *pifq* Seedlings at WL0 (50 Genes).

Supplemental Data Set 16. Expression Data and Primary Analysis for All Shade-Induced Genes Identified in Supplemental Figure 4B Online (626 Genes).

Supplemental Data Set 17. Expression Data and Primary Analysis for All Shade-Repressed Genes Identified in Supplemental Figure 4C Online (644 Genes).

Supplemental Data Set 18. SSTF FR1-Induced Genes Arrayed by Dependence on PIF by Fold Induction Ratio and Sorted by G-Box Presence/Absence and Functional Category (103 Genes).

Supplemental Data Set 19. Expression Data and Primary Analysis for All Non-PIF-Dependent Early Shade-Induced Genes.

Supplemental Data Set 20. Primary Data for Meta-Analysis Derived from Deetiolation and Shade Response Studies (22,207 Genes; ATH1 Array).

Supplemental Data Set 21. Deetiolation Class 7/Meta-Analysis Class M: Genes That Display Robust, Rapid, Reciprocal Responsiveness to the Onset of Light (Rc) and Shade (Supplemental FRc) Signals, Respectively (14 Genes).

Supplemental Data Set 22. Deetiolation Class 7/Meta-Analysis Class L: Genes That Display Robust, Rapid Responsiveness to the Onset of Light (Rc) but No Reciprocal Response to Shade (Supplemental FRc) Signals (42 Genes).

Supplemental Data Set 23. Deetiolation Class 7/Meta-Analysis Class R: Genes That Display Robust, Rapid Responsiveness to the Onset of Shade (Supplemental FRc) Signals but Do Not Show a Reciprocal Responsiveness to the Onset of Light (Rc) (89 Genes).

Supplemental Data Set 24. Deetiolation Class 3/Meta-Analysis Class M: Genes That Display Robust, Rapid, Reciprocal Responsiveness to the Onset of Light (Rc) and Shade (Supplemental FRc) Signals, Respectively (Eight Genes).

Supplemental Data Set 25. Deetiolation Class 3/Meta-Analysis Class L: Genes That Display Robust, Rapid Responsiveness to the Onset of Light (Rc) but No Reciprocal Response to Shade (Supplemental FRc) Signals (37 Genes).

Supplemental Data Set 26. Deetiolation Class 3/Meta-Analysis Class R: Genes That Display Robust, Rapid Responsiveness to the Onset of Shade (Supplemental FRc) Signals but Do Not Show a Reciprocal Responsiveness to the Onset of Light (Rc) (95 Genes).

Supplemental Data Set 27. Deetiolation Class 6/Meta-Analysis Class M: Genes That Display Robust, Rapid, Reciprocal Responsiveness to the Onset of Light (Rc) and Shade (Supplemental FRc) Signals, Respectively (Eight Genes).

Supplemental Data Set 28. Deetiolation Class 6/Meta-Analysis Class L: Genes That Display Robust, Rapid Responsiveness to the Onset of Light (Rc) but No Reciprocal Response to Shade (Supplemental FRc) Signals (21 Genes).

Supplemental Data Set 29. Deetiolation Class 6/Meta-Analysis Class R: Genes That Display Robust, Rapid Responsiveness to the Onset of Shade (Supplemental FRc) Signals but Do Not Show a Reciprocal Responsiveness to the Onset of Light (Rc) (95 Genes).

Supplemental Data Set 30. Deetiolation Class 1/Meta-Analysis Class M: Genes That Display Robust Repression with Respect to Dark Controls under Continuous Light Conditions (Rc) and Rapid Induction in Response to Shade (Supplemental FRc) Signals, Respectively (18 Genes).

Supplemental Data Set 31. Deetiolation Class 1/Meta-Analysis Class L: Genes That Display Robust Repression with Respect to Dark Controls under Continuous Light Conditions (Rc) and Do Not Respond to Shade (Supplemental FRc) signals, respectively (322 Genes).

Supplemental Data Set 32. Deetiolation Class 1/Meta-Analysis Class R: Genes That Do Not Display Responsiveness with Respect to Dark Controls under Continuous Light Conditions (Rc) and Are Rapidly Induced in Response to Shade (Supplemental FRc) Signals, Respectively (85 Genes).

Supplemental Data Set 33. Deetiolation Class 4/Meta-Analysis Class M: Genes That Display Robust Repression with Respect to Dark Controls under Continuous Light Conditions (Rc) and Rapid Induction in Response to Shade (Supplemental FRc) Signals, Respectively (11 Genes).

Supplemental Data Set 34. Deetiolation Class 4/Meta-Analysis Class L: Genes That Display Robust Repression with Respect to Dark Controls under Continuous Light Conditions (Rc) and Do Not Respond to Shade (Supplemental FRc) Signals, Respectively (243 Genes).

Supplemental Data Set 35. Deetiolation Class 4/Meta-Analysis Class R: Genes That Do Not Display Responsiveness with Respect to Dark Controls under Continuous Light Conditions (Rc) and Are Rapidly Induced in Response to Shade (Supplemental FRc) Signals, Respectively (92 Genes).

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AUTHOR CONTRIBUTIONS

P.L. and J.M.T. designed and performed the research, contributed new analytical tools, analyzed the data, and wrote the article. M.M.C., E.M., B.A.-S., and E.E. performed the research and analyzed the data. P.H.Q. designed the research and wrote the article.

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