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Functional Profiling Reveals That Only a Small Number of Phytochrome-Regulated Early-Response Genes in *Arabidopsis* Are Necessary for Optimal Deetiolation [W]

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In previous time-resolved microarray-based expression profiling, we identified 32 genes encoding putative transcription factors, signaling components, and unknown proteins that are rapidly and robustly induced by phytochrome (phy)-mediated light signals. Postulating that they are the most likely to be direct targets of phy signaling and to function in the primary phy regulatory circuitry, we examined the impact of targeted mutations in these genes on the phy-induced seedling deetiolation process in *Arabidopsis thaliana*. Using light-imposed concomitant inhibition of hypocotyl and stimulation of cotyledon growth as diagnostic criteria for normal deetiolation, we identified three major mutant response categories. Seven (22%) lines displayed statistically significant, reciprocal, aberrant photoresponsiveness in the two organs, suggesting disruption of normal deetiolation; 13 (41%) lines displayed significant defects either unidirectionally in both organs or in hypocotyls only, suggesting global effects not directly related to photomorphogenic signaling; and 12 (37%) lines displayed no significant difference in photoresponsiveness from the wild type. Potential reasons for the high proportion of rapidly light-responsive genes apparently unnecessary for the deetiolation phenotype are discussed. One of the seven disrupted genes displaying a significant mutant phenotype, the basic helix-loop-helix factor–encoding *PHYTOCHROME-INTERACTING FACTOR3-LIKE1* gene, was found to be necessary for rapid light-induced expression of the photomorphogenesis- and circadian-related *PSEUDO-RESPONSE REGULATOR9* gene, indicating a regulatory function in the early phy-induced transcriptional network.

INTRODUCTION

Reverse genetics disruption of target genes provides a centrally important tool for examining the consequences of the loss of gene function. This strategy has been used in multiple model eukaryotes in a range of contexts, from investigation of specific individual genes to global systematic assessment of all genes in the genome (Krysan et al., 1999; Giaever et al., 2002; Kamath and Ahringer, 2003; Carpenter and Sabatani, 2004; Kuepfer et al., 2005; Sönnichsen et al., 2005). The majority of genome-scale studies reported to date have focused on probing the functional roles of multiple sequence-related members of selected gene families of interest. In yeast, however, genes identified by microarray-based, genome-wide expression profiling as exhibiting significant increases in expression in response to different imposed stress conditions have been assessed in parallel for their functional necessity to the cell's capacity to respond and grow optimally under those conditions (Giaever et al., 2002). This was assessed by coculturing all members of a genome-wide set of gene-disruption mutants under each stress condition and monitoring the fitness of each mutant for growth and survival using gene bar code tags. Strikingly, only 0.3 to 7% of the genes that displayed stress-induced expression changes were functionally necessary for optimal growth under the relevant stress imposed (Giaever et al., 2002). Genome-wide RNA interference screens have been conducted in *Caenorhabditis elegans* to identify genes whose silencing causes observable growth phenotypes, but no correlation with the expression of these genes was examined (Kamath and Ahringer, 2003; Sönnichsen et al., 2005). In *Arabidopsis thaliana*, the available disruptional mutant collections (Syngenta Arabidopsis Insertion Library [SAIL] and SIGnAL) have been used to assess the functional relevance of numerous individual genes to a variety of cellular and developmental processes as well as to explore the potential functions of the multiple sequence-related members of some of the various gene families present in plants (Kranz et al., 1998; Meissner et al., 1999; Okushima et al., 2005). However, except for a limited study of four ethylene-response genes (Alonso et al., 2003) and three salicylic acid–induced genes (Wang et al., 2005), there have been no reports of the systematic global analysis of genes identified by expression profiling as coordinately induced by specific environmental or hormonal signals for functional relevance to the

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plant's response to these signals. Only very recently, one expression profiling study examined 16 genes in *Arabidopsis* that respond to developmental cues associated with secondary cell wall formation and found that 7 of these genes are apparently functionally involved in secondary cell wall biosynthesis by reverse genetics (Brown et al., 2005).

The *Arabidopsis* phytochrome (phy) family (designated phyA to phyE) of red (R) and far-red (FR) light sensory photoreceptors regulates growth and developmental transitions in response to light signals throughout the plant life cycle (Quail, 2002a, 2002b). Studies with mutants lacking functional photoreceptor proteins have shown that the different phy family members exhibit differential, albeit partially overlapping, photosensory and/or physiological functions in controlling plant light responses (Whitelam and Devlin, 1997; Devlin et al., 1998; Quail, 1998; Tepperman et al., 2004, 2006). Although phyA is exclusively responsible for photoresponsiveness to continuous monochromatic far-red (FRc) light, phyB has been considered to be predominantly responsible for photoresponsiveness to continuous monochromatic red (Rc) light (Quail et al., 1995; Fankhauser and Chory, 1997; Whitelam and Devlin, 1997; Quail, 1998; Whitelam et al., 1998; Neff et al., 2000; Chen et al., 2004; Franklin and Whitelam, 2004). Strikingly, however, recent microarray expression profiling has revealed that phyA, and not phyB, dominates in transducing Rc signals to rapidly responding genes at the initiation of seedling deetiolation (Monte et al., 2004; Tepperman et al., 2006)

Seedling deetiolation is marked by concomitant light-induced inhibition of hypocotyl cell elongation and reciprocal promotion of cotyledon cell expansion (Quail, 2002a). Genetic screens for mutant seedlings exhibiting hyposensitivity or hypersensitivity to light inhibition of hypocotyl cell elongation in response to FRc and Rc light have identified numerous loci putatively involved in phy signaling pathways (Quail, 2002a, 2002b; Gyula et al., 2003; Kaczorowski and Quail, 2003; Wang and Deng, 2003; Chen et al., 2004; Franklin and Whitelam, 2004; Huq and Quail, 2005; Zhou et al., 2005). These forward genetics screens have been very useful but appear to have been carried to near saturation, perhaps because in practice they are limited to identifying mutations that cause relatively large phenotypic differences that are easily observable when comparing hypocotyl length. However, mutations in the *Arabidopsis* genome representing all of the genes necessary to the optimal functioning of the phy signaling pathway may be far from saturated. Disruptions of some genes may result in obscured phenotypes or may exhibit only weak phenotypes as a result of possible partial functional redundancy with other closely related gene family members (Zhou et al., 2005). Reverse genetics can help overcome this problem by targeting genes of interest and studying the effects of loss of function independently or in combination with the loss of function of one or more other family members (Ostergaard and Yanofsky, 2004).

Using Affymetrix ATH1 microarray genome-scale expression profiling (22,000 genes), we have identified a set of genes regulated by the phy system in response to light signals during seedling deetiolation (Monte et al., 2004; Tepperman et al., 2006). These data confirm and extend the list of genes identified as robustly light-responsive in earlier studies using the more limited 8000-gene Affymetrix microarray (Tepperman et al., 2001, 2004). Based on the rationale that genes responding most rapidly to the light signals are those most likely to be direct targets of phy signaling and to have a functionally significant role in regulating downstream responses, we have focused on genes that respond within 1 h of initial exposure of etiolated seedlings to light (defined as early-response genes) for further analysis. Of the large number of genes identified as responding rapidly to light in a statistically significant manner by limma analysis at a false discovery rate P value of 0.05 (Gentleman et al., 2004; Smyth, 2004; see http://www.bioconductor.org/), we have chosen to focus on the subset exhibiting the quantitatively greatest response to the light signal (defined as greater than twofold induction or repression relative to unirradiated controls). Sequential application of these dual statistical and response-magnitude criteria, coupled with RNA gel blot validation of multiple selected examples, has resulted in the identification of ~250 early-response genes defined as robustly light-regulated (Monte et al., 2004; Tepperman et al., 2006). The data show that phyA dominates in eliciting the early-light-induced responsiveness of these genes (Tepperman et al., 2006).

To begin to determine whether these genes have functionally relevant roles in transducing phy signals in the overall, global process of seedling deetiolation, we have selected here an initial subset of 32 genes categorized as having potential transcriptional-regulatory or signaling activity, as well as several unannotated sequences, for systematic analysis by reverse-genetic disruption. For this purpose, we have isolated and/or characterized for photomorphogenic defects insertional-disruption or other known mutants in these genes. These include five previously reported mutants characterized to date only for hypocotyl length, and not cotyledon expansion, and therefore lacking definitive phenotypic evidence of involvement at early steps in the complete seedling deetiolation process.

RESULTS

Isolation of T-DNA Insertional Mutants in Early-Response Genes

Table 1 lists the genes studied here and the information relevant to the mutant *Arabidopsis* lines obtained for each of these loci. Supplemental Figure 1 online shows the early Rc light–regulated expression by RNA gel blot of two genes (induced, At3g21150; repressed, At3g21330) that were not identified in these studies because they are not present on the microarrays used previously but were included here for analysis (Tepperman et al., 2006).

The presence of a disrupting T-DNA sequence was confirmed for each previously unreported mutant by PCR. Cosegregation of the T-DNA with the phenotype (where detectable) was established, and homozygous mutant lines were selected. The homozygous mutant lines isolated in this study were subjected to RT-PCR or RNA gel blot analysis to confirm the disruption of gene expression caused by the inserted T-DNA (see Supplemental Figures 2 and 5 online; summarized in Table 1). No transcripts were detected in any except three of the mutant lines tested. Two of these lines displayed aberrant (larger) transcripts at levels lower than or about the same as those found in the wild-type seedlings (gene 25, At3g21330; gene 26, At4g16780; see Supplemental Figure 2 online), whereas the third displayed greatly reduced mRNA levels (gene 11, At5g37260; see Supplemental

Table 1. Functional Profiling of phy-Regulated Early-Response Genes for Photomorphogenic Phenotypes

(Class) Numbers	Arabidopsis Genome Initiative Number	Protein Name Designation	Functional Category	T-DNA Line (in This Study)	Mutant Line Designation	Insertion/ Transcript	Hypocotyl Length FRc	Hypocotyl Length Rc	Cotyledon Area (Rc)	Reported Function for This Locus
(I) Aberrant photomorphogenic phenotype displayed										
1	At2g46790^{AI}	APRR9	Transcription		*prr9-1*[1]	null[1]	None	Tall	Small	Circadian rhythms
2	At2g46970^{AR}	PIL1	Transcription	SALK_025372	*pil1-2*	Null^	Tall	Tall	Small	
					pil1-1[2]	Null[2]	Hyposensitive in R, FR, and Low R/FR			Shade avoidance[2]
3	At5g11260^{AI}	HY5	Transcription	SAIL_19_G04	*hy5-101*	S+26^	Tall	Tall	Small	
					hy5-1[3,4]	Null[3,4]	Hyposensitive in R, FR, B, WL			Transcription
4	At2g02950^{AI}	PKS1	Signaling	SAIL_828_H11	*pks1-101*	−552^	Short	Short	Large	
					pks1[5]	Null[5]	phyA VLFR in FRp			phyA signaling[5]
5	At2g46340^{AI}	SPA1	Signaling		*spa1-3*^{RLD,6,7}	Null[6,7]	Short	Short	Large	Suppressor of phyA[6,7]
6	At2g37970^{AI}	SOUL family	Unknown	SAIL_1280_E03	*soul1*	−35^	None	Short	Large	ND
7	At2g40080^{AI}	ELF4	Signaling	SAIL_1244_G01	*elf4-101*[8]	Null[8]	None	Tall	Small	phyB signaling[8]
(II) Parallel, unidirectional defect in both hypocotyl and cotyledon photoresponsiveness displayed										
8	At3g10910^{AI}	RING-ZFN	Transcription	SAIL_256_C02		418^	None	Short	Small	ND
9	At3g21150^{AI}	B-box type ZF	Transcription	SALK_059534		670^	None	Short	Small	ND
10	At5g24120^{AI}	SIGE	Transcription	SALK_141383		383^	Short	Short	Small	ND
11	At5g37260^{AI}	CCA1-L	Transcription	SALK_074896		1192^	Tall	Tall	Large	ND
12	At1g78820^{AI}	Putative EP1	Signaling	SAIL_639_F10		1110^	Short	Short	Small	ND
13	At2g20750^{AR}	β-Expansin	Growth	SAIL_345_G11		519^	None	Short	Small	Loosen cell wall
14	At1g61890^{AI}	MATE	Unknown	SAIL_749_F06		−211^	Short	Short	Small	ND
(III) Aberrant photoresponsive phenotype in hypocotyl only displayed										
15	At1g01060^{AI}	LHY	Transcription		*lhy-101*	Null	Short	Short	None	
					lhy-11, *-12*, *-13*^{le,9}	LoF[9]	Same as wild-type in LD conditions			Circadian rhythms
16	At2g31380^{AI}	STH (ZF3)	Transcription	SAIL_786_F08		−442^	Short	Short	None	ND
17	At3g47500^{ABI}	Dof-type ZF	Transcription	SAIL_434_G09		651^	None	Short	None	ND
18	At4g25480^{AI,BI}	CBF3	Transcription	SAIL_244_D02		48^	None	Short	None	Cold tolerance[10]
19	At2g42870^{AR}	Expressed	Unknown	SAIL_668_E10		S+10^	Short	Short	None	ND
20	At4g14690^{AI}	ELIP1	Unknown	SAIL_667_C04		1316^	Short	Short	None	Photoprotection[11]
(IV) No aberrant seedling phenotype displayed										
21	At1g18330^{AI}	EPR1[12]	Transcription	SAIL_195_F11		602^	None	None	None	Slave oscillator[12]
22	At2g44910^{AR}	ATHB4[13]	Transcription	SALK_104843		1365^	None	None	None	Shade avoidance[13]
23	At2g46830^{AI}	CCA1	Transcription		*cca1-1*^{w,14}	Null[14]	None	None	None	Circadian rhythms[14]
24	At3g02380^{AI}	COL2[15]	Transcription	SAIL_70_F03		−258^	None	None	None	Circadian-regulated[15]
25	At3g21330^{AR}	bHLH087[16]	Transcription	SALK_066339		487^	None	None	None	ND
26	At4g16780^{AR}	HAT4[17]	Transcription	SALK_106790		162^	None	None	None	Development[17]
27	At5g44260^{AR}	Putative ZF	Transcription	SAIL_1277_D05		360^	None	None	None	ND
28	At1g78070^{AI}	WD-40 protein	Signaling	SAIL_1264_G10		−4^	None	None	None	ND
29	At2g30040^{AI}	Putative PK	Signaling	SAIL_1175_F12		981^	Short*	None	None	ND
30	At4g26850^{AI}	VTC2	Unknown	SAIL_769_H05		620^	None	None	None	ND
31	At4g27520^{AI}	Plastocyanin-L	Unknown	SAIL_437_B03		−206^	None	None	None	ND
32	At5g52250^{AI}	COP1-L	Unknown	SALK_060638		298^	None	None	None	ND

List of 32 genes analyzed, including Arabidopsis Genome Initiative loci and designated protein names. The corresponding mutant lines isolated in this work (SAIL or SALK lines) and previously identified mutant lines are indicated. The predicted T-DNA insertion sites within the respective genes, relative to the start codon, are shown. In some mutant lines, the T-DNA insertion sites were upstream of the start codon (−) or downstream of the stop codon (S+). Based on the statistical significance (see Supplemental Figures 3 and 4 online) of differences observed in hypocotyl length and cotyledon area between wild-type and mutant seedlings grown in Rc light, genes were grouped into four classes, as indicated. Functions previously reported for some of the genes are listed. One of the lines (line 29) displays a short-hypocotyl phenotype only in FRc light (asterisk). ^, transcript not detected in the mutant (see Supplemental Figures 2 and 5 online). AI, phyA early-induced genes; AR, phyA early-repressed genes; BI, phyB early-induced gene; ABI, phyA/B early-induced gene; R, red light; FR, far-red light; B, blue light; WL, white light; FRp, far-red pulse; VLFR, very-low-fluence response; LD, long day (16-h-light/8-h-dark cycles); Lof, loss of function; ND, not determined; *le*, Landsberg *erecta* (wild type); rld, RLD (wild type); w, Wassilewskija (wild type). References are designated as follows: 1, Eriksson et al. (2003); 2, Salter et al. (2003); 3, Koornneef et al. (1980); 4, Ang et al. (1998); 5, Lariguet et al. (2003); 6, Hoecker et al. (1998); 7, Hoecker et al. (1999); 8, Khanna et al. (2003); 9, Mizoguchi et al. (2002); 10, Gong et al. (2002); 11, Hutin et al. (2003); 12, Kuno et al. (2003); 13, Carabelli et al. (1993); 14, Green and Tobin (1999); 15, Ledger et al. (2001); 16, Bailey et al. (2003); 17, Schena et al. (1993).

Figure 2 online; Table 1). Mutant lines for genes 25 and 26 exhibited no detectable seedling phenotypes. The larger sizes of the transcripts detected in both of these mutant lines are consistent with the predicted T-DNA insertion sites at 487 and 162 bp downstream from the start codons, respectively (Table 1; see Supplemental Figure 2 online), coupled with transcriptional read-through into the T-DNA sequence. Therefore, these mutant lines likely lack functional proteins. Of the remaining 29 lines, 6 (*pseudo-response regulator9* [*prr9-1*], *pil1-1*, *spa1-3*, *elf4-101*, *lhy-11*, and *cca1-1*) have been reported previously as null for

gene expression (Table 1). Analysis of the remaining 23 lines showed no detectable transcript for any, suggesting that these are also likely null mutants (see Supplemental Figure 2 online; Table 1). Wild-type siblings were maintained and used for initial phenotypic characterizations using T3, T4, and T5 populations. Any phenotypes observed in homozygous lines but not observed in their wild-type siblings were compared and confirmed with ecotype Columbia (Col-0; wild-type) seedlings.

Functional Profiling of phy-Regulated Early-Response Genes

The 32 mutated loci investigated here were analyzed for statistically significant differences from the wild type in hypocotyl and cotyledon responsiveness to light signals. These statistical data are summarized in the volcano plots in Supplemental Figures 3 and 4 online. We defined seedling deetiolation phenotypes as concomitant, reciprocal deviations in light-regulated hypocotyl and cotyledon cell expansion relative to the wild type. Seedlings exhibiting both longer hypocotyls and smaller cotyledons than the wild type in light were classified as having a hyposensitive deetiolation phenotype in response to the light signal, whereas those exhibiting both shorter hypocotyls and larger cotyledons than the wild type in light were classified as having a hypersensitive deetiolation phenotype in response to the light signal. Seedlings exhibiting parallel, unidirectional deviant growth rates in both organs (i.e., both shorter hypocotyls and smaller cotyledons or taller hypocotyls and larger cotyledons) in light were considered to be more globally defective in cell expansion processes not necessarily specifically involved in the light signaling pathways that differentially regulate the rate and extent of the normal cell expansion process in these different organs.

Of these 32 loci, mutations in 20 genes caused apparent seedling phenotypes, at least in hypocotyl length, that ranged in magnitude from moderate to marginal. The primary quantitative data for these 20 mutant lines are shown in Figure 1. Of these 20 genes, disruptions of 7 caused concomitant, reciprocal defects in light-induced hypocotyl and cotyledon responses (class I, Table 1), another 7 caused nonreciprocal (global) defects in cell expansion processes in both hypocotyls and cotyledons (class II, Table 1), and 6 caused hypocotyl-only defects (class III, Table 1). The remaining 12 lines showed no detectable seedling phenotypes (class IV, Table 1). The visible seedling photomorphogenic phenotypes for the 7 class I mutants are shown in Figure 2. The data for all 32 mutant lines are summarized in Figure 3, which plots hypocotyl length against cotyledon area in Rc light for each line normalized to the wild type set at unity. Also included to provide a frame of reference for this plot are data for dark-grown wild-type and light-grown *phyB*-null mutant seedlings demarcating the values for the fully etiolated state and the maximally deetiolation-defective Rc phenotype reported to date, respectively, as well as data for a phyB-overexpressor (estimated from the data of Wagner et al., 1991), depicting values expected of a strong hypersensitive deetiolation phenotype. These data demonstrate the clear reciprocal relationship between hypocotyl and cotyledon cell expansion in the wild type in response to Rc light and the potential extremes in hyposensitive and hypersensitive light-responsive phenotypes that might be expected for severe mutations in the central light-signaling machinery.

The data in Figure 3 depict the three main categories of phenotypes observed for the T-DNA insertional mutant lines. (1) The 12 lines (including line 29, which exhibits a phenotype only in FRc light) with values clustered tightly around the wild type, at unity on both scales, showing no statistically significant effects on Rc responsiveness (see class IV, lines 21 to 32, in Table 1 for gene list). (2) The 13 lines showing non-organ-reciprocal effects on seedling growth, either globally on both hypocotyl and cotyledon (class II, Table 1) or on hypocotyl alone (class III, Table 1). Of these, 12 lines display significantly shorter hypocotyls than the wild type in Rc light coupled with either significantly smaller cotyledons (six lines) (class II, Table 1) or no significant difference from the wild type in cotyledon area (six lines) (class III, Table 1). The former six lines suggest general light-induced seedling growth defects. The latter six lines do not appear to represent lesions at early, central steps in the phy signaling system but could be involved in hypocotyl-specific defects either in phy signaling or in general growth processes not related to phy signaling. One line (line 11, class II, Table 1), displays significantly taller hypocotyls coupled with significantly larger cotyledons than the wild type, suggesting a generally enhanced cellular expansion in both organs, not necessarily related to phy signaling. (3) The seven lines displaying either hyposensitive (four lines) or hypersensitive (three lines) reciprocal phenotypes in hypocotyls and cotyledons in response to Rc light (class I, Table 1), indicative of a disruption early in the normal, overall deetiolation process.

Of the seven early-response loci defined here as having functions in the overall deetiolation process, six (*PRR9*, *PIL1*, *HY5*, *PKS1*, *SPA1*, and *ELF4*) had either been reported previously, or were reported during this study, to be involved in light signaling (Koornneef et al., 1980; Ang et al., 1998; Hoecker et al., 1998, 1999; Fankhauser et al., 1999; Eriksson et al., 2003; Khanna et al., 2003; Lariguet et al., 2003; Salter et al., 2003), whereas the *SOUL-1* locus is newly identified here. Despite these early reports, with the exception of our work on *ELF4* (Khanna et al., 2003), no quantitative measurements of the effects of mutations at these loci on concomitant hypocotyl and cotyledon light-responsiveness have been documented. These previous studies described light-dependent defects in hypocotyl cell elongation only. Insertional mutations in *PRR9* have been reported to result in tall (hyposensitive) hypocotyls in response to Rc light (Eriksson et al., 2003). PIL1 and HY5 have been shown to be necessary for optimal responsiveness in hypocotyl elongation to both FRc and Rc light treatment (Koornneef et al., 1980; Ang et al., 1998; Salter et al., 2003). PKS1 and SPA1 have been implicated in phyA signaling (Table 1) (Hoecker et al., 1998, 1999; Fankhauser et al., 1999; Lariguet et al., 2003). We have isolated new mutant alleles at three of these loci, *pil1-2*, *hy5-101*, and *pks1-101*, and have obtained the previously reported mutants *prr9-1* and *spa1-3* (Hoecker et al., 1998; Eriksson et al., 2003). The effects of disruptions at these five loci on cotyledon area expansion, as well as hypocotyl elongation, are reported in Table 1, Figures 1 to 3, and Supplemental Figures 3 and 4 online. The new mutant alleles, *pil1-2*, *hy5-101*, and *pks1-101*, show photomorphogenic phenotypes in both FRc and Rc light, in addition to

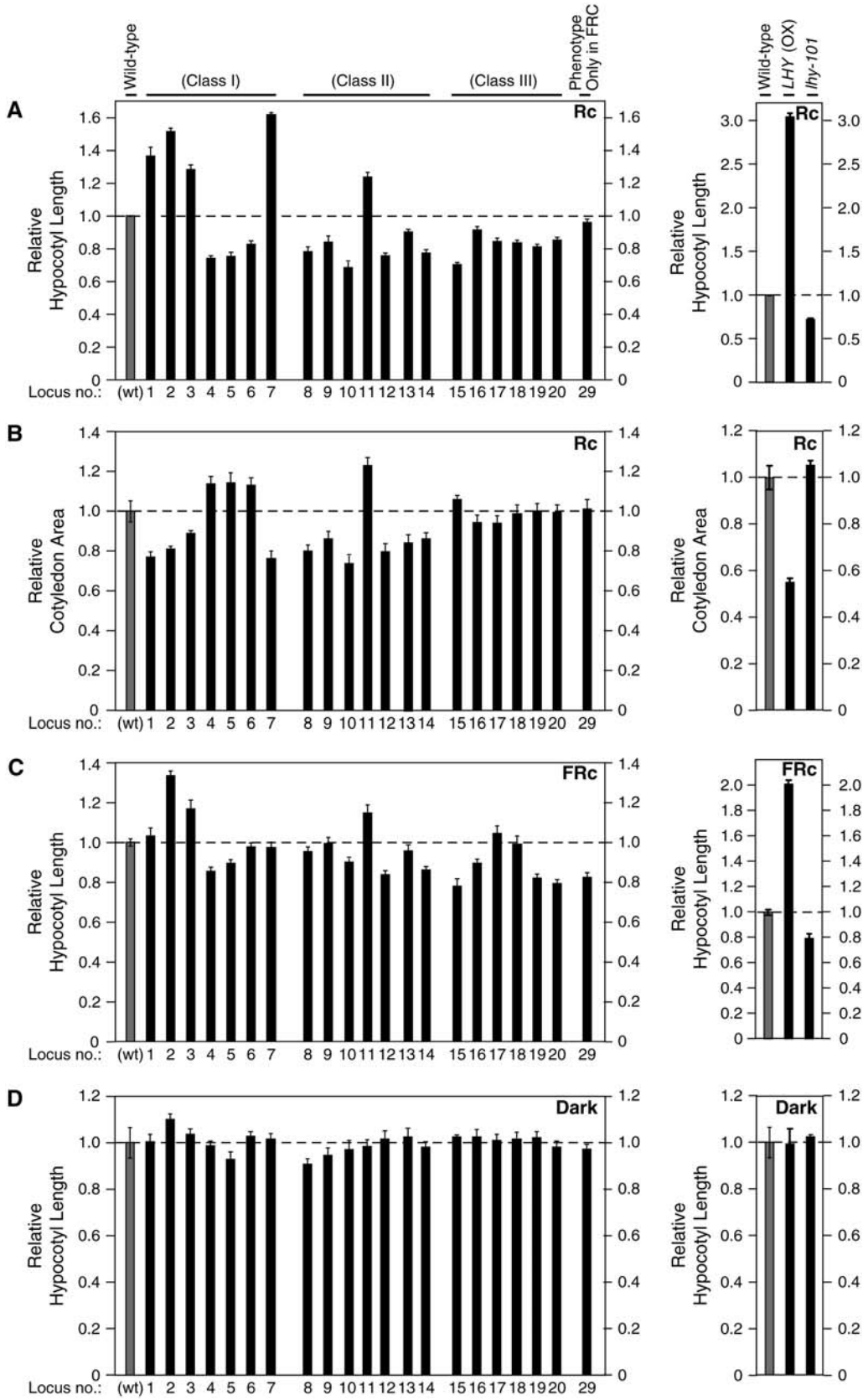

Figure 1. Hypocotyl Length and Cotyledon Area Displayed by 4-d-Old Mutant Seedlings Relative to the Wild Type.

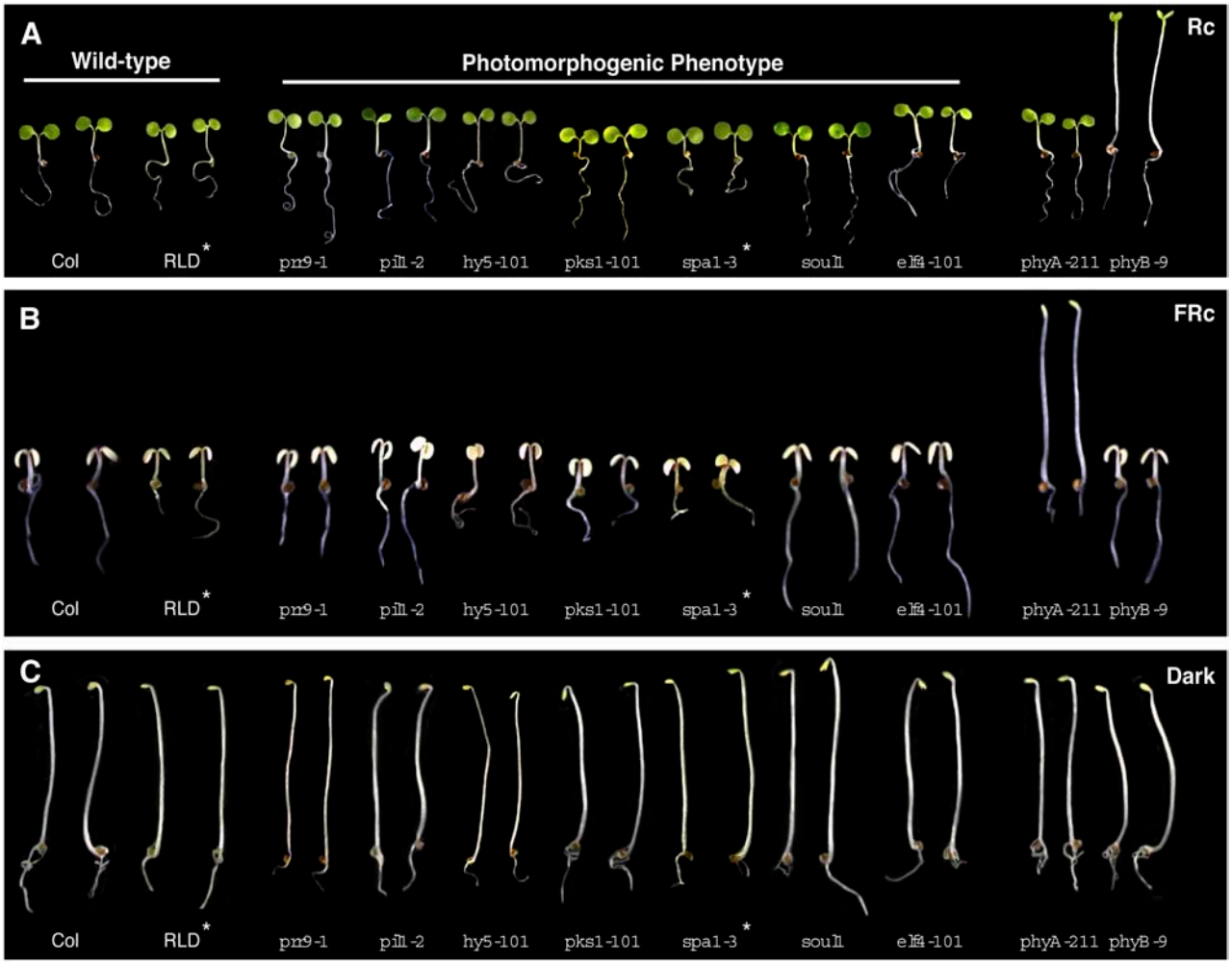

Figure 2. Wild-Type and Mutant Seedlings Grown for 4 d in Rc Light, FRc Light, or Darkness.

The *phyA-211* and *phyB-9* mutants are shown for reference for comparison with the extent of the phenotypes observed in the other mutant seedlings. Note that the *spa1-3* mutation is in the RLD background (asterisks), whereas the remainder are in the Col background.
(A) Rc light.
(B) FRc light.
(C) Darkness.

and/or consistent with previously reported phenotypes (Table 1, Figures 1 to 3; see Supplemental Figures 3 and 4 online). One exception is that the previously examined *pks1* allele was reported to exhibit no phenotype when irradiated hourly with R light pulses (Lariguet et al., 2003). Because both alleles appear to be null, this discrepancy might have arisen from the difference in irradiation regimes in the two studies, as we used Rc light in this study.

We have isolated and characterized a new T-DNA insertional mutant allele for another previously studied gene, *lhy-101*, and have reexamined this mutant along with an original allele for *LHY*,

Figure 1. (continued).

(A) Relative hypocotyl lengths of mutant seedlings grown in Rc light.
(B) Relative cotyledon areas of mutant seedlings grown in Rc light.
(C) and **(D)** Relative hypocotyl lengths of mutant seedlings grown in FRc light **(C)** or darkness **(D)**.
Thirty seedlings for each line were used for measurements, values were normalized to Col, and SE values were determined. Data are shown for mutant seedlings categorized as belonging to classes I, II, and III (see Table 1 for definitions, gene list, and locus numbers). The *lhy-1* mutant (actually an overexpressor of *LHY*) is included for comparison with the new allele, *lhy-101* (right panels).

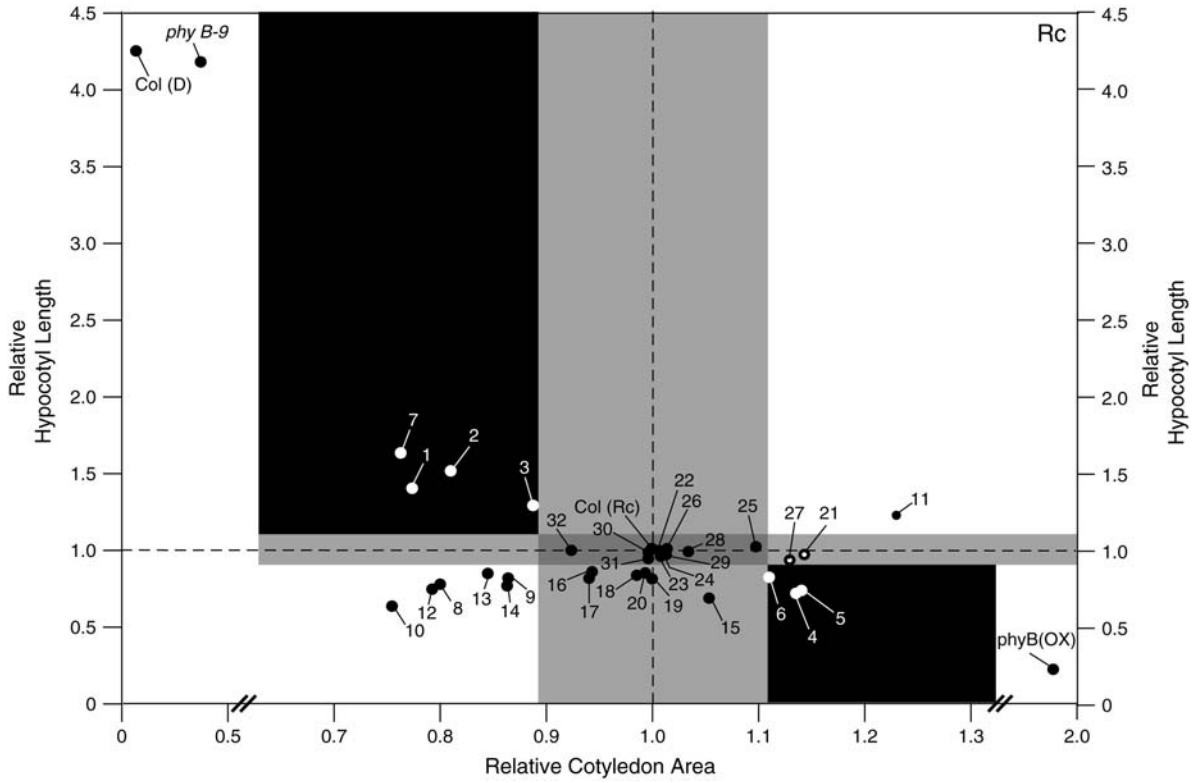

Figure 3. Quantitative Classification of Seedling Phenotypes Observed in All Mutant Lines.

Mean values of hypocotyl length (relative to the wild type set at unity) were plotted against the mean relative cotyledon areas (wild type set at unity) for each mutant line grown for 4 d in Rc light to ascertain the relationship between the cell elongation phenotypes displayed in the two different organs in response to the light signal. The statistical significance of differences in hypocotyl length and cotyledon area was determined by *t* test analysis of data from 30 seedlings (see Supplemental Figure 3 online). Mutant lines lacking statistically significant differences from the wild type (falling within the gray area) are indicated. Mutant lines that exhibit statistically significant, concomitant reciprocal defects in hypocotyl and cotyledon (photomorphogenic defects) are shown in white on black background. Three major categories of responses among mutant lines are observed: (1) seven lines (class I, Table 1) display aberrant deetiolation phenotypes (white on black background), of which 4 are hyposensitive (lines 1, 2, 3, and 7) and 3 are hypersensitive (lines 4, 5, and 6) in response to Rc light; (2) 13 lines (classes II and III, Table 1) display aberrant Rc light responsiveness that lacks the coordinated, reciprocal disruption of both hypocotyl and cotyledon cell expansion that defines normal deetiolation (including six lines [lines 8 to 10 and 12 to 14] that display significant parallel inhibition of both hypocotyl and cotyledon cell expansion, one line [line 11] that displays significant parallel enhancement of both hypocotyl and cotyledon cell expansion, and six lines [lines 15 to 20] that show significant inhibition of hypocotyl growth with no significant effect on cotyledon); (3) 12 lines (lines 20 to 32) display no significant effects of mutation on Rc light responsiveness (class IV, Table 1). Note that lines 21 and 27 (open circles) have greater than wild-type mean values for cotyledon area, although these values lack statistical significance for enhanced cotyledon cell expansion for these two lines (see Supplemental Figure 3 online). See Table 1 for gene list and assigned locus numbers.

lhy-1, for photomorphogenic phenotypes in response to Rc and FRc light (Table 1). Our data show that *lhy-1* (which actually overexpresses constant high levels of *LHY* transcript; Schaffer et al., 1998) is markedly hyposensitive to both FRc and Rc light treatments (Figure 1), consistent with previous reports (Schaffer et al., 1998). By contrast, our new *lhy-101* null mutant allele is hypersensitive in hypocotyl elongation and exhibits a small reciprocal enlargement in cotyledon area, albeit not statistically significant (Figures 1 to 3). The MYB-like factor CCA1 causes hyposensitive phenotypes under long-day growth conditions when overexpressed (Schaffer et al., 1998; Wang and Tobin, 1998). In our study, *cca1-1* did not exhibit any significant differences in seedling deetiolation from the wild type. A recent analysis of hypocotyl lengths of *lhy-11 cca1-1* double mutants showed that these seedlings developed significantly shorter hypocotyls under short-day growth conditions (Mizoguchi et al., 2005). However, no analysis of reciprocal defects in cotyledon expansion was reported in that study.

One of the genes tested, *At2g30040* (putative protein kinase), exhibited a short-hypocotyl phenotype only in FRc and not in Rc light (line 29, class IV, Table 1, Figures 1 and 3; see Supplemental Figures 3 and 4 online) and thus is potentially involved in phyA-mediated FRc light–induced seedling deetiolation. Disruptions in three genes cause phenotypes only in Rc light and not in FRc light (*PRR9*, *SOUL-1*, and *ELF4*, class I, Table 1, Figures 1 to 3; see Supplemental Figures 3 and 4 online). This set includes the previously reported *ELF4* (line 7). We previously isolated two independent T-DNA insertional mutant

lines, *elf4-101* and a weaker allele, *elf4-102*, during the initial phase of this work and determined that ELF4 is involved in Rc signaling (Khanna et al., 2003). Unlike *elf4-101*, a disruption mutation at the new locus *SOUL-1* resulted in hypersensitivity to Rc light (Table 1, Figures 1 to 3; see Supplemental Figures 3 and 4 online).

Further Characterization of *pil1* Mutants

PIL1 is exceptional in several ways among the early-light-responsive genes. First, whereas the majority of early-response genes are induced by the light signal, *PIL1* expression is repressed. Second, the rate and magnitude of this repression (≥10-fold reduction in transcript levels within 1 h; Figure 4C) is strikingly exceptional among this group. Finally, *PIL1* encodes a basic helix-loop-helix (bHLH) protein closely related in sequence to the phy-interacting bHLH, PIF3 (Toledo-Ortiz et al., 2003). Therefore, we initiated a more in-depth analysis of the potential functional role of this locus in phy-regulated development. In an independent study, Salter et al. (2003) identified *PIL1* as a negative shade-responsive gene in light-grown plants involved in circadian clock action, as well as in seedling deetiolation, based on studies with a single T-DNA insertion allele, *pil1-1*. Our own studies here on two additional independently isolated *pil1* alleles, *pil1-2* and *pil1-3* (see Supplemental Figure 5 online), in addition to the SAIL *pil1-1* allele used by these workers, confirm and extend their findings.

RNA gel blot analysis shows that all three alleles are likely null for *PIL1* expression (see Supplemental Figure 5 online). Fluence rate response curves show that all three alleles display both reduced hypocotyl inhibition and reduced cotyledon expansion in both Rc and FRc wavelengths compared with the wild type, indicating a moderate positive role in phy-induced seedling deetiolation (see Supplemental Figure 6 online; G. Toledo-Ortiz and P.H. Quail, unpublished data). Examination of *pil1* mutant development at other stages of the life cycle revealed that PIL1 might also have a function in regulating both vegetative and reproductive development in light-grown plants. The data show that *pil1* mutants display markedly longer petioles than wild-type plants when grown under low-intensity white light (see Supplemental Figure 7A online) and delayed flowering under short-day photoperiods (see Supplemental Figure 7B online). Because these data suggest that PIL1 may function in multiple phyB-regulated responses, we examined the possibility that PIL1 may exert its activity indirectly by regulating phyB levels. Protein gel blot analysis showed identical levels of phyB in *pil1* and wild-type seedlings (see Supplemental Figure 8 online), arguing against this possibility.

Because PIL1 is a bHLH protein, it is predicted to be localized to the nucleus. Transient expression of a PIL1:β-glucuronidase (GUS) fusion protein in leek (*Allium porrum*) epidermal cells showed that this protein localizes to the nucleus, consistent with this prediction (see Supplemental Figure 9 online). Based on its sequence, PIL1 is also predicted to bind the G box DNA sequence, CACGTG, found in many light-regulated genes (Toledo-Ortiz et al., 2003). To begin to define potential molecular phenotypes in *pil1* mutant seedlings, we selected a small subset of early-light-response genes with G box motifs in their promoters. Quantification of RNA gel blot assays showed that the *pil1* mutation does not significantly alter the expression patterns of the central oscillator genes *CCA1*, *LHY*, or *TOC1* (see Supplemental Figure 10 online). By contrast, this mutation does reduce the level of rapid, Rc light–induced expression of the phy-regulated early-response gene *PRR9* (Figure 4), a member of the *PRR/TOC1* family (Ito et al., 2003). These data suggest that PIL1 is involved in the phy-mediated induction of *PRR9* in Rc light. The rapid, reciprocal regulation of *PIL1* and *PRR9* expression (Figure 4C) may be consistent with such involvement (see Discussion).

DISCUSSION

The primary goal of this analysis was to determine whether and to what extent genes that respond rapidly to, and are therefore potential primary targets of, the light-induced phy signaling system exhibit overt functional necessity to the visibly monitorable seedling deetiolation process, as determined by targeted reverse genetics disruption of the gene. Genes identified as phy-regulated, early-response genes and assigned to transcriptional, signaling, or unknown functional categories in our microarray analyses (Tepperman et al., 2001, 2004, 2006; Monte et al., 2004) were selected as those most likely to have regulatory functions in phy signaling and transcriptional networks, and all available T-DNA insertional mutants for these loci were obtained. The well-established, coordinated reciprocal growth responses of hypocotyl and cotyledon cells induced by light signals (Quail, 2002a) were stringently applied as diagnostic criteria in defining loci most likely to be involved in early steps in the overall deetiolation process. Only mutants displaying statistically significant, light-induced, reciprocal deviations of hypocotyl and cotyledon cell expansion rates from those of the wild type (class I mutants, Table 1) are considered to provide evidence of potential defects in early steps of the phy signaling or transcriptional networks. Conversely, mutants displaying parallel, unidirectional perturbations of cell expansion responses in the two organs (class II mutants, Table 1; here, exhibiting significant inhibition [six mutants] or enhancement [one mutant] of both hypocotyl and cotyledon growth in light compared with the wild type) are considered more likely to be affected in general cell growth processes than in the upstream events specific to phy signaling. Mutants displaying statistically significant deviations in light-regulated growth of only one of the two organs (class III mutants, Table 1; here, all exhibiting stronger inhibition of hypocotyl extension than the wild type but no difference in cotyledon expansion) could represent organ-specific components involved in either normal phy signaling or more general cell growth processes. Distinguishing between these and other possible alternatives will require more extensive investigation. These mutants, therefore, were not included as representing likely contributors to phy signaling in this study.

The data indicate that only four of the genes newly mutagenized in this study exhibit significant perturbation of the synchronized parameters of the light-induced deetiolation process (class I, Table 1). One of these, *PKS1*, had been implicated previously in

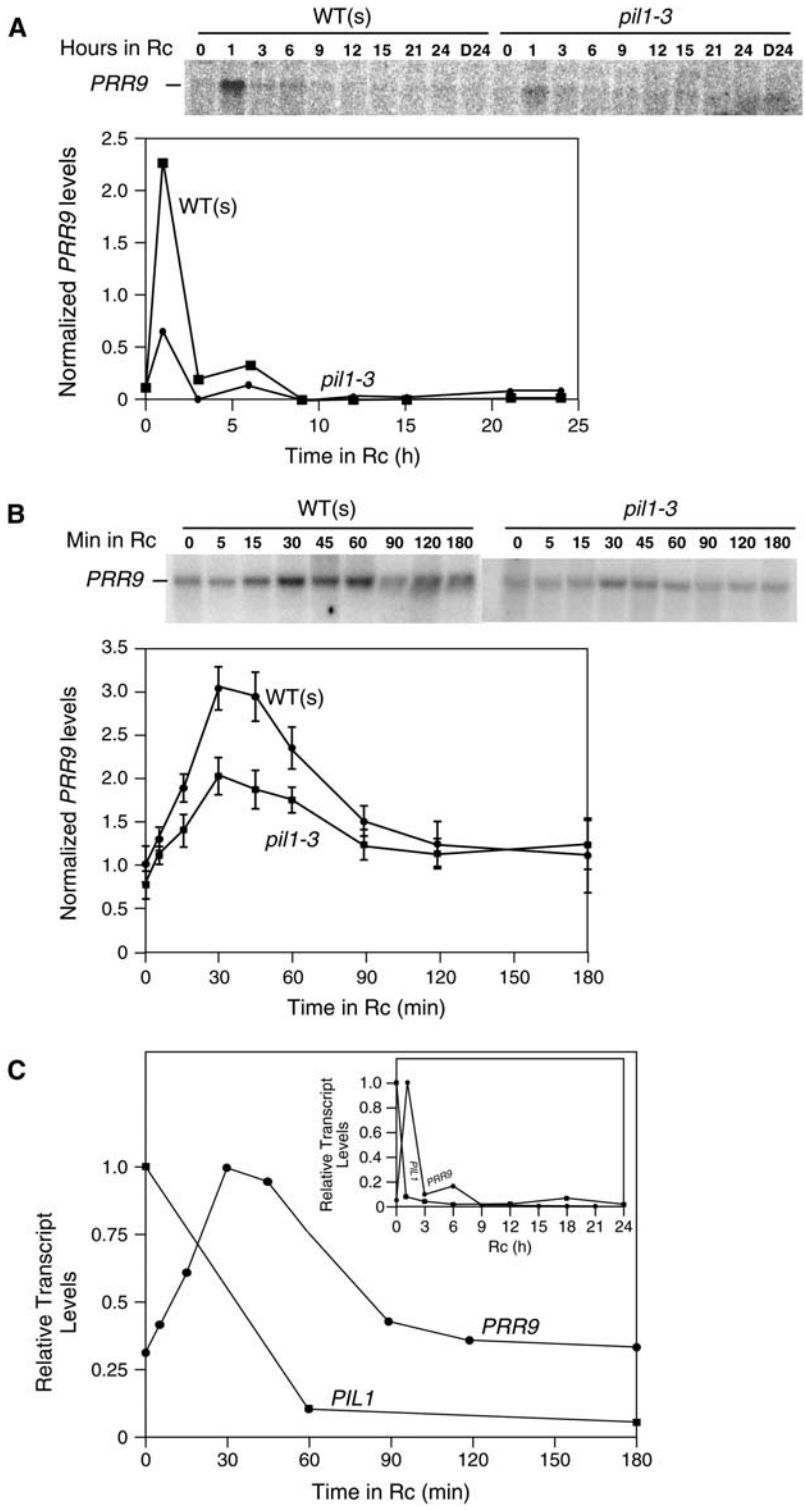

Figure 4. Rapid Rc Light–Induced Increase in *PRR9* Transcript Levels Is Reduced in *pil1* Mutant Seedlings.

(A) Time course of *PRR9* expression, as determined by RNA gel blot analysis, in seedlings treated with Rc light from 0 to 24 h after transfer from dark.
(B) Higher resolution, shorter time course (0 to 3 h of Rc light). *pil1* and wild-type sibling seedlings were grown for 96 h in darkness and then transferred to Rc light ($\sim$8 μmol·m^{-2}·s^{-1}). Tissue was collected at the indicated times, including a dark control at 24 h (D24). Average values of two biological replicates are plotted (normalized for loading using 18S rRNA), and error bars represent ranges. Representative RNA gel blots are shown.
(C) Comparative time course of Rc light–induced changes in relative transcript levels of *PIL1* and *PRR9* in wild-type seedlings after initial exposure to Rc light (0 to 3 h and 0 to 24 h [inset]). Values for *PRR9* transcript levels were obtained from **(A)** and **(B)**, and those for *PIL1* were obtained from Tepperman et al. (2004). Relative transcript levels were determined by normalizing to the highest value for each gene.

phy signaling by Fankhauser et al. (1999) based on overexpression, a notion supported more recently by *pks1* mutant data (Lariguet et al., 2003). Two more of these genes, *ELF4* (Khanna et al., 2003) and *PIL1* (Salter et al., 2003), both of which were initially annotated as hypothetical/unknown, were separately characterized while this study was in progress and evidence was presented for each being involved in phy-regulated responses (see below). The fourth gene, At2g37970 (designated *SOUL1* here for convenience), also initially unannotated, encodes a SOUL family putative heme binding protein not previously implicated in phy signaling. Interestingly, the *elf4* and *pil1* mutants exhibit reduced sensitivity to light signals, whereas *pks1* and *soul1* mutants show enhanced sensitivity. In addition to these four genes, two of the other early-response genes identified by microarray (Monte et al., 2004; Tepperman et al., 2006), *HY5* and *SPA1*, had been identified even earlier in forward genetics screens as having important roles in phy-regulated seedling deetiolation (Koornneef et al., 1980; Hoecker et al., 1998, 1999). In addition, another early-response gene identified by microarray (Tepperman et al., 2001, 2004; Monte et al., 2004), *PRR9*, was more recently identified as important for optimal sensitivity of responses to red and blue light (Eriksson et al., 2003). It is notable that the phenotypes caused by disruption of the four new loci, *elf4*, *pil1*, *pks1*, and *soul1*, are relatively moderate compared with the most extreme hyposensitive and hypersensitive phenotypes observed for *phyB*-null mutants and phyB overexpressors, respectively (Figure 3). This observation indicates that although these loci appear to have a level of functional importance in light-induced deetiolation, none is singularly essential sufficiently early in the phy-induced signaling cascade to pleiotropically affect the full development of the deetiolation response.

The subset of six new mutant loci, *lhy-101*, *sth* (*zf3*), *dof*, *cbf3*, *At2g42870*, and *elip1*, that exhibit statistically significant hypersensitivity in hypocotyl inhibition in Rc light but no significant difference in cotyledon expansion (class III, Table 1), do not provide evidence of functional importance in early, central regulatory events in phy signaling. On the other hand, the possibility of organ-specific activity in hypocotyl cells cannot be ruled out. It is noteworthy that the data for the *lhy-101* allele isolated in this work document a seedling-deetiolation phenotype for a monogenic loss-of function mutant at this locus, in contrast with previous studies that used an overexpressor allele (Schaffer et al., 1998). This latter report showed that *LHY* overexpression results in hypocotyl hyposensitivity to Rc light (Schaffer et al., 1998), the converse of what was observed for *lhy-101*, strengthening the notion that LHY may indeed function in mediating phy signaling in these cells. A more recent study with a *cca1 lhy* double mutant under light/dark cycling conditions supports the role of LHY as a component of the central circadian oscillator (Mizoguchi et al., 2005).

One of the seven class II mutants, *cca1-like*, displays enhanced cell expansion in both hypocotyl and cotyledon, whereas the other six class II mutants, *ring-zfn*, *b-box type zf*, *sigE*, *put.ep1*, β-*exp*, and *mate*, display hypersensitive inhibition of cell expansion in both hypocotyl and cotyledon. These loci are either important to the normal cell expansion process in general or defects in these genes make the seedlings sensitive to the deleterious effects of light, such as through photooxidative damage to cellular components (at least for the six hypersensitive mutants). Care is needed in studies of potential photomorphogenic mutants to avoid interpreting short hypocotyls alone (without cotyledon measurements) in light-grown seedlings as evidence of a specific defect in the photoreceptor perception and signaling systems (Quail, 2002a).

Why do only a limited proportion of genes that respond rapidly to light signals through the phy system display clear seedling deetiolation phenotypes when disrupted (Figure 5)? The data show that only 7 genes (22%; including previously evaluated *HY5* and *SPA1*) of the 32 genes identified by microarray analysis as phy-regulated early-response genes (Tepperman et al., 2001, 2004, 2006; Monte et al., 2004) and investigated here significantly perturb global deetiolation when mutated (Table 1, Figure 5). These results with a temporally selected, relatively small subset of photoresponsive genes are consistent with the genome-wide functional profiling in yeast, in which only 7% or less of responsive genes were identified as functionally necessary for optimal growth under the relevant imposed environment (Giaever et al., 2002). Possible explanations for these findings in our study include the following.

First, it is possible that the microarray-based expression measurements were not sufficiently reliable or that the induced changes are quantitatively functionally insignificant to the organism. Inspection of microarray expression data makes it clear that changes declared significantly different by statistical methods identify large numbers of genes for which these changes are quantitatively minor (Monte et al., 2004; Tepperman et al., 2006). Although the initial selection criteria used here for

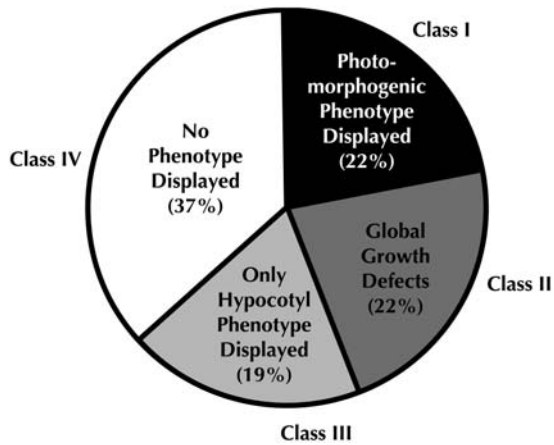

Figure 5. Only a Limited Fraction of phy Early-Response Genes Are Necessary for Optimal Deetiolation.

Seven lines (22%) display concomitant, reciprocal disruption of normal hypocotyl and cotyledon cell expansion in response to Rc light (class I). Seven lines (22%) display significant, parallel (nonreciprocal), global defects in Rc light responsiveness in both hypocotyl and cotyledon cell expansion (class II). Six lines (19%) show significant enhanced, Rc light–imposed inhibition of only hypocotyl cell expansion, with no effect on normal cotyledon cell expansion (class III). And 12 lines (37%) display no detectable aberrant seedling phenotypes (class IV).

the early-response genes imposed, in addition, a minimal quantitative change threshold (induction or repression) of twofold within 1 h of the start of the light signal, it is possible that these criteria are insufficiently stringent to identify genes with biologically meaningful light-induced changes in expression.

Second, it is possible that the rapid changes in expression measured after the initial transfer of seedlings from dark to light are transient and are only functionally important to an early transitional process that has no observable longer term impact on the overall deetiolation process under our conditions. This possibility might be consistent with the observations of Spalding and coworkers (Parks and Spalding, 1999; Parks et al., 2001; Parks, 2003), who observed that phyA acts only transiently to inhibit hypocotyl growth upon initial exposure of seedlings to light and that this has little or no measurable impact on hypocotyl expansion determined in end point analyses after prolonged (4 to 5 d) growth under light conditions. On the other hand, defects in some such genes could have negative selective impact for seedlings germinated under natural conditions that are not apparent for seedlings germinated on agar plates in the laboratory.

Third, because we only examined the phenotypes at one fluence rate for each wavelength, we cannot rule out the possibility that a phenotype might be observed at another fluence rate. However, the fluence rates selected (Rc light, 7 $\mu mol \cdot m^{-2} \cdot s^{-1}$; FRc light, 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were similar to those used for the microarray analyses and in our hands approach, but are not at, maximum effectiveness for the wild type; therefore, they are expected both to maximize the opportunity for the display of hyposensitive mutants and to leave sufficient latitude for the detection of hypersensitivity.

Fourth, it cannot be excluded that the light-induced changes for some genes are functionally irrelevant to the deetiolation process altogether, perhaps being an inevitable consequence of selection pressures on light-regulated events at other stages of the life cycle.

Finally, the commonly offered explanation for the lack of apparent phenotypes from reverse genetics disruption of target loci is functional redundancy (Ostergaard and Yanofsky, 2004; Cutler and McCourt, 2005; Kuepfer et al., 2005). The assessment of this possibility can in principle be approached through systematic construction of higher order combinations of candidate mutant loci. However, in this case, it is not immediately apparent which of the coordinately light-regulated loci that define the molecular phenotype are likely to function redundantly in determining the visible deetiolation phenotype. Although some of the 78% of early-response genes that do not exhibit a coordinated seedling-deetiolation phenotype when mutated are potentially sequence-related (e.g., *CCA1* and *LHY*; *ATHB4* and *HAT4*), the majority do not appear to be so related and therefore are more difficult to reconcile with any postulated redundant molecular or biochemical activity. One possibility, in the case of the transcriptional regulators, is that multiple different classes of these factors might mediate the phy-regulated expression of the same single target genes through the presence of multiple cognate *cis* elements for these factors arrayed in the individual promoters of each of these genes. Thus, compared with studies focused on multigene families in which sequence-relatedness may be a more reliable predictor of functional redundancy, identifying targets for mutant combinations here offers additional challenges. On the other hand, it is possible that broadening or refinement of the parameters used to assess the visible deetiolation phenotype might reveal previously undetected defects for some of these loci. Furthermore, a systematic assessment of light-regulated gene expression in the mutants might reveal defects not visible at the phenotypic level.

Despite the relatively low proportion of genes targeted here that were found to display insertional mutant phenotypes, some new loci not identified previously in forward genetics screens as involved in phy-regulated deetiolation were found. Although mutations in most of these loci resulted in relatively modest phenotypes, we nevertheless consider that the results indicate that the strategy has merit, particularly in the quest to define the full complement of genes involved in light-regulated development.

This point is exemplified by *ELF4* and *PIL1*. Each was targeted for potential functional involvement in deetiolation at the outset of this analysis, and each was also subsequently identified in separate studies as involved in other phy-regulated processes, namely, flowering (Doyle et al., 2002) and shade avoidance (Salter et al., 2003). The additional *pil1* mutant analysis here provides evidence of further functional roles for PIL1 in the phyB-imposed suppression of petiole extension during vegetative development and the induction of floral development. Importantly, in addition, the data also suggest that PIL1 may have a regulatory role in the phy-induced expression of the *PRR9* gene. The evidence shows that PIL1 is necessary for normal maximum *PRR9* expression upon first exposure of dark-grown seedlings to light. Together with a comparison of the temporal patterns of rapid light-induced changes in *PIL1* and *PRR9* expression (Figure 4C), these data suggest the possibility of transcriptional regulation of *PRR9* by PIL1 in a light-modulated manner. *PIL1* expression is high in dark-grown seedlings but is rapidly repressed within 60 min to a new steady state basal level upon light exposure. By contrast, *PRR9* is rapidly induced in the light, but only transiently, with a sharp maximum 30 to 45 min after initial exposure (Figures 4B and 4C). A potential model consistent with these data would suggest that PIL1 is necessary for light-induced *PRR9* expression. According to this model, the light-induced expression of *PRR9* would be supported by the initially high levels of PIL1 at the dark-to-light transition, but because *PIL1* itself is rapidly negatively regulated by the light signal, PIL1 protein abundance would decrease rapidly, thereby reversing the initial increase in *PRR9* expression and rendering the overall temporal pattern of induction transient. Because PRR9 is proposed to have a function in circadian regulation (Eriksson et al., 2003) and PIL1 is proposed to interact with the circadian clock in regulating shade avoidance (Salter et al., 2003), these data may provide insight into the possible signaling mechanisms involved. It is important to note that the absence of significant effects of *pil1* mutations on the light-induced initiation of oscillations of the central oscillator components, *CCA1*, *LHY*, and *TOC1* (see Supplemental Figure 10 online), which occurs upon first exposure of etiolated seedlings to light (Kikis et al., 2005), provides evidence against the possibility that *PRR9* is regulated indirectly by *PIL1* via the circadian clock (Harmer and Kay, 2005).

In summary, the data presented here document the functional profiling of a plant signaling system responsive to an external environmental stimulus, based on genome-scale identification of responsive genes in a transcriptional network by microarray expression analysis coupled with targeted reverse genetics assessment of the functional necessity of each gene to the induced morphogenic response. The evidence from these monogenic mutants shows that only a low proportion of genes that respond rapidly to phy-mediated light signals during the initiation of seedling deetiolation appear to be functionally necessary for the normal development of the visibly measurable deetiolation phenotype, consistent with analogous genome-wide studies in yeast. Although functional redundancy may provide an explanation for the majority of the rapidly light-responsive genes that appear to be functionally unnecessary for deetiolation, this possibility is difficult to reconcile with the absence of any obvious sequence-relatedness among most of these genes. Alternative possible explanations include a lack of dependence of full deetiolation on initial light-induced expression changes of some genes, organ- or cell-specific functional activity of some genes, and statistically defined expression changes that are either quantitatively too small to be important to the phenotype or simply functionally irrelevant to the organism. These findings may be relevant to other plant studies using reverse genetics strategies to determine gene function in *Arabidopsis*.

METHODS

Plant Material, Growth Conditions, and Measurements

Gene annotations shown in Table 1 were obtained using the National Center for Biotechnology Information gene search (http://www.ncbi.nlm.nih.gov/entrez/) and the *Arabidopsis thaliana* insertion database (http://www.atidb.cshl.org). We identified T-DNA insertion lines in the ecotype Col background by searching the databases of SAIL and the SIGnAL collection (Alonso et al., 2003). Homozygous T-DNA insertion lines were isolated using PCR with T-DNA– and gene-specific primers flanking the T-DNA insertion sites. A wild-type sibling of each mutant line was used for initial evaluations of the seedling phenotypes. Col was used as the control after confirmation of phenotype. The consequence of T-DNA insertion on the expression of the affected gene was checked by RT-PCR (see Supplemental Figure 2 online). For mutants in genetic backgrounds other than Col, *spa1-3* (RLD) and *cca1-1* (Wassilewskija), the respective wild-type seedlings were used as controls (as indicated in Table 1).

Sterilized seeds were plated on growth medium plates as described (Tepperman et al., 2004), stratified for 3 d at 4°C, synchronized by a 3-h white light treatment followed by a 21-h dark treatment at 21°C, and then either maintained in darkness or transferred to various light conditions as specified. Seedlings were irradiated with either FRc light (740 nm, 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or Rc light (660 nm, 7 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The light sources used were as described (Tepperman et al., 2001, 2004), and the fluence rates were monitored using a spectroradiometer (model L1–1800; LI-COR). Hypocotyl and cotyledon measurements were performed 96 h after germination using a digital camera (Coolpix 990; Nikon) and NIH Image software (National Institutes of Health).

Statistical Analyses

Hypocotyl length and cotyledon area measurements of 30 seedlings for each mutant genotype and its respective wild type were analyzed using Excel (Microsoft). Mean values were used to calculate relative differences between mutant and wild-type seedlings (wild-type values set to unity). P values were determined by Student's *t* test (homoscedastic, two-tailed distribution) analysis of data from 30 seedlings for each genotype. Values below $P = 0.05$ were considered statistically significant for differences in hypocotyl length and cotyledon area between the wild-type and mutant lines.

RNA Isolation and Analysis

Total RNA was isolated from 4-d-old seedlings using RNeasy plant mini kits (Qiagen). For dark-to-red light transition experiments, seedlings were grown in darkness for 4 d before transfer to Rc light (8.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$; time 0) for various periods as indicated before extraction for RNA analysis. For RNA gel blot analysis, 5 μg of RNA was loaded per lane and transferred to a nylon membrane for hybridization. Gene-specific probes were amplified by PCR and labeled by random priming. Hybridization signal was quantified with a Storm 860 PhosphorImager (Molecular Dynamics) using ImageQuant software, and loading was normalized to 18S rRNA levels. Mean values from three biological replicates were plotted with SE. For RT-PCR analysis, gene-specific primers were used to detect transcripts in wild-type (Col) and mutant seedlings.

Identification of *pil1-3* Mutants

The Maxygen collection of fast-neutron deletion mutants (Li et al., 2001) was screened by PCR for mutants in the *PIL1* gene. Genotyping was performed by PCR analysis using genomic DNA extracted from leaf tissue from individual plants. Homozygous plants were backcrossed twice to their corresponding wild types, and the F2 population was reselected as described above. Wild-type siblings were used as controls.

Petiole Length and Flowering Time Measurements

Plants were grown under dim white light (2.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 3 or 4 weeks to measure petioles of the longest leaf. Measurements were done using NIH Image software. For flowering time determination, plants were maintained in Enconair chambers at 21°C. Seeds were germinated under long-day conditions (16 h of light/8 h of dark) or short-day conditions (8 h of light/16 h of dark). After 2 weeks, seedlings were transplanted to soil.

Nuclear Localization Experiments

The *PIL1* open reading frame was amplified by PCR from cDNA using primers containing the *Cla*I and *Xba*I restriction sites and cloned into the modified pRLT2-GUS/NiaDBam vector described by Hoecker et al. (1999). Leek (*Allium porrum*) epidermal peels were bombarded with 35S::PIL1:GUS or 35S::GUS, incubated in the dark for 24 h, and assayed as described by Ni et al. (1998).

Immunoblot Analysis

Total protein was isolated from 4-d-old seedlings grown in darkness or in Rc light. Thirty micrograms of total extract protein was loaded on an 8% SDS-PAGE gel. Immunoblot analysis was performed as described by Martínez-García et al. (2000). Monoclonal antibodies B1 and B7 (1:500 dilution each) were used to detect phyB protein, as described by Hirschfeld et al. (1998).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are listed in Table 1.

Supplemental Data

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

Supplemental Figure 1. Changes in the Expression of *At3g21150* and *At3g21330* Genes in Response to a 1-h Rc Light Treatment.

Supplemental Figure 2. Assessment of the Effect of T-DNA Insertion on Gene Expression for the Homozygous Mutant Lines Analyzed in This Study.

Supplemental Figure 3. Statistical Significance of the Differences in Hypocotyl Length and Cotyledon Area between 4-d-Old Wild-Type and Mutant Seedlings Grown in Rc Light.

Supplemental Figure 4. Statistical Significance of the Differences in Hypocotyl Length between 4-d-Old Wild-Type and Mutant Seedlings Grown in FRc Light or in Darkness.

Supplemental Figure 5. RNA Gel Blot Analysis of *pil1* Allele Expression.

Supplemental Figure 6. *pil1* Seedlings Are Hyposensitive to Rc Light.

Supplemental Figure 7. Effect of *pil1* on Petiole Elongation and Flowering Time.

Supplemental Figure 8. PHYB Protein Levels Are Not Affected in *pil1* Mutants.

Supplemental Figure 9. PIL1 Is Localized to the Nucleus.

Supplemental Figure 10. *pil1* Does Not Alter the Light-Induced Expression Pattern of Central Circadian Oscillator Genes *CCA1*, *LHY*, and *TOC1*.

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