phyA dominates in transduction of red-light signals to rapidly responding genes at the initiation of Arabidopsis seedling de-etiolation

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Summary

Contrary to expectations based on the visible phenotypic behavior of seedlings undergoing de-etiolation in response to continuous red light (Rc), previous gene expression profiling showed that one or more of the five-membered phytochrome (phy) family of Arabidopsis, other than phyB, is predominantly responsible for transducing the Rc signals to light-responsive genes. To begin to identify which phys are involved, and to define potential primary targets of phy signaling, we have examined the genome-wide expression profiles of genes responding to Rc within 1 h (early response genes) of initial exposure of dark-grown wild-type, phyA, phyB and phyAphyB double mutant seedlings to the light signal. The data show that phyA has a quantitatively dominant role in Rc-induced expression of these early response genes, that phyB has minimal detectable regulatory activity in the presence of phyA, but assumes a quantitatively larger role in its absence, and that phyA and phyB combined are responsible for the full extent of Rc responsiveness of 96% of these genes. No evidence was obtained of a significant role for the remaining family members, phyC, phyD or phyE, in this process. In striking contrast, Rc-imposed repression of early response gene expression remains quantitatively strong in the phyAphyB double mutant, as well as the monogenic mutants, suggesting a significant role for one or more of the other three phys in this response. Examination of the established or predicted functional roles of the early response genes indicates that genes encoding transcription factors represent the largest single category, at a frequency three times their prevalence genome-wide. This dominance is particularly striking among those genes responding most robustly to the Rc signal, where >50% are classified as involved in transcriptional regulation, suggesting that these may have potentially primary regulatory roles at the interface between phy signaling and the light-responsive transcriptional network. Integration of the present data with those of a previous genome-scale transcriptional analysis of a pif3 mutant, suggests a complex network involving perception and transduction of inductive Rc signals by both phyA and phyB through both PIF3 and other undefined signaling partners to early response genes.

Keywords: microarray, phytochromes, photosensory perception, signaling, transcriptional networks.

Introduction

Plants use the phytochrome (phy) family of sensory photoreceptors (phyA to phyE in Arabidopsis) to track red (R) and far-red (FR) light signals in the environment in order to adjust their growth and development in accord with the prevailing conditions (Chen et al., 2004; Franklin and Whitelam, 2004; Schaefer and Nagy, 2006). Photoconversion of the initially cytoplasmically localized molecule from the red-absorbing inactive Pr form to the far red-absorbing active form triggers an intracellular signaling process that culminates in alterations in nuclear gene transcription that are detectable within minutes of the light signal (Lissemore and Quail, 1988). There is evidence that this signaling process involves rapid translocation of the photoactivated phy molecule into the nucleus (Nagatani, 2004) where it interacts with signaling partners such as the basic helix-loop-helix (bHLH) transcription factors, PHYTOCHROME-INTERACT-
ING FACTOR 3 (PIF3) and PIF1, inducing changes in the expression of target genes (Duek and Fankhauser, 2005; Huq et al., 2004; Khanna et al., 2004; Quail, 2002). The precise mechanism by which these changes are induced has not yet been definitively determined, but appears to include phy-induced degradation of the PIF3 and PIF1 proteins via the ubiquitin-26S proteosome system (UPS) (Bauer et al., 2004; Kim et al., 2003; Monte et al., 2004; Shen et al., 2005). Recent evidence indicates that rapid, phy-induced phosphorylation of PIF3 initiates this process by flagging the transcription factor for recognition and degradation by the UPS system (Al-Sady et al., 2006).

A major goal of efforts to understand the mechanisms by which plants respond to their environment is to define the genome-wide transcriptional networks responsible for regulating the downstream gene expression patterns that elaborate the appropriate cellular and organismal responses. Microarray-based expression profiling provides the opportunity not only to define the complete set of genes induced or repressed in wild-type plants in response to any given stimulus (the molecular phenotype), but also, using time-resolved expression analysis, to identify the genes responding most rapidly to the stimulus, and, therefore, potentially direct transcriptional targets of the primary signal transduction process. When combined with mutants disrupted in candidate receptors, signaling intermediates or transcriptional regulators, this technology provides the opportunity to define subsets of genes within the overall responsive population of genes that are subject to regulation by these individual components. This strategy thus provides the potential for mapping the hierarchy, branching and interactions in the transcriptional network that orchestrates the response, and for positioning putative upstream signaling intermediates in the circuitry linking receptor molecules to responsive genes.

Several studies aimed at defining the spectrum of photoresponsive genes regulated by phyA and phyB have now used microarrays to examine the changes in expression profiles elicited by continuous red (Rc) or continuous far-red (FRc) irradiation of Arabidopsis seedlings. One set of studies employed glass-slide, spotted-cDNA or spotted-oligonucleotide arrays to measure presumptive end-point, steady-state transcript profiles after prolonged (5- to 6-day) FRc or Rc irradiation (Jiao et al., 2005; Ma et al., 2001, 2002, 2005). The other set of studies used Affymetrix oligonucleotide microarrays to follow the time-course of changes in expression over the first 24 h of irradiation of dark-grown seedlings (Tepperman et al., 2001, 2004) or focused specifically on genes responding to the light signal within 1 h of initial exposure of previously dark-grown seedlings to Rc (Monte et al., 2004). The earlier studies (Ma et al., 2001, 2002; Tepperman et al., 2001, 2004; Wang et al., 2002) were performed with arrays of only about 6000–8000 genes (‘8K’ microarrays), representing 25–30% of the Arabidopsis genome, whereas the more recent studies have been done with arrays that provide closer to full-genome coverage (>80%), namely the Affymetrix ATH1 array (Monte et al., 2004) and an Operon spotted oligoarray (Jiao et al., 2005; Ma et al., 2005). These reports have cataloged the molecular phenotype of wild-type seedlings during and following completion of de-etiolation in Arabidopsis in response to Rc and FRc light signals, and have begun to examine the photosensory functions of phyA and phyB in this process using phyA and phyB null mutants. In addition, the expression of genes in seedlings germinated in darkness has also recently been reported (Mazzella et al., 2005).

The 8K-microarray data verify that phyA is exclusively responsible for regulation of the genes that respond to FRc signals in these seedlings, at least for those represented on the arrays (Ma et al., 2001; Tepperman et al., 2001). However, the majority of Rc-responsive genes on these arrays were found, unexpectedly, to remain relatively strongly responsive to this light signal in the phyB null mutant, suggesting that one or more of the remaining phy family members is predominantly responsible for perception and transduction of this signal (Ma et al., 2001; Tepperman et al., 2004). The large majority of phy-regulated genes on the 8K arrays (~90%) did not display changes in expression until 3 h or more after the onset of the light signal (‘late-response’ genes). However, the remaining minority (10%) exhibited altered expression within 1 h of the signal (‘early response’ genes; Tepperman et al., 2001, 2004).

The more recent studies with the ATH1 (Monte et al., 2004) and Operon (Jiao et al., 2005) microarrays have established the genome-scale molecular phenotypes of wild-type seedlings in response to Rc and FRc light, but the responsiveness of the phyA and phyB mutants genome-wide is yet to be reported, and the phy family member(s) predominantly responsible for Rc-regulated gene expression during seedling de-etiolation has not been identified. We have addressed these questions here by defining the genome-scale expression profiles of phyA and phyB mono- genic, and phyAphyB double mutants in response to Rc using the Affymetrix ATH1 microarray. Moreover, in order to identify those genes most likely to be early, if not direct, targets of phy signaling, we focused this analysis on those genes responding relatively rapidly to the light signal.

**Results**

**Definition of Rc-regulated, phy-dependent early response genes**

To identify early response genes and define the potential roles of phyA and phyB in this process, we performed microarray analysis of light-induced changes in expression in wild-type, phyA, phyB and phyAphyB mutant seedlings after initial transfer of dark-grown seedlings to Rc for 1 h.
The primary data and all statistical parameters generated in this analysis are presented for all approximately 22,000 genes on the ATH1 microarray in Table S1. This analysis identified a total of 1,459 genes (Table S2) that were either induced (1,031) or repressed (428) in the wild type by 1 h Rc, in a statistically significant fashion (see Experimental procedures and Figure 1a). Further analysis of the expression of these genes in the phyA, phyB and phyAphyB mutants identified those genes whose response to 1 h Rc differed in a statistically significant fashion from that of the wild type (Figure 1a, Table S2).

For these genes, the magnitude of the contribution of each phy, alone or in combination, to the Rc responsiveness of each gene was defined as the fold-induction ratio (FIR) for induced genes, or the fold-repression ratio (FRR) for repressed genes (Monte et al., 2004; Tepperman et al., 2004; see Experimental procedures). This parameter quantitatively measures the magnitude of the change in expression (fold-induction (FI) or fold-repression (FR)) induced by the Rc treatment in the wild type compared to each phy mutant genotype, expressed as a ratio (wild-type/phy mutant). The FI, FR, FIR and FRR values for each light-responsive gene are listed in Table S2 and its derivatives. A ratio of 1.0 signifies no detectable contribution of that phy photoreceptor species to the light-induced response, independently of whether the underlying response to light is intrinsically large or small for that gene. Deviations from 1.0 provide a quantitative measure of the robustness of the contribution of that phy family member to the rapid Rc-induced response. We defined those genes with FIR and FRR values of $\geq 1.5$ as robustly ($\geq 2.0$) to moderately ($1.5–2.0$) dependent on the relevant phy for Rc responsiveness, and those with values $\leq 1.5$ as marginally ($1.25–1.5$) to minimally ($1.0–1.25$) dependent (Figure 1a, Note S1, Figures S1a–S6a).

Finally, to identify those genes responding intrinsically, quantitatively most robustly to the light signal, we defined those genes with FI or FR values $\geq 2.0$ in the wild type as robustly to moderately Rc responsive, whereas those with values $\leq 2.0$ were defined as marginally to minimally Rc responsive (Figure 1a, Figures S1b–S6b). The genes with FI or FR values $\geq 2.0$ were assigned to functional categories, and the percentage of the annotated genes falling into each category are displayed in Figures S1c–S6c. The functional category assignments for each of these genes are listed in Table S3.

**Figure 1.** Flow charts showing the numbers of genes identified at sequential steps in the microarray expression analysis performed in this study. (a) Analysis sequence 1. These steps involved, first, identification, within the approximately 22,000 genes on the Affymetrix ATH1 microarray, of those genes whose expression change (either induced or repressed) in a statistically significant fashion in wild-type (WT) seedlings in response to 1-h Rc compared with unirradiated dark-control WT seedlings. Within this set of 1,459, those genes were identified that displayed a statistically significant difference in expression (induction or repression) between the WT and each of the phy mutant genotypes (phyA, phyB or phyAphyB) in response to 1-h Rc. Within each of these sets, those genes were identified that changed $\geq$ twofold in WT seedlings in response to 1-h Rc (induced or repressed) compared with unirradiated dark-control seedlings. Within each of these sets, those genes were identified that displayed a quantitatively robust to moderate dependence on phyA, phyB or phyA plus phyB (combined), as defined by a 1-h Rc-induced fold-change (FC; either induction (FI) or repression (FRI)), that was 1.5-fold greater in WT seedlings than in the respective phy mutant genotypes (phyA, phyB or phyAphyB), i.e. a fold-change ratio (FCR) of $\geq 1.5$, where $\text{FCR} = [\text{FC in WT}] / [\text{FC in phyX}].$

(b) Analysis sequence 2. These steps involved, first, identification, of statistically Rc-responsive genes as in (a). Within this set of 1,459, those genes were identified whose expression change (induced or repressed) $\geq$ twofold in WT seedlings in response to 1-h Rc (induced or repressed) compared to unirradiated dark-control seedlings. Within this set of 251, those genes were identified that displayed a statistically significant difference in expression (induction or repression) between WT and each of the phy mutant genotypes (phyA, phyB or phyAphyB) in response to 1-h Rc. Within each of these sets, those genes were identified that displayed a quantitatively robust to moderate dependence on phyA, phyB or phyA plus phyB (combined), as defined by FCR of $\geq 1.5$ as in (a). Percentage values represent the percentage of total induced (206) or repressed (45) genes in each category.
Table 1 Number of Rc-regulated, early response genes categorized by functional class in wild-type seedlings, and the number of these genes moderately to robustly dependent on phyA, phyB or both phyA and phyB for full Rc-responsiveness

<table>
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Genes represented on the ATH1 microarray whose transcript abundance was either increased (induced) or decreased (repressed) in response to 1-h Rc, both in statistically significant fashion and by greater than twofold, in wild-type seedlings, were defined as Rc-regulated, early response genes, and classified into the broad functional categories shown, according to established or putative function. Dependence on the designated phy(s) for Rc responsiveness was as defined in the text (see Figure 1).

Overall, the analysis identified a total of 251 genes that are statistically and robustly to moderately changed (≥ twofold) in expression in wild-type seedlings by 1-h Rc (Figure 1b, Table 1, Table S3). Of these, 206 genes are induced and 45 are repressed by the Rc signal. Figure 2 provides a visual summary of the Rc-responsive expression of all 251 of these early response genes in all four genotypes, as well as their classification into functional categories (see also Table 1). The expression levels detected by this microarray analysis were validated for selected genes by RNA blot analysis (Figure 3).

Induced genes

Figure 4(a) shows the means of the expression levels of all 206 Rc-induced genes for all four genotypes, and Figures S7 displays the fold-induction for each individual gene in each genotype arrayed by descending fold-induction in wild type. Examination of the expression profiles of the individual genes across these genotypes shows that, of these 206, 60% (124 genes) are statistically and robustly to moderately dependent on phyA for that Rc responsiveness (FIR ≥ 1.5; Figure 1b, Figure S1, Table 1, Table S3), and another 14% (29 genes) marginally dependent (FIR 1.25–1.5). Together these data indicate that 74% (153 genes) of the early response genes that are robustly to moderately induced by Rc are robustly to marginally regulated by phyA during the first 1 h of Rc (Table S3). By contrast, only 4% (nine genes) of these genes display moderate to robust dependence on phyB (FIR ≥ 1.5) for rapid Rc induction and a total of only 10% (20 genes) marginal to robust dependence (FIR ≥ 1.25; Figure S2, Table 1, Table S3).

The magnitude of this differential effect between phyA and phyB for each individual gene is depicted more directly in Figure 4(b), which compares the FIR values for each gene in the phyA and phyB monogenic mutants. These data show that the vast majority of these genes (>90%) display greater dependence on phyA than phyB for the full, wild-type level of responsiveness to the Rc signal. Thus phyA is necessary for the principal quantitative component of the early light responsiveness of most of these Rc-responsive genes. Other phy(s), including phyB, are apparently unable to substitute for phyA in Rc signaling to these genes. Conversely, the data suggest that phyB is not necessary for the near-full Rc-light responsiveness for most of these genes, as only a small (7%) reduction in mean expression level of all 206 Rc-responsive genes is observed in the phyB mutant (Figure 4a).

On the other hand, 96% (198) of these genes are robustly (80%) to moderately (16%) reduced in their Rc responsiveness (FIR ≥ 1.5) in the absence of both photoreceptors in the phyAphyB double mutant (Figure 1b, Figure S3, Table 1, Table S3). This is evident in Figure 4(a) which shows that, on average, the residual Rc-responsive expression in the phyA mutant is almost entirely eliminated in the phyAphyB mutant, and in Figure S7 which arrays the fold-induction for the individual genes. The magnitude of this differential effect for each individual gene is more directly depicted in Figure 4(c) and (d). These data reveal that a large proportion of these genes do display a detectable to substantial dependence on phyB for the residual Rc responsiveness in the phyA mutant.

Taken together these data reveal the striking dominance of phyA in the induction of early response genes during initial exposure to Rc, with phyB playing only a minor role in this process in the presence of phyA. On the other hand, comparison of the single and double mutants shows that phyB does contribute significantly to Rc-induced expression in the absence of phyA. Figure S8 presents examples, in
were transferred to Rc (8), hormones (H); stress/defense (S/D); metabolism (M); signaling (S); transport (TR); growth/development (G/D); Functional category: transcription (TX); photosynthesis/chloroplast (P/C); categories, expressed as a percentage of the total annotated genes in each set.

Figure 2. Visual summary of the relative contributions of phyA and phyB to induction (red) and repression (green) of gene expression during initial induction of seedling de-etiolation in response to 1-h Rc.

Four-day dark-grown wild-type, phyA, phyB and phyAphyB mutant seedlings were transferred to Rc (8 μmol m⁻² sec⁻¹) or retained in darkness for 1 h before extraction of RNA and determination of gene expression levels, genome-wide, by microarray analysis. Relative expression levels are depicted for the 251 genes defined as robustly (>twofold) responsive to the 1-h Rc signal (see Figure 1b). Relative expression levels for each gene in each genotype are expressed as the fold-induction (a) or fold-repression (b) in Rc relative to the level in the dark-grown control seedlings of the same genotype set at unity. Pie charts show the distribution of these genes among functional categories, expressed as a percentage of the total annotated genes in each set. Functional category: transcription (TX); photosynthesis/chloroplast (P/C); metabolism (MI); signaling (IS); transport (TR); growth/development (G/D); hormones (H); stress/defense (S/D).

addition to those in Figure 3, of expression profiles of individual genes representing the range of response patterns exhibited across the arrays. A detailed discussion of these patterns is presented in Note S2. Together these patterns suggest a range of quantitative contributions of phyB to Rc responsiveness, from additive to synergistic with that of phyA.

It is notable that the majority of the 206 Rc-induced genes display little or no residual responsiveness to the Rc signal in the phyAphyB double mutant. This is evident in Figure 4(a) which shows that the mean residual expression across all these genes is only about 5% of the wild-type response level. Most strikingly, however, statistical analysis indicates that only three (1.5%) of these genes (APRR9, AT2G46790; STO, AT1G06040; AT3G12320) display statistically significant Rc induction compared to the dark control, and this is quantitatively small in each case compared with wild type.

Figure 5 provides a simplified schematic summary of the overall analysis of the 206 Rc-induced early response genes described above. The data suggest that whereas phyA is able to (almost) fully compensate for the absence of (i.e. is functionally redundant with) phyB, phyB is not able to compensate for the absence of (i.e. is not functionally redundant with) phyA in transducing these signals. On the other hand, the data indicate that phyB does have the capacity to share this early Rc-signaling activity with phyA to varying degrees (i.e. is partially redundant). Moreover, together phyA and/or phyB are robustly to moderately responsible for transducing Rc signals to 198 (96%) of the 206 Rc-induced genes (Table S3), with only eight genes (4%) displaying the possibility of quantitatively minor involvement of phyC, phyD or phyE in this process.

Preliminary analysis suggests a high degree of overlap of the genes rapidly regulated by phyA in response to Rc (present study) and FRc light (Tepperman et al., 2001). Northern blot analysis verifies this observation for a selected set of representative genes (Figure 3), suggesting that phyA targets the same gene set through both photosensory modes. However, because of the differences in microarray platforms used in these studies, comprehensive genome-scale comparisons are not currently possible.

The 206 Rc-induced genes identified here are grouped into functional categories and arrayed by fold-induction in Table S4, and the subsets of these that are dependent on phyA, phyB, and phyA plus phyB combined for this Rc responsiveness are similarly categorized in Tables S5, S6 and S7, respectively. The data indicate that putative or established transcription-factor-encoding genes comprise the largest single class of the currently annotated genes in this induced set (27%), and are significantly over-represented relative to their occurrence on the ATH1 microarray and in the Arabidopsis genome (9% of ATH1 GeneChip; http://arabidopsis.med.ohio-state.edu/AtTFDB). This dominance is even more striking within the most strongly induced subset of these genes where 50% of the 22 most strongly Rc-responsive genes are in this category (Figure 6). Putative signaling-component genes are also a prominent class (15% of annotated). Inspection of the individual genes in the transcription factor and signaling categories indicates the presence of several that have been previously implicated in phy-mediated light responses, including HY5, CCA1, LHY, APRR9, APRR5, HYH, SPA1, PKS1, ELF4, GI and FKF1, as well as numerous additional potential regulators, such as SIGE.
Figure 3. RNA blot validation of microarray data.
Expression analysis of Rc-light- and FRC-light-regulated phy-dependent genes.
Left column: Representative RNA blots (from biological triplicates) of extracts from 4-day-old dark-grown wild-type (WT) and phy mutant (phyA, phyB or phyAphyB) seedlings maintained in the dark (D) or irradiated with either Rc (8 μmol m⁻² sec⁻¹) or FRC (2 μmol m⁻² sec⁻¹) for 1 h, hybridized with specific probes for the following genes: (a) Rc-light-induced phy-dependent genes: SIGE (AT5G24120), ELIP (AT4G14690), HY5 (AT5G11260), PKS1 (AT2G02950), SPA1 (AT2G46340), ABC TRANSPORTER (AT5G44110), APRR5 (AT5G24470) and COP1-L (AT5G52250); (b) Rc-light-repressed genes ATGRF7 (AT5G53660), SAUR-AC1 (AT4G13790) and AUX/IAA (AT432280); (c) constitutive, non-light-regulated genes ACTIN2 (AT3G18780).
Affymetrix GeneChip results for Rc-irradiated seedlings (center column of histograms) are flanked by quantitative measurements from triplicate RNA blots (means ± SE) for seedlings irradiated with Rc for 1 h (left) or with FRC for 1 h (right).
Integration of phy- and PIF3-regulated genes

To begin to investigate the possibility of selective channeling of signals from individual phy family members to different downstream signaling partners in the transcriptional network, we compared the Rc-induced genes identified here as dependent on phyA and/or phyB for responsiveness, with those identified previously as dependent on either PIF3 or other presumptive PIFs for such Rc responsiveness (Monte et al., 2004). The analysis identified 143 genes in common between the two independent experiments, as exhibiting robust to moderate (P > twofold), statistically significant induction of expression by 1 h of Rc in wild-type seedlings in both cases (Figure 7a and b, Table S8). Thus, about 70% of the 206 genes identified here as Rc-induced, early response genes were reproducibly thus induced in the two separate experiments as defined by these criteria. Of these 143 common genes, 26 (18%) are dependent on PIF3 for Rc expression.

Figure 4. PhyA dominates transduction of Rc signals to induced early response genes.
(a) Mean expression levels for all 206 Rc-responsive genes induced > twofold by 1 h Rc. Data are shown for dark-grown (black) and 1 h Rc-irradiated WT (blue), phyA (dark red), phyB (red) and phyAphyB (medium-red) seedlings, normalized to the WT dark-control value set at unity.
(b) All 206 induced genes arrayed by FIR value for the monogenic mutants, phyA (blue) and phyB (red). Vertical lines divide the array into bins according to FIR value. Inset: Histogram showing numbers of genes in each bin.
(c) All 206 genes arrayed by FIR value for the double mutant, phyAphyB (blue) and the monogenic mutant, phyA (red). Vertical lines divide the array into bins according to FIR value. Inset: Histogram showing numbers of genes in each bin.
(d) Significant contribution of phyB to Rc-induced gene expression revealed by phyAphyB double mutant. All 206 robustly Rc-light-induced genes arrayed by percentage contribution (see Experimental Procedures) of phyA and phyB to the total Rc responsiveness in the phyAphyB double mutant, in rank order of percentage contribution of phyA (phyA, blue; phyB, red). Vertical lines divide the array into bins according to values of percentage contribution. Inset: Histogram showing numbers of genes in each bin.

Figure 5. Simplified schematic summary depicting phyA dominance in perception and transduction of Rc signals to induced early response genes.
Of the 206 robustly Rc-induced early response genes, 124 (60%) display robust to moderate dependence on phyA in the presence of phyB (solid arrow), but only nine (4%) display such dependency on phyB in the presence of phyA (dashed arrow). Collectively, 198 (96%) of the 206 genes display robust to moderate dependence on phyA and/or phyB combined for rapid Rc responsiveness.
responsiveness, whereas 114 (80%) display little or no dependence on PIF3 for such responsiveness (Figure 7c). PhyA dominates in signaling to both sets of genes, whereby 95 genes (66%) are robustly to moderately dependent on this family member, and the remainder exhibit a range of shared dependence on phyB in the absence of phyA (Figure 7b). Taken together the data indicate that the dominant Rc-light signaling pathway to induced early response genes occurs through phyA and signaling intermediates other than PIF3 [79 genes (55%)], with a second smaller phyA-dominant pathway through PIF3 [16 genes (11%)]. In the absence of phyA, phyB also participates in Rc signaling, likewise predominantly through intermediates other than PIF3 [35 genes (25%)], with a small set of genes [10 genes (7%)] being regulated through PIF3 (Figure 7b). Because the early response genes regulated through PIF3 include a high proportion of chloroplast-related genes (Monte et al., 2004), it appears that phyA has a dominant role in early chloroplast biogenesis. On the other hand, because the larger non-PIF3 pathway regulates a gene-set dominated by transcription-factor genes, with very few chloroplast-related genes (Monte et al., 2004), it appears that phyA may also channel signals to regulate other facets of seedling de-etiolation through PIFs other than PIF3. Overall the data suggest that Rc-signal perception and transduction is shared at the level of the phy photoreceptor molecules, albeit dominated by phyA in early de-etiolation, with targeting of predominantly common primary signaling partners, and that signal channeling to early response genes may be determined at the level of these primary signaling partners, such as PIF3 (Figure 7c).

Repressed genes

As indicated above, of the total of 251 genes that are statistically and robustly to moderately changed (≥twofold) in

Figure 6. Transcription-factor genes dominate the most robustly Rc-induced early response genes.
(a) All 206 Rc-induced genes arrayed by fold-induction in WT seedlings and divided into bins by FI value (vertical lines). Inset: Histogram showing the numbers of genes in each bin.
(b) Distribution of genes among functional categories expressed as a percentage of the total annotated genes within each bin. TX, transcription; P/C, photosynthesis/chloroplast; M, metabolism; S, signaling; TR, transport; H, hormone-related; S/D, stress and defense.

Figure 7. Integration of phy-mutant and pif3-mutant microarray expression data.
(a) Scatterplot of fold-induction values for robustly (≥twofold) Rc-induced early response genes in WT seedlings in this experiment (x-axis) and that described in Monte et al. (2004) (y-axis), identifying 143 genes that reproducibly meet the statistical and quantitative-robustness criteria in both experiments. The twofold induction threshold for each experiment is indicated.
(b) Summary of the comparison of the roles of phyA, phyB and PIF3 in Rc-induced expression of the 143 genes common to both experiments. ‘Common’, ≥twofold-induced genes identified here as statistically dependent on phyA, phyB or phyaphyB combined, and their relationship to genes that are either dependent (+) or not (−) on PIF3 for their responsiveness to Rc (from Monte et al., 2004).
(c) Model depicting proposed integration and channeling of Rc signals at early steps in the phy signaling network.
expression in wild-type seedlings by 1-h Rc (Figure 1b, Table 1, Table S3), 45 genes (18%) are repressed by this signal. Figure 8(a) shows the means of the expression levels of all 45 of these Rc-repressed genes for all four genotypes, and Figure S9 displays the fold-repression for each individual gene in each genotype, arrayed by descending fold-repression in wild type. Examination of the expression profiles of the individual genes across these genotypes shows that, of these 45, only 7% (three genes) are statistically and robustly to moderately dependent on phyA for that Rc responsiveness (FRR ≥ 1.5; Figure 1b, Figure S4, Table 1, Table S3). These data indicate that in contrast to the Rc-induced genes, the majority of the early response genes that are robustly to moderately repressed by Rc are not robustly regulated by phyA during the first hour of Rc. Yet more striking, only one of these genes (2%) displays moderate dependence on phyB (FRR ≥ 1.5) for rapid Rc repression. These data are reflected in the mean expression levels of all 45 genes shown in Figure 8(a), and in the arrayed expression levels for the individual genes in Figure S9, indicating that neither phyA nor phyB is individually necessary for the near-full, negative Rc-light responsiveness for most of these genes.

On the other hand, 64% (29) of these genes are robustly to moderately reduced in their Rc responsiveness (FRR ≥ 1.5) in the absence of both photoreceptors in the phyAphyB double mutant (Figure 1b, Figure S6, Table 1, Table S3), and another 18% (eight genes) marginally reduced (FRR 1.25–1.5). These data indicate, therefore that, overall, 82% (37 genes) of the early response genes that are robustly to moderately repressed by Rc are robustly to marginally regulated in redundant fashion by phyA and phyB combined, during the first hour of Rc (Table S3). This is also evident in Figure 8(a), which shows that, on average, the extent of Rc-imposed repression of expression observed in the wild type, phyA or phyB monogenic mutants, is reduced by about 50% in the phyAphyB mutant, and in Figure S9 which arrays the fold-repression for the individual genes. The magnitude of this differential effect between genotypes for each individual gene is more directly depicted in Figure 8(b), which compares the FRR values for each gene in the phyAphyB double mutant with the phyA and phyB monogenic mutants.

Taken together these data show that, in striking contrast to induced genes, there is an absence of dominance of either phyA or phyB alone in mediating the Rc-imposed repression of early response genes, and less than full control by these two family members in combination. The majority of the 45 repressed genes display apparent residual responsiveness to the Rc signal in the phyAphyB double mutant (Figure 8a). Indeed, statistical analysis indicates that 18 (40%) of these genes display statistically significant Rc repression compared with the dark control (see EXPERIMENTAL PROCEDURES and Table S3). Thus, the evidence suggests that one or more of the remaining three phy family members, phyC, phyD

Figure 8. Phy-family member(s) apart from phyA and phyB contribute strongly to transduction of Rc signals to repressed early response genes. (a) Mean expression levels for all 45 Rc-responsive genes repressed ≥2-fold by 1-h Rc. Data are shown for dark-grown (black) and 1-h Rc-irradiated WT (blue), phyA (dark red), phyB (red) and phyAphyB (medium-red) seedlings, normalized to the WT dark-control value set at unity. (b) Redundant partial contribution of phyA and phyB to Rc-repressed gene expression revealed by comparison of monogenic and double mutants. All 45 robustly Rc-light-repressed genes arrayed by FRR value (phyAphyB, blue; phyA, red; phyB, green). (c) Significant contribution of phyC, phyD and/or phyE to Rc-repressed gene expression revealed by phyAphyB double mutant. All 45 robustly Rc-light-repressed genes arrayed by percentage contribution of phyA plus phyB combined to the total Rc responsiveness in the WT (blue), compared with the residual percentage contribution of phyC, phyD and/or phyE combined (red). Vertical lines divide the arrays into bins according to percentages of contribution.
and/or phyE, contribute significantly to the residual Rc responsiveness of these genes. The percentage contribution of the phyA/phyB pair compared with the presumptive residual contribution of phyC, phyD and/or phyE to the Rc-imposed repression of each gene is arrayed in Figure 8(c). Together, these data indicate that phyA and phyB act in mutually redundant fashion to partially repress these genes with varying degrees of combined effectiveness, but that in addition, one or more of the phyC/phyD/phyE trio also act partially redundantly, or additively, with the phyA/phyB pair, again to varying degrees, to exert full Rc-imposed repression of expression. It should be noted, however, that, in the absence of a quintuple phy mutant, the formal possibility remains that other as yet unidentified photoreceptor(s) mediate this response.

Figure S10 presents examples of expression profiles of individual Rc-repressed genes representing the range of response patterns observed across the arrays. Although some extreme patterns, such as that of GT2-L, are present, in general the Rc-repressed genes display considerably greater uniformity of response pattern than the Rc-induced genes (Figure 3, Figure S8).

The 45 Rc-repressed genes identified here are grouped into functional categories and arrayed by fold-repression in Table S9, and the subsets of these dependent on phyA, phyB and phyA and phyB combined for this Rc responsiveness are similarly categorized in Tables S10, S11 and S12, respectively. As for the Rc-induced genes, the data indicate that putative or established transcription-factor-encoding genes comprise the largest single class of the currently annotated genes in this set (41%), once again strongly over-represented relative to their occurrence in the Arabidopsis genome (9% of ATH1 GeneChip; http://arabidopsis.med.ohio-state.edu/AtTFDB). Also notable is a high representation of hormone-related genes in the repressed set (18% of annotated) compared with the induced set (1.5%). Most striking, however, is the dominance of these two functional categories in the set of 18 repressed genes identified as retaining statistically significant Rc responsiveness in the phyAphyB double mutant, where 60% of the currently annotated genes are classified as transcription-related and 27% as hormone-related (Figure 9). Inspection of the individual genes in the transcription-factor category indicates the presence of several that have been previously implicated in phy-mediated light responses, including HAT4 and ATHB4. Interestingly, auxin-related genes dominate both repressed and induced sets of hormone-related genes, representing 78% of the total hormone-related, early response genes, with the remainder split between the ethylene, cytokinin and ABA pathways.

Discussion

The data presented here establish that phyA and phyB combined are responsible for mediating the full extent of Rc-induced expression of 96% of the induced early response genes defined in this study. No evidence of substantial involvement of any of the remaining family members, phyC, phyD or phyE, in this process was obtained. Moreover, the data show that phyA, and not phyB, dominates in transducing the Rc signals to these early response genes, both quantitatively, in terms of the magnitude of the light-regulated expression, and numerically, in terms of the number of genes regulated. This finding may appear initially surprising given the frequent simplified portrayal in the literature of the differential photosensory functions of phyA and phyB, whereby phyA is often referred to as being exclusively responsible for the perception of FRc signals and phyB is described as being predominantly responsible for the perception of Rc signals (Chen et al., 2004; Franklin and Whitelam, 2004; Quail, 2002; Whitelam et al., 1998). This portrayal is based primarily on the well-established visible phenotypes of phyA and phyB null-mutant Arabidopsis seedlings following prolonged (4–5 days) growth under continuous irradiation with Rc or FRc wavelengths (von Arnim and Deng, 1996; Quail, 2002; Tepperman et al., 2004; Whitelam et al., 1998). Under these conditions, phyA null mutants exhibit no detectable responsiveness to FRc, but...
appear visibly indistinguishable from wild-type seedlings in Rc, whereas, conversely, phyB mutants display strongly reduced responsiveness to Rc, but are indistinguishable from wild-type in FrC.

However, this portrayal is oversimplified in several respects relevant to the gene-expression responses reported here. First, the available evidence indicates that although phyB dominates, or is exclusively responsible for, long-term, Rc-imposed suppression of hypocotyl cell elongation, other phys (especially phyA), in addition to phyB, have a significant role in the apical-zone responses of hook opening, cotyledon separation and cell expansion and chloroplast biogenesis (Franklin et al., 2003; Reed et al., 1993, 1994; Tepperman et al., 2004; Whitelam et al., 1998). Secondly, it is likely that the mRNA used for microarray analysis here is derived predominantly from the smaller, less vacuolated apical zone (hook, cotyledon) cells so that the expression patterns observed probably reflect the light-induced responses of these tissues, rather than the hypocotyls (Tepperman et al., 2004). Consequently, the phyA-dominated regulation of Rc-induced, early response gene expression is probably focused predominantly, if not exclusively, in the apical zone cells of the de-etiolating seedling. Third, it might be argued a priori that the rapid changes in gene expression induced after the initial transfer of dark-grown seedlings to Rc light are transient and only functionally relevant to an early transitional process that has no measurable impact on the visible phenotype observed after 4–5 days of Rc. The rapid light lability of phyA (Hirschfeld et al., 1998; Somers et al., 1991) and the observations of Spalding and co-workers (Parks, 2003; Parks and Spalding, 1999; Parks et al., 2001) that phyA acts only transiently to inhibit hypocotyl elongation over the first 3 h of light exposure, with no detectable impact after 4–5 days of Rc (McCormac et al., 1993; Parks, 2003; Reed et al., 1994; Whitelam et al., 1998), might be consistent with this possibility. This question is discussed in more detail in Note S3.

Rc-induced genes constitute 82% of the early response genes, consistent with broad involvement in early facets of the seedling de-etiolation process. Examination of the functional categories into which these genes are classified confirms this notion. Genes putatively involved in signaling, chloroplast biogenesis and transport are well represented. However, genes encoding putative or established transcriptional regulators constitute the single most prominent category, particularly among those most robustly responsive to the Rc signal. This finding confirms and extends to the genome scale similar conclusions reached earlier from the more limited 8K-microarray studies (Tepperman et al., 2001, 2004). These factors are drawn from a diversity of transcriptional-regulator classes, including zinc-finger, MYB, bZIP and bHLH factors, some of which, such as HY5, CCA1 and LHY, have been previously implicated in phy regulation. Of particular interest is SIGE, a nuclear-encoded sigma factor, considered likely to function as a regulatory subunit of plastid-localized RNA polymerase (Allison, 2000) and therefore to have a potentially key, and possibly global, role in regulating phy-induced expression of genes in the chloroplast genome. Collectively, these data indicate that these transcription-factor genes are early, if not primary, direct targets of phy signaling, and, therefore, have the potential to function as regulators of multiple downstream target genes in the phy-regulated transcriptional network.

Rc-repressed genes comprise only 18% of the total number of early response genes. However, in striking contrast to the Rc-induced genes, phyA and phyB have a markedly less dominant role in transducing Rc signals to these down-regulated genes. Instead, the strong residual responsiveness in the phyAphyB double mutant suggests that one or more of the remaining family members, phyC, phyD and/or phyE, contribute significantly to this photosensory function. These data thus reveal a previously unrecognized dichotomy in signaling to induced and repressed genes within the phy family. It would appear that whereas phyA and phyB dominate in transducing signals to genes whose products are needed in higher abundance to facilitate de-etiolation, phyC, phyD and/or phyE contribute substantially, in addition to the phyA/phyB pair, in regulating genes whose products are active in dark-grown seedlings, but which need to be removed or decreased in abundance to permit de-etiolation.

Like the Rc-induced genes, the Rc-repressed, early response genes include a high proportion (41% of the currently annotated genes) that encode established or putative transcriptional regulators. These represent a diversity of classes, including zinc-finger, bHLH and trihelix factor-encoding genes, as well as the growth factor gene, AtGRF7 (AT5G53660). Interestingly, the most prevalent class are the homeodomain-leucine zipper (HD-ZIP) class, including ATHB2 (HAT4) and ATHB4, both of which have been previously implicated in phy-regulated responses (Morelli and Ruberti, 2002; Salter et al., 2003). This gene set could, in principle, represent either negative regulators that need to be downregulated to permit light-induced derepression of downstream targets, or positive regulators of dark-induced genes that need to be downregulated during de-etiolation. Also notable is the relatively high proportion of hormone-related genes (18% of annotated) in the Rc-repressed, early response set, compared to the Rc-induced set (2% of annotated). Yet more striking, is the prominence of auxin-related genes within both the induced and repressed hormone-related sets. These genes, which include SAUR (AT4G38840) and GH3-L (AT4G03400) (induced), and GH3-L (AT2G23170), SAUR-AC1 (AT4G13790), AUX/IAA (AT4G32280), IAA19 (AT3G15540) and HOOKLESS (HLS) (AT4G37580) (repressed), comprise 78% of the total annotated, hormone-related, early Rc-responsive genes. This observation raises the possibility...
that these auxin-related genes may be direct targets of phy signaling, perhaps representing a mechanism by which the phy rapidly modulate cell expansion rates in hypocotyls and/or cotyledons via the auxin system in response to Rc.

A central goal of photosensory signaling research is to define the structure, and dissect the regulatory circuitry, of the primary transcriptional network responsive to the incoming light signals (Quail, 2002; Tepperman et al., 2001). A powerful approach to this problem is to identify specific genes or subsets within the genome-wide population of light-responsive genes whose photoresponsiveness is compromised by genetic disruption of signaling or transcriptional-regulatory components putatively positioned in the transduction pathway between the photoreceptors and the primary response genes. Using this approach, we have previously identified a subset of genes responding to Rc within 1 h of initial exposure that are dependent on the phy-interacting bHLH factor, PIF3, for this rapid Rc responsiveness (Monte et al., 2004). A high proportion of these genes are chloroplast-related, suggesting that PIF3 has a key role in regulating light-induced chloroplast biogenesis. On the other hand, a larger subset of the Rc-responsive genes in that study displayed no dependence on PIF3 for this responsiveness, suggesting the involvement of other phy-interacting transducing components (PIFs) in this process. Moreover, this gene set includes very few chloroplast-related genes, suggesting that they have functional roles predominantly in other aspects of the de-etiolation process. Comparison of these previous microarray data with those presented here shows that about 70% of the Rc-induced early response genes are reproducibly induced in statistically significant and quantitatively robust (≥twofold) fashion in wild-type seedlings between the two independent experiments (Figure 7a).

Further analysis of this group, integrating the phy and pif3 mutant expression profile data (Figure 7b), suggests the existence of a complex sequential dichotomy of signaling, both at the photoreceptor and signaling-partner levels. This is illustrated schematically in Figure 7(c), whereby Rc signals are perceived by both phyA and phyB, which both then transduce this information to both PIF3 and other presumptive PIFs, which in turn regulate different subsets of rapidly Rc-induced genes. However, this process is strongly skewed at both levels, whereby phyA dominates the perception and primary transduction of the Rc signal, and non-PIF3 PIFs dominate further transduction of this information to early response genes (Figure 7c). Collectively, these data suggest the existence of a photosensory system that employs quantitatively graded, partial redund-

### Experimental procedures

#### Plant material and growth conditions

Wild-type RLD and phyA101, phyB1 and phyAphyB mutant seed were plated on growth medium (−sucrose) (Valvekens et al., 1988) and transferred to the dark at 4°C for 5 days. After a 3-h white light treatment, the plates were wrapped in foil and held in the dark at 21°C for 4 days. Seedlings were then irradiated with Rc (8 μmol m−2 s−1) for 1 h or retained in darkness as controls.

#### RNA isolation, cRNA synthesis, and microarray hybridizations

Three different biological replicates of each treatment (designated D-1, D-2 and D-3 for dark controls, and R1-1, R1-2 and R1-3 for 1-h Rc-irradiated samples) were grown separately under the same conditions, and extracted, processed and analyzed independently. Total RNA isolation, cRNA synthesis and microarray hybridizations were performed as described in Monte et al. (2004). Probe synthesis was performed as described in the GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com). After first- and second-strand cDNA synthesis, reactions were tested for amount of template by PCR using EIF-4-specific primers (Monte et al., 2004). Hybridization and washes were performed as described by Affymetrix (Santa Clara, CA, USA).

#### Data analysis

Each chip was scanned using an Agilent scanner and the image was analyzed using Microarray Suite 5.0 software (Affymetrix Inc.) to generate array CEL data files that contain probe intensity data. A log scale robust multiarray analysis (RMA) was applied to the raw data to calculate expression values for each probe set (Irizarry et al., 2003) using the open source Bioconductor software analysis package (Gentleman et al., 2004).

#### Statistically significant differential expression

To define statistically significant differential gene expression between light treatments and genotypes the limma method (Smyth, 2004; PLM and LIMMA packages, http://www.bioconductor.org/) was used as previously described (Monte et al., 2004). This method was developed specifically to identify differentially expressed genes in large microarray studies and uses a moderated t-statistic which produces p values adjusted for false discovery rate (FDR; Benjamini and Hochberg, 1995; Storey, 2002). After compiling a data set using RMA, specific subsets of triplicate samples were compared with identify genes with statistically different levels of expression. This method was used to identify: (i) Rc-light-responsive genes in wild type and in phyAphyB double mutants. Statistically light responsive genes are defined as those with p.values (adjusted for FDR) of ≤0.05 when triplicate dark control values are compared with those of samples taken after 1-h Rc (see Tables S2 and S3). (ii) Phy-dependent genes. To identify genes statistically significantly differentially expressed in the phyA, phyB or phyAphyB mutant genotypes compared to the wild type, the same method (limma; Smyth, 2004) was used. The light values for the wild type were compared with the light values for each mutant genotype and phy-dependent genes were defined as those with an adjusted p value < 0.05.
Quantification of the magnitude of differential expression (FIR and FRR)

To provide a quantitative measure of the robustness of the contribution of phyA, phyB or phyA + phyB to the light-induced response of each gene, we calculated a FIR value for induced genes, and a FRR value for repressed genes. FIR is defined as the ratio of the FI values for the wild type and each individual mutant (phyA, phyB, phyA-phyB; FIR = FI for wild type)/(FI for phy mutant). The FRR is defined as the ratio of the FR values for the wild type and each individual phy mutant: FRR = (FR for wild type)/(FR for phy mutant).

Percentage contribution of individual phys to light responsiveness

The percentage contribution of phyA (PCphyA) to the Rc-induced change in expression levels of each gene in wild-type (WT) seedlings (Figure 4d) was determined as follows:

\[
PC_{\text{phyA}} = 1 - \left( \frac{M}{W} - 1 \right) \times 100
\]

where PC is the percentage contribution, W is the FI value for the WT and M is the FI value for the phyA mutant. The PC for phyB (Figure 4d) was determined as follows. First, the PC for phyA and phyB combined in the phyAphyB double mutant was calculated as above, except using the FI values for the phyAphyB double mutant. The PC for phyB (in the absence of phyA) was then defined as the difference between these two values for the phyA and phyAphyB double mutants.

The percentage contribution of phyA, phyB (PCphyA,phyB) to the Rc-repression of expression of each gene in WT seedlings (Figure 8c) was determined as follows:

\[
PC_{\text{phyA,phyB}} = 1 - \left( \frac{X}{R} - 1 \right) \times 100
\]

where PC is the percentage contribution, R is the FR value for the WT and X is the FR value for the phyAphyB double mutant. The percentage contribution of phyC,D,E (PCphyC,D,E) was calculated as follows:

\[
PC_{\text{phyC,D,E}} = 100 - PC_{\text{phyA,phyB}}
\]

Functional category assignments

Genes were assigned to functional categories based on the gene descriptions provided by Affymetrix and TIGR404 (http://www.tigr.org/tdb/e2k1/ath1/ath1.shtml last accessed 2 October 2006) and the classification provided by the Munich Information Center for Protein Sequences (MIPS; http://mips.gsf.de/projects/funct). The following supplementary material is available for this article online:

Note 1. Quantitative robustness of statistically defined light responses.
Note 2. Range of Rc-induced response patterns in individual genes.
Note 3. Differential photosensory functions of phyA and phyB.
Figure S1. PhyA function in early Rc-induced gene expression.
Figure S2. PhyB function in early Rc-induced gene expression.
Figure S3. PhyAphyB function in early Rc-induced gene expression.
Figure S4. PhyA function in early Rc-repressed gene expression.
Figure S5. PhyB function in early Rc-repressed gene expression.
Figure S6. PhyAphyB function in early Rc-repressed gene expression.
Figure S7. PhyA dominates transduction of Rc signals to induced early response genes.
Figure S8. Examples of expression profiles of individual Rc-induced early response genes representing the range of response patterns encountered across the arrays.
Figure S9. Phy-family member(s) apart from phyA and phyB contribute strongly to transduction of Rc signals to repressed early response genes.
Figure S10. Examples of expression profiles of individual Rc-repressed early response genes representing the range of response patterns encountered across the arrays.
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Table S2 All statistically defined light-responsive genes (1459)
Table S3 All twofold Rc-light-responsive genes in wild type (251)
Table S4 Rc-induced genes in wild type (206 genes)
Table S5 PhyA-dependent, Rc-induced genes (154 genes)
Table S6 PhyB-dependent, Rc-induced genes (20 genes)
Table S7 PhyA and phyB (combined)-dependent, Rc-induced genes (203 genes)
Table S8 Genes common to phy and pif3 experiments (143 genes)
Table S9 Rc-repressed genes in wild type (45 genes)
Table S10 PhyA-dependent, Rc-repressed genes (four genes)
Table S11 PhyB-dependent, Rc-repressed genes (three genes)
Table S12 PhyA and phyB (combined)-dependent, Rc-repressed genes (36 genes)
Table S13 Primers for PCR amplification of probes used for RNA blot analysis (Figure 3)

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Supplementary Material

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This material is available as part of the online article from http://www.blackwell-synergy.com and also at http://www.pgec.usda.gov/Quail/TPJ_2006_SM/TPJ_2006.html.

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phyA dominates in early red-light signaling 741


