Dynamics of the Shade-Avoidance Response in Arabidopsis[1][W]

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Shade-intolerant plants perceive the reduction in the ratio of red light (R) to far-red light (FR) as a warning of competition with neighboring vegetation and display a suite of developmental responses known as shade avoidance. In recent years, major progress has been made in understanding the molecular mechanisms underlying shade avoidance. Despite this, little is known about the dynamics of this response and the cascade of molecular events leading to plant adaptation to a low-R/FR environment. By combining genome-wide expression profiling and computational analyses, we show highly significant overlap between shade avoidance and deetiolation transcript profiles in Arabidopsis (*Arabidopsis thaliana*). The direction of the response was dissimilar at the early stages of shade avoidance and congruent at the late ones. This latter regulation requires *LONG HYPOCOTYL IN FAR RED1/SLENDER IN CANOPY SHADE1* and phytochrome A, which function largely independently to negatively control shade avoidance. Gene network analysis highlights a subnetwork containing *ELONGATED HYPOCOTYL5* (*HY5*), a master regulator of deetiolation, in the wild type and not in *phytochrome A* mutant upon prolonged low R/FR. Network analysis also highlights a direct connection between *HY5* and *HY5 HOMOLOG* (*HYH*), a gene functionally implicated in the inhibition of hypocotyl elongation and known to be a direct target of the HY5 transcription factor. Kinetics analysis show that the *HYH* gene is indeed late induced by low R/FR and that its up-regulation depends on the action of HY5, since it does not occur in *hy5* mutant. Therefore, we propose that one way plants adapt to a low-R/FR environment is by enhancing HY5 function.

Plants have evolved two opposing strategies in response to competition for light: shade tolerance and shade avoidance. Angiosperms have an impressive capacity to avoid shade. Daylight contains roughly equal proportions of red light (R) and far-red light (FR), but within a vegetation community, that ratio is lowered as a result of R absorption by photosynthetic pigments. The reduction in the R/FR ratio is perceived as an early signal of neighbor proximity, resulting in a suite of developmental responses known as shade avoidance. The most dramatic response to low R/FR is the stimulation of elongation growth that is remarkably rapid, with a lag phase of a few minutes. In dicotyledonous plants, elongation growth induced by low R/FR is often associated with a reduction of leaf development. In the long term, low R/FR exposure leads to early flowering with a reduced seed set, which is considered an escape mechanism because it shortens generation time. All of these responses occur both in natural dense communities and in shade simulations (low R/FR). Furthermore, similar responses are induced by exposing plants to horizontal FR radiation with white light from above. This is because shade-avoiding plants are able to perceive light reflected by neighboring plants as partially depleted of the R wavelengths, and they can activate responses to avoid shade even before canopy closure and actual shading occurs (Franklin, 2008; Ruberti et al., 2012; Casal, 2013). However, at high canopy density, multiple light signals control the shade-avoidance response (Ballaré, 1999). There is evidence that both low R/FR and reduced blue light are required for full expression of shade avoidance in plant canopies. Interestingly, the blue light responses seem to be mediated through pathways that showed only limited overlap with those activated by low R/FR (Keller et al., 2011; Keuskamp et al., 2011).

Changes in the R/FR ratio of light occurring within a vegetation community are detected by the phytochrome (phy) family of R/FR photoreceptors (phyA–phyE in Arabidopsis [*Arabidopsis thaliana*]). Phytochromes are photochromic biliproteins that exist in two photoconvertible isoforms: Pr and Pfr. They are synthesized in their inactive Pr form; upon absorption of R, Pr is converted

into the biologically active Pfr form, which can absorb FR and switch back to Pr, resulting in a dynamic photoequilibrium between the two forms of phytochrome. Following conversion to the Pfr form, phytochromes translocate to the nucleus. phyA is the only phytochrome that is rapidly degraded in its Pfr form and can signal during rapid photoconversion between the Pr and Pfr forms. All the other phytochromes are relatively stable in the Pfr form (Bae and Choi, 2008; Franklin and Quail, 2010; Casal, 2013).

Among the light-stable phytochromes, phyB plays a key role in shade avoidance. Arabidopsis *phyB* mutants constitutively display shade-avoidance traits such as elongated hypocotyl, stem, petioles, and leaves, acceleration of flowering, and higher apical dominance under high R/FR (Reed et al., 1993). However, other light-stable phytochromes (phyD and phyE) also contribute to shade avoidance (Smith and Whitelam, 1997; Devlin et al., 1998, 1999). By contrast, phyA seems to attenuate the elongation response induced by low R/FR (Johnson et al., 1994; Devlin et al., 2003; Wang et al., 2011).

In the nucleus, phytochromes physically interact with a subfamily of basic helix-loop-helix (bHLH) proteins, the PHYTOCHROME-INTERACTING FACTORS (PIFs), controlling several aspects of photomorphogenesis (Castillon et al., 2007; Jiao et al., 2007; Leivar and Quail, 2011). This interaction in turn leads to PIF's phosphorylation, ubiquitination, and degradation via the 26S proteasome, providing an elegant mechanism for the rapid regulation of gene expression in response to changes in the light environment (Bauer et al., 2004; Park et al., 2004; Shen et al., 2005, 2007; Al-Sady et al., 2006; Nozue et al., 2007).

PIF1, PIF3, PIF4, PIF5, and PIF7 have been demonstrated to directly contribute to shade avoidance (Lorrain et al., 2008; Hornitschek et al., 2012; Leivar et al., 2012; Li et al., 2012). They all interact physically with phyB through the conserved N-terminal sequence, called the active phyB-binding motif (Leivar and Quail, 2011). As a result, PIF1, PIF3, PIF4, and PIF5 become phosphorylated and degraded via the ubiquitin-proteasome system, with degradation half-times in the range of 5 to 20 min (Leivar and Quail, 2011). PIF3, PIF4, and PIF5 protein levels increase rapidly in photoautotrophic seedlings upon exposure to low R/FR (Lorrain et al., 2008; Leivar et al., 2012). Unlike its close relatives, PIF7 is not rapidly degraded in high R/FR (Leivar et al., 2008a). However, this PIF protein accumulates in its dephosphorylated form in shade, suggesting the existence of a protein phosphatase and a protein kinase whose activities or availability are regulated by light quality changes (Li et al., 2012). Shade-induced elongation response is significantly attenuated in *pif4 pif5* and, to an even greater extent, in *pif1 pif3 pif4 pif5* quadruple (*pifq*) and *pif7* mutants (Lorrain et al., 2008; Leivar et al., 2012; Li et al., 2012). Conversely, PIF4- and PIF5-overexpressing seedlings have constitutively long hypocotyls and petioles (Lorrain et al., 2008).

Consistent with the rapidity of the elongation growth response to low R/FR and its reversibility upon perception of high R/FR, changes in gene expression are very rapid and reversible (Carabelli et al., 1996; Salter et al., 2003). The transcript levels of the homeodomain-leucine zipper (HD-Zip) II *ARABIDOPSIS THALIANA HOMEOBOX2* (*ATHB2*) and bHLH *PIF3-LIKE1* (*PIL1*) transcription factor genes, functionally implicated in the elongation response provoked by neighbor detection (Steindler et al., 1999; Salter et al., 2003), increase within a few minutes of low R/FR exposure (Carabelli et al., 1996; Salter et al., 2003). Significantly, *ATHB2* and *PIL1* transcript levels fall very rapidly after transfer from low R/FR to high R/FR (Carabelli et al., 1996; Salter et al., 2003). *ATHB2* and *PIL1* induction by low R/FR does not require de novo protein synthesis (Roig-Villanova et al., 2006) and is significantly reduced in loss-of-function *pif* mutants (*pif1 pif3, pif4 pif5*, and *pif7*; Lorrain et al., 2008; Hornitschek et al., 2009; Leivar et al., 2012; Li et al., 2012). There is evidence that *PIL1* and *ATHB2* are recognized in vivo by PIF4 and PIF5 (de Lucas et al., 2008; Hornitschek et al., 2009, 2012), and physical interaction between the *PIL1* promoter and PIF7 has also been reported (Li et al., 2012).

An ever-increasing body of evidence shows that auxin biosynthesis, signaling, and transport are all critical for shade avoidance (Morelli and Ruberti, 2000; Kanyuka et al., 2003; Carabelli et al., 2007; Tao et al., 2008; Keuskamp et al., 2010; Sassi et al., 2013), and links between this hormone and transcription factors triggering plant responses to low R/FR (PIF and HD-Zip II proteins) have been established (Steindler et al., 1999; Hornitschek et al., 2012; Li et al., 2012; Turchi et al., 2013). PIF4 and PIF5 physically interact with the promoter of *YUCCA8* (*YUC8*), which encodes a rate-limiting enzyme in auxin synthesis (Hornitschek et al., 2012), and PIF7 in its dephosphorylated form binds G-boxes of the auxin biosynthetic genes *YUC5*, *YUC8*, and *YUC9* and increases their expression, thus directly linking the perception of a low R/FR signal to changes in free INDOLE-3-ACETIC ACID (IAA) required for shade-induced growth (Li et al., 2012). By contrast, how HD-Zip II proteins promote auxin response and transport remains to be investigated.

By exploiting mutant analysis in combination with genome-wide expression profiling, Sessa et al. (2005) uncovered a negative regulatory mechanism active in low R/FR that involves *HYPOCOTYL FAR RED1/ SLENDER IN CANOPY SHADE1* (*HFR1/SICS1*). The *HFR1/SICS1* gene is rapidly induced by low R/FR, and there is evidence that its promoter is recognized in vivo by PIF5 (Sessa et al., 2005; Hornitschek et al., 2009). *HFR1/SICS1* encodes an atypical bHLH protein and acts as a helix-loop-helix inhibitor. Upon prolonged exposure to low R/FR, HFR1/SICS1 accumulates and interacts with PIF4 and PIF5, forming non-DNA-binding heterodimers, thus limiting PIF-mediated gene expression (Hornitschek et al., 2009). Consistent with this, several genes rapidly and transiently induced by low R/FR are significantly up-regulated in *hfr1/sics1* loss-of-function mutants upon prolonged exposure to simulated shade (Sessa et al., 2005). Another atypical bHLH protein gene, *HELIX LOOP HELIX1/*

PHYTOCHROME RAPIDLY REGULATED1 (*HLH1/PAR1*; Sessa et al., 2005; Roig-Villanova et al., 2006), is also rapidly regulated by low R/FR, and its induction does not require de novo protein synthesis (Roig-Villanova et al., 2006). HLH1/PAR1 has also been involved in the negative regulation of shade-induced elongation and proposed to act as a dominant negative antagonist of conventional bHLH transcription factors (Roig-Villanova et al., 2007; Galstyan et al., 2011; Hao et al., 2012).

Despite the significant advances over the last decade in understanding shade avoidance, little is known about the dynamics of this response. Here, the shade-avoidance response was examined by genome-wide expression profiling in wild-type and genetically altered plants exposed to low R/FR for different times. Functional associations by response overlap (FARO) between shade avoidance transcript profiles and available microarray gene expression data revealed highly significant overlap with genes regulated in deetiolation experiments. Remarkably, the direction of the response was dissimilar at the early stages of shade avoidance and congruent at the late ones. This latter regulation involves both HFR1/SICS1 and phyA, which function largely independently to control the shade-avoidance response. Computational gene network analysis identified a subnetwork containing *ELONGATED HYPO-COTYL5* (*HY5*), a key regulator of the transcriptional cascades promoting seedling photomorphogenesis (Lau and Deng, 2010), in wild-type seedlings and not in *phyA* mutants upon prolonged exposure to low R/FR. Based on these data, we propose that one way in which plants adapt to a low R/FR environment is by modulating the HY5 pathway.

RESULTS

Simulated Shade Environment

Several laboratories use white light supplemented with FR to study early events of the shade-avoidance response (neighbor detection). The light conditions utilized are quite different one from the other both in terms of photosynthetic active radiation (PAR) and R/FR ratio, and PAR is usually quite low (Tao et al., 2008; Hornitschek et al., 2009; Wang et al., 2011). For several years, we have been using a different experimental setup to simulate a shade environment (Sessa et al., 2005; Carabelli et al., 2007; Ciarbelli et al., 2008; Ruberti et al., 2012). To study changes in gene expression underlying early and late responses to a low-R/FR environment, we set up simulated shade conditions in which R is reduced and FR is increased, maintaining total light quantity (400–800 nm) constant (Sessa et al., 2005) and thus making entirely negligible the well-known influence of this parameter on the stability of several transcription factors involved in photomorphogenesis (Henriques et al., 2009). Varying R/FR while maintaining total light quantity constant inevitably implies a nonconstant supply of PAR (400–700 nm).

Therefore, control experiments were performed lowering PAR without changing the ratio between R and FR. Columbia (Col-0) seedlings were grown for 7 d in a light/dark (L/D) cycle (16/8 h) in high R/FR$_{high\ PAR}$ (PAR 111) and then either maintained in the same regimen or exposed to high R/FR$_{low\ PAR}$ (PAR 27) or to low R/FR$_{low\ PAR}$ (PAR 27) for 1, 2, 4, 8, and 12 h. No significant difference was observed in the expression of several genes rapidly induced by low R/FR, such as *ATHB2* (Carabelli et al., 1996), *PIL1* (Salter et al., 2003), and *HFR1/SICS1* (Sessa et al., 2005), between high R/FR$_{high\ PAR}$ and high R/FR$_{low\ PAR}$, thus demonstrating that these genes are specifically regulated at the transcriptional level by light quality changes under our simulated shade environment (Supplemental Fig. S1A). Hypocotyl elongation rate upon exposure to high R/FR$_{low\ PAR}$ was also measured. To this end, Col-0 seedlings were grown for 4 d in a L/D cycle in high R/FR$_{high\ PAR}$ and then either maintained in the same regimen or exposed to high R/FR$_{low\ PAR}$ or to low R/FR$_{low\ PAR}$ for 1 d. No significant difference was observed in hypocotyl elongation in high R/FR$_{low\ PAR}$ relative to high R/FR$_{high\ PAR}$, indicating that the increase in elongation rate observed upon exposure to low R/FR$_{low\ PAR}$ is induced by the change in the ratio of R to FR (Supplemental Fig. S1B).

Genome-Wide Expression Profiling during the Shade-Avoidance Response

To analyze the dynamics of the shade-avoidance response at a genome-wide scale, we inspected gene expression global profiles upon brief (1 and 4 h) and prolonged (1 d) exposure to low R/FR by means of Affymetrix Arabidopsis Genome GeneChip array (ATH1) analyses on 8-d-old Arabidopsis seedlings. A total of 399 genes were identified as differentially regulated by low R/FR (Supplemental Tables S1–S3). Among them, we could find a number of genes whose FR-rich light regulation has been previously demonstrated. To this group, among others, belong the genes encoding the HD-Zip transcription factors ATHB2 and ATHB4, the atypical bHLH HLH1/PAR1, the auxin efflux carrier PIN3, the IAA proteins IAA1 and IAA3, and the floral inducer FLOWERING LOCUS T (FT; Carabelli et al., 1993, 1996; Devlin et al., 2003; Sessa et al., 2005; Roig-Villanova et al., 2006; Tao et al., 2008; Keuskamp et al., 2010; Leivar et al., 2012). Moreover, although we could not observe *PIL1* transcriptional regulation in our experiments because the Affymetrix ATH1 GeneChip lacks a probe set for this gene (Salter et al., 2003), its homolog *PIL2*, previously shown to be regulated by light quality changes (Salter et al., 2003), was found to be significantly induced upon 4 h of exposure to low R/FR (Supplemental Tables S1–S3).

To gain insights into the large number of low-R/FR-regulated genes, we proceeded to cluster them into different functional groups according to the putative or established gene functions in the plant, taking

advantage of the Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional annotation clustering (Huang et al., 2009). For each time point, the most enriched groups (top 10 ranked with EASE score [a modified Fisher Exact P-Value] greater than 1) were identified and described by means of a Gene Ontology (GO) term (descriptor). Intriguingly, the relative abundance of each functional class varies significantly between genes early and late regulated by low R/FR (Supplemental Tables S4–S6).

Among the very early-regulated genes (1 h of low R/FR), transcription factor- and hormone-related genes were significantly more abundant than other functional classes. In agreement with the major role of auxin in shade avoidance (Steindler at al., 1999; Carabelli et al., 2007; Tao et al., 2008; Keuskamp et al., 2010; Li et al., 2012), DAVID analysis evidenced a highly significant overrepresentation of "response to auxin stimulus" and "auxin-mediated signaling pathway" functional classes, whereas the importance of transcriptional regulation at the early stages of shade avoidance (Carabelli et al., 1993, 1996; Salter et al., 2003; Sessa et al., 2005; Roig-Villanova et al., 2006; Sorin et al., 2009) was highlighted by the strong enrichment of "transcription regulator activity" and "basic helix-loop-helix dimerization region" class descriptors. Furthermore, consistent with evidence on the existence of cross talk between auxin and brassinosteroid signal transduction pathways active in the modulation of plant growth and tropic responses (Bao et al., 2004; Nemhauser et al., 2004; Nakamoto et al., 2006; Keuskamp et al., 2011), besides R and FR signaling pathway overrepresentation, we found that "brassinosteroid metabolic process" and "developmental growth involved in morphogenesis" classes were also enriched among genes rapidly regulated by simulated shade (Supplemental Table S4).

After 4 h of low R/FR, DAVID analysis revealed a behavior partially similar to that observed at the very early stages of shade avoidance, further highlighting the involvement of transcription factors ("basic helix-loop-helix dimerization region") and auxin pathways ("response to auxin stimulus" and "auxin-mediated signaling pathway") in plant responses to light quality changes. However, overrepresentation of these functional classes among low-R/FR-regulated genes was weaker, and some differences emerged (Supplemental Table S5). Among them is a significant overrepresentation of ethylene signaling genes ("regulation of ethylene-mediated signaling pathway"), supporting recent evidences on the function of this hormone in the shade-avoidance response (Pierik et al., 2009) and suggesting a strict temporal coordination of hormone interplay. Moreover, DAVID analysis highlighted a role for sulfur metabolism, probably related to secondary pathways ("regulation of glycosinolate biosynthetic process" and "sulfotransferase activity"; Supplemental Table S5).

Among the late-regulated genes (1 d of low R/FR), class enrichment scores appeared to be, on average, lower than among the early ones. The most overrepresented functional classes were related to light signaling ("response to red or far-red light" and "phototransduction"; Supplemental Table S6). Moreover, in agreement with the down-regulation of the jasmonic acid response by prolonged exposure to low R/FR (Moreno et al., 2009; Robson et al., 2010), the "response to jasmonic acid stimulus" class descriptor was also overrepresented. Finally, at late stages of shade avoidance, DAVID analysis highlighted a rearrangement of cellular metabolism and circadian rhythm pathways (Supplemental Table S6).

The finding that the gene expression pattern is dynamic in a low-R/FR environment suggests that the elongation growth response to simulated shade might change over time. To investigate whether this is the case, the effect of low R/FR on hypocotyl growth during days 1 and 2 of exposure to light enriched in FR was measured. Elongation occurs during both days 1 and 2. However, the growth response is promoted significantly more during day 1 than day 2 of exposure to low R/FR (Fig. 1).

Deriving Functional Associations between Shade-Avoidance Transcript Profiles and Available Microarray Gene Expression Data

The DAVID functional analysis of the genes regulated by low R/FR indicated that shade-driven transcriptional alterations are highly dynamic, involving profound changes in the regulation of gene expression over time. However, the analysis of some functional classes (e.g. "response to red or far-red light") suggests that DAVID may be not entirely effective in describing the dynamics of light-regulated genes. To overcome this limitation, we inspected in more detail the genes regulated by low R/FR by means of the FARO Web tool, which compares genome-wide expression profiles of a query (our experiments) with a large number of studies ("factors") from a microarray data repository ("response compendium") to find a significant functional overlap (Nielsen et al., 2007). For each time point (1 h, 4 h, and 1 d), we defined significantly overlapping groups as the top 15 ranked ones with more than 25% of genes shared with repository experiments. A large majority of top-ranked associated factors by FARO turned out to be composed of light- and hormone-related experiments, which were selected for further analysis (Supplemental Tables S7–S9). Remarkably, we found an opposite regulation of genes regulated in deetiolation experiments with respect to early shade-regulated genes and consistent regulation for late-regulated ones (Fig. 2; Supplemental Table S7). In fact, among the 12 factors showing significant overlap with genes regulated in the wild type upon 1 h of exposure to low R/FR, 10 of them pertain to deetiolation experiments and always display a congruence lower than 30% (Fig. 2; Supplemental Table S7). A similar result was observed for genes regulated upon 4 h of exposure to low R/FR (Fig. 2; Supplemental Table S8). By contrast, FARO analyses highlighted a strong congruence between genes late regulated by
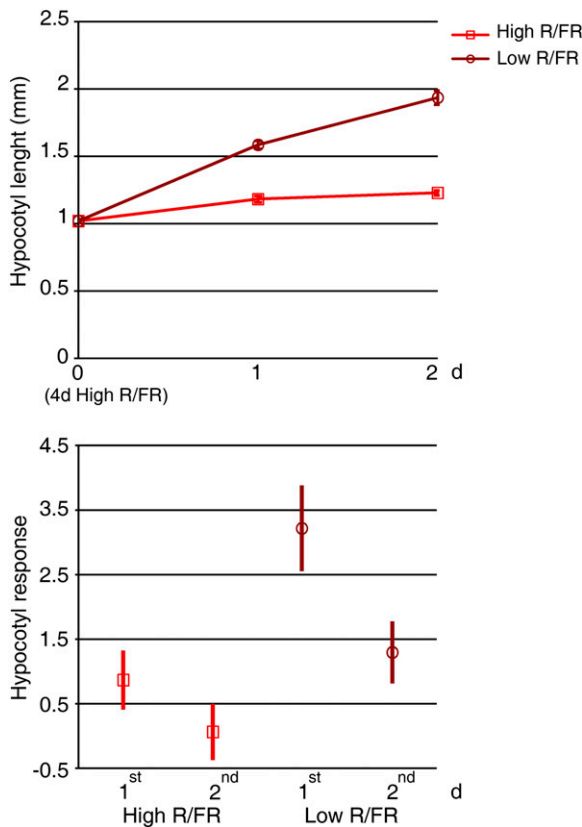
Figure 1. The growth elongation response to low R/FR decreases over time. Col-0 seedlings were grown in a L/D cycle (16/8 h) for 4 d in high R/FR (control) and then maintained in high R/FR or transferred to low R/FR for 1 and 2 d under the same light regimen. The top graph shows hypocotyl lengths (means ± SE) of wild-type seedlings grown as described. At least 30 seedlings were analyzed for each condition. The bottom graph shows the strength of the hypocotyl elongation response of wild-type seedlings during days 1 and 2 in low R/FR. High-R/FR controls are also shown. Symbols (squares and circles) and vertical lines depict the standardized effect size values and 95% confidence intervals, respectively (Hedges and Olkin, 1985).

low R/FR (1 d) and deetiolation experiments (Fig. 2; Supplemental Table S9). Moreover, a clear positive correlation of an early shade-avoidance response with auxin- and ethylene-responsive genes was also shown by FARO (Fig. 2).

Inference of Functional Gene Networks in the Shade-Avoidance Response

For each functional cluster obtained by DAVID analysis of the genes differentially expressed in the wild type after exposure to low R/FR for different times, the genes associated with corresponding functional annotations were extracted to infer gene regulatory networks by means of the GeneMANIA prediction server, utilizing a large set of available genomics and proteomics data (protein, physical and genetic interactions, coexpression, and colocalization data) stored in

its database, and assigning an equal weight to all different data sets. Each cluster was extended with associated genes considering the 10 top-scored ones by GeneMANIA. Then, we removed the genes poorly connected (e.g. networks composed of less than three nodes) to reduce prediction bias. Hence, starting from these extended clusters, we ran a further GeneMANIA algorithm on a single time-point level. This strategy globally resulted in three different gene networks depicting the shade-avoidance response during time (Figs. 3 and 4; Supplemental Fig. S2).

In the very early stages of shade avoidance, gene networks underscore the connections among the phy-PIF signaling pathway (Bae and Choi, 2008; Leivar and Quail, 2011), transcription factors (e.g. HD-Zip II; Ruberti et al., 2012), and hormones (Fig. 3). The genes involved in phytochrome transduction pathways form a clear subnetwork, connected also with *GIBBERELLIN INSENSITIVE* (*GAI*) and *SLEEPY1* (*SLY1*), key gibberellin signaling components (Hartweck, 2008; Harberd et al., 2009). Moreover, this subnetwork is mostly related to the auxin signaling pathway (Chapman and Estelle, 2009), further supporting the central role of this hormone during early phases of the shade-avoidance response. Another subnetwork is composed of HD-Zip genes and highlights the interaction among family II members with a documented role in the shade-avoidance response (*ATHB2*, *ATHB4*, *HOMEOBOX ARABIDOPSIS THALIANA1* [*HAT1*], *HAT2*, *HAT3*; Carabelli et al., 1996; Steindler et al., 1999; Sessa et al., 2005; Ciarbelli et al., 2008, Sorin et al., 2009) and family I members (*ATHB5*, *ATHB7*, and *ATHB12*; Henriksson et al., 2005). Finally, GeneMANIA evidenced a tight cluster of genes involved predominantly in sulfur primary metabolism (*ADENOSINE-5'-PHOSPHOSULFATE REDUCTASES* [*APRs*] and *ADENOSINE-5'-PHOSPHOSULFATE KINASES* [*APKs*]; Kopriva et al., 2009; Mugford et al., 2011; Fig. 3).

After 4 h of low R/FR, networks show a higher number of genes linked in two major interwoven subnetworks. The first one is related to phytochrome signaling pathways similar to 1 h; however, beyond the connection with *GAI* and *SLY1* emerges the association with ethylene signaling (Lin et al., 2009; Yoo et al., 2009). The second one is constituted of genes known to be involved in sulfate assimilation pathways, with a prominent role of secondary metabolism (*APRs-APKs-SULFOTRANSFERASES*; Kopriva et al., 2009; Mugford et al., 2011; Supplemental Fig. S2).

Interestingly, upon prolonged exposure to low R/FR (1 d), GeneMANIA analysis describes a different scenario with respect to the early phases of the shade-avoidance response: the phytochrome transduction pathways do not involve *PIFs*, whereas *HY5*, a crucial regulator of photomorphogenesis (Bae and Choi, 2008), gains a central position in this subnetwork (Fig. 4). The results also suggest casein kinases (*CKA1* and *CKBs*) and central circadian oscillator (*CCA1*) as points of convergence of different cues (Portolés and Más, 2010; Fig. 4). Furthermore, the analysis highlights smaller subnetworks containing genes belonging to metabolic
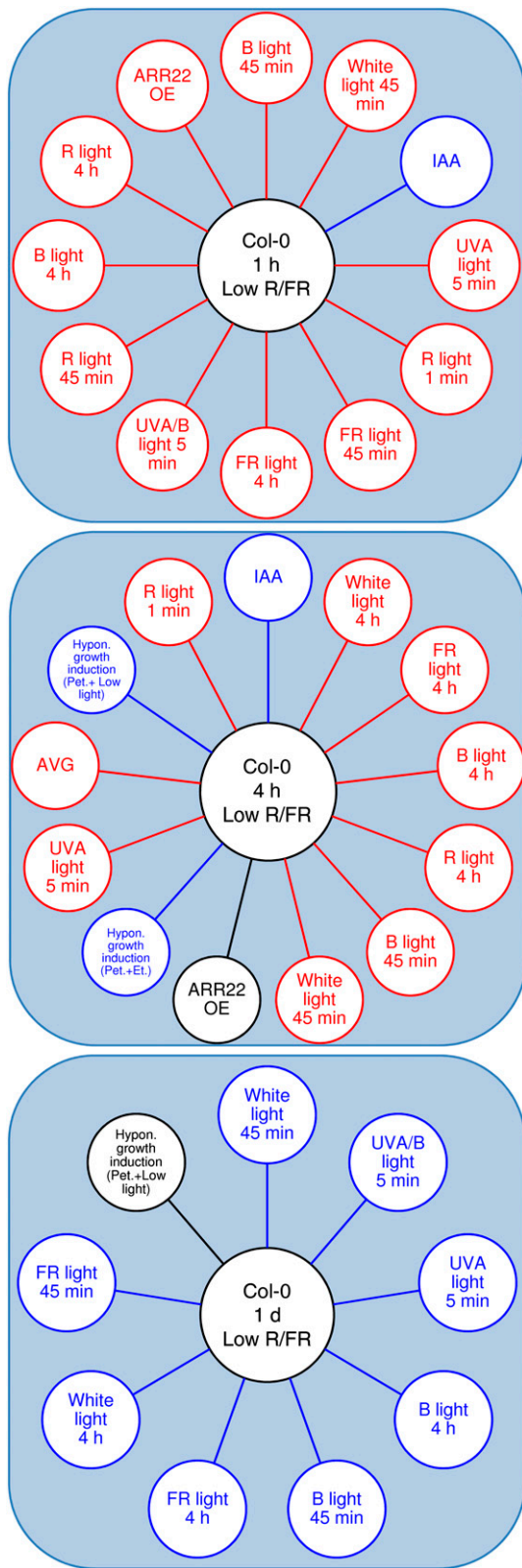
Figure 2. FARO of Arabidopsis seedlings exposed to low R/FR for different times. Low-R/FR-regulated genes involved in light and hormone pathways are visualized by means of the FARO Web tool, which compares genome-wide expression profiles of a query (our microarray

pathways, mainly in sulfur primary metabolism (APRs-APKs; Kopriva et al., 2009; Mugford et al., 2011; Fig. 4).

Promoter cis-Element Analysis of Genes Regulated at Different Times during Shade Avoidance

To gain further insights on transcriptional gene regulation during the shade-avoidance response, cis-acting element enrichment analysis was carried out by means of the Athena Web tool (O'Connor et al., 2005). We focused on the promoter regions (−2,000 bp, truncated if they overlap with an upstream gene) of the genes regulated by low R/FR upon 1 h, 4 h, or 1 d. For each promoter, we identified the top 30 enriched motifs with $P < 0.05$: upon score calculation, we displayed cis-elements nonredundantly by means of a heat map to highlight their overrepresentation at different time points (Fig. 5).

Promoter analyses further highlighted the complex, dynamic transcriptional regulation taking place during the shade-avoidance response. In fact, cis-elements related to hormonal pathways, mainly those of auxin ("ARF [for auxin response factor] binding site motif"; Liu et al., 1994; Ulmasov et al., 1995) and gibberellin ("GAREAT"; Ogawa et al., 2003), display a rapid and transient enrichment (Fig. 5). A similar behavior is depicted for HD-Zip II transcription factor-binding sites (e.g. "ATHB2 binding site motif," "ATHB6 binding site motif," and "ATHB5ATCORE"; Sessa et al., 1993, 1997), whereas the opposite is true for the DNA elements recognized by the key regulator of photomorphogenesis, HY5 ("HY5AT"; Chattopadhyay et al., 1998), and CHAL-CONE SYNTHASE (CHS; "MRE motif in CHS" and "ACE promoter motif"; Hartmann et al., 1998), which are significantly enriched only in late-regulated genes (Fig. 5). Moreover, consistent with DAVID analyses, cis-elements involved in phytochrome and light signaling, such as E-box/G-box (Toledo-Ortiz et al., 2003) or I-box/GATA (Giuliano et al., 1988; Terzaghi and Cashmore, 1995), display an overrepresentation both upon brief and prolonged low R/FR treatment (Fig. 5).

Genome-Wide Functional Analyses of Negative Regulators of the Shade-Avoidance Response

To further explore, at a genome-wide scale, the dynamics of the plant response to light quality changes,

experiments) with a large number of studies (factors) from a microarray data repository (response compendium) to find a significant functional overlap. The large majority of top-ranked associated factors by FARO were composed of experiments related to light and hormone signaling, which were selected for functional analysis (for details, see "Materials and Methods"). Genes differentially regulated in Col-0 after 1 h, 4 h, or 1 d of low-R/FR exposure are displayed in the central circles. In the outer circles, experimental factors with strong associations to the wild type exposed to low R/FR are shown. The factors showing overlap with the opposite direction (i.e. congruence less than 30%) with respect to our experiments are depicted in red; those showing the highest congruence (greater than 70%) are depicted in blue.
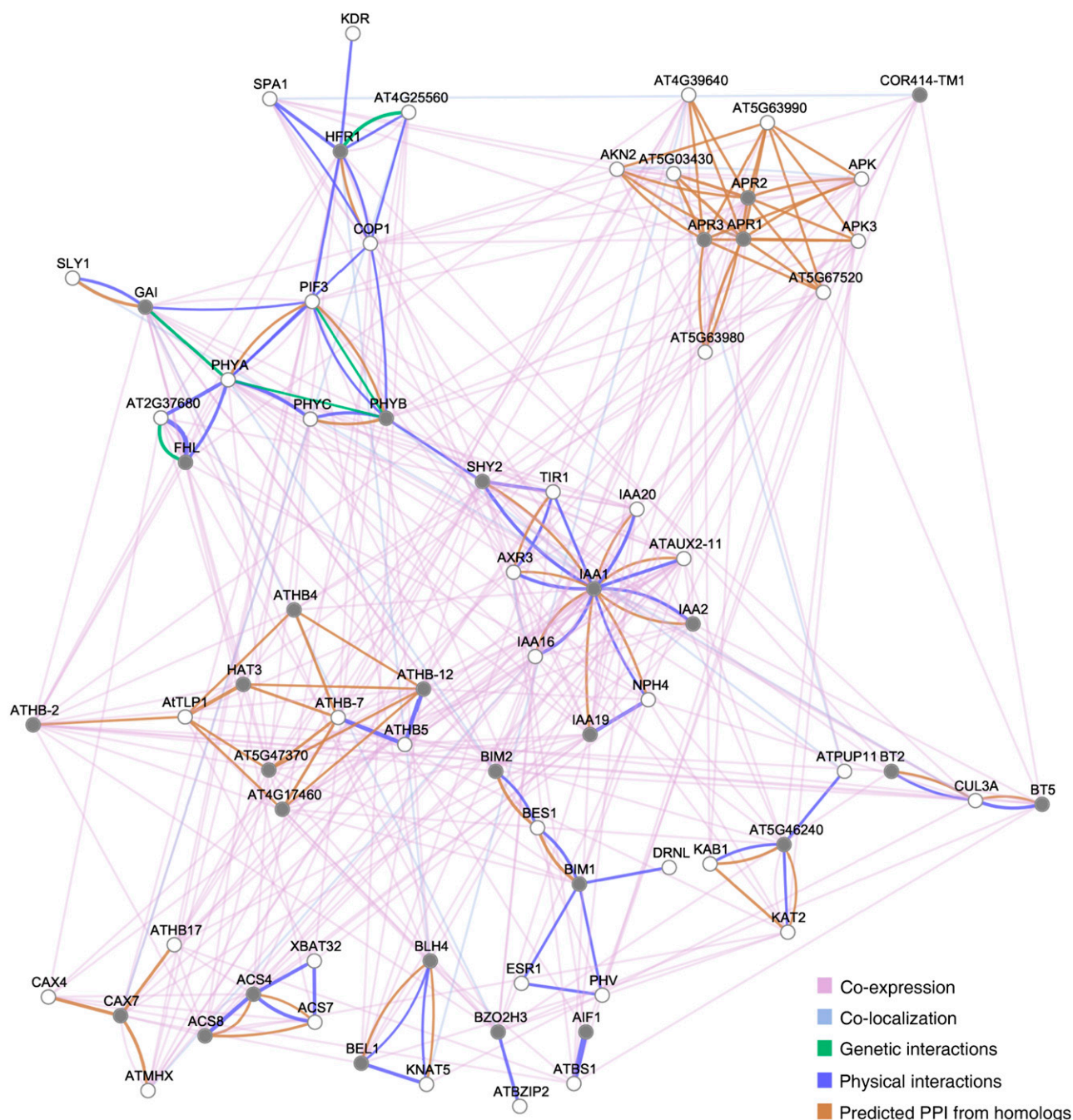
Figure 3. Network analysis of genes regulated very early in the shade-avoidance response. DAVID functional clusters of genes regulated upon 1 h of exposure to low R/FR were utilized to infer gene networks by means of the GeneMANIA server using available genomics and proteomics data. Networks are represented as graphs, in which the nodes correspond to genes and the edges correspond to interactions between them. Nodes colored with gray indicate significant differential gene expression with respect to the control in our array data (Supplemental Table S1; for details, see "Materials and Methods"). PPI, Protein-protein interactions.

we took advantage of loss-of-function mutants in negative regulators of shade avoidance (*hfr1/sics1* and *phyA*).

HFR1/SICS1, originally described as a downstream component of phyA and CRYPTOCHROME1 in the deetiolation process (Fairchild et al., 2000; Fankhauser

and Chory, 2000; Soh et al., 2000; Duek and Fankhauser 2003; Bae and Choi, 2008), has been subsequently demonstrated to be specifically regulated by light quality changes and to act as a master negative regulator of the shade-avoidance response (Sessa et al.,
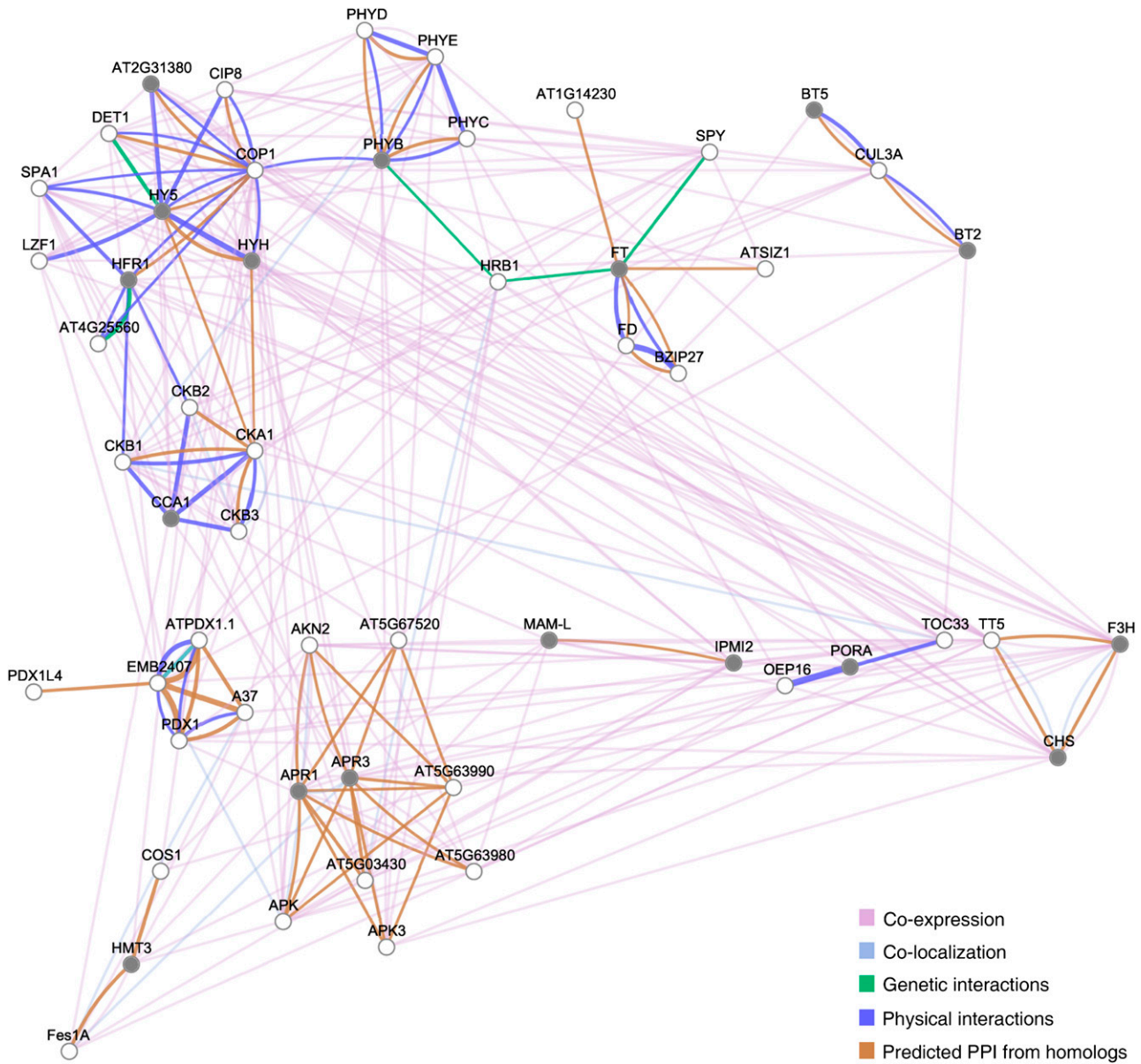
Figure 4. Network analysis of genes regulated late in the shade-avoidance response. DAVID functional clusters of genes regulated upon 1 d of exposure to low R/FR were utilized to infer gene networks by means of the GeneMANIA server using available genomics and proteomics data. Networks are represented as graphs, in which the nodes correspond to genes and the edges correspond to interactions between them. Nodes colored with gray indicate significant differential gene expression with respect to the control in our array data (Supplemental Table S3; for details, see "Materials and Methods"). PPI, Protein-protein interactions.

2005; Hornitschek et al., 2009). The rapid induction of *HFR1/SICS1* by low R/FR as well as its reversibility by high R/FR have strongly suggested the involvement of type II phytochromes in the regulation of this gene during shade avoidance (Sessa et al., 2005). Consistent with this hypothesis, *HFR1/SICS1* is significantly up-regulated in *phyB-9* seedlings in high R/FR (Lorrain et al., 2008; Supplemental Fig. S3A); the expression level of *HFR1/SICS1* in *phyB-9* in high R/FR is comparable to that observed in the wild type briefly exposed

to low R/FR (Supplemental Fig. S3A), implying a major role for phyB in the regulation of *HFR1/SICS1* expression by light quality changes. To investigate whether the up-regulation of *HFR1/SICS1* in the *phyB-9* mutant is functionally relevant, we constructed the double mutant *hfr1-4/sics1-1 phyB-9* and analyzed its phenotype in high R/FR. As expected, *phyB-9* is extremely elongated relative to the wild type (Reed et al., 1993; Supplemental Fig. S3B). However, in accordance with HFR1/SICS1 acting as a negative regulator of elongation

Figure 5. Overrepresented DNA motifs on promoters of genes regulated in Col-0 seedlings upon exposure to low R/FR. The heat map displays the *P* values of significantly overrepresented cis-elements (top-ranked 30 motifs with *P* < 0.05, calculated using a hypergeometric distribution) in the wild type upon low-R/FR exposure for different times, identified by means of the Athena Web tool (http://www.bioinformatics2.wsu.edu/Athena).

Motif Name	Col-0 1 h Low R/FR	Col-0 4 h Low R/FR	Col-0 1 d Low R/FR	Sequence
MYCATERD1				CATGTG
AtMYC2 BS in RD22				CACATG
CARGCW8GAT				CWWWWWWWWG
MYB4 binding site motif				AMCWAMC
GAREAT				TAACAAR
Ibox promoter motif				GATAAG
ABRE-like binding site motif				BACGTGKM
TATA-box Motif				TATAAA
ARF binding site motif				TGTCTC
CACGTGMOTIF				CACGTG
MYB1AT				WAACCA
SV40 core promoter motif				GTGGWWHG
W-box promoter motif				TTGACY
UPRMOTIFIAT				CCACGTCA
TGA1 binding site motif				TGACGTGG
DRE core motif				RCCGAC
MYB binding site promoter				MACCWAMC
AtMYB2 BS in RD22				CTAACCA
GBF1/2/3 BS in ADH1				CCACGTGG
MYB2AT				TAACTG
BoxII promoter motif				GGTTAA
T-box promoter motif				ACTTTG
ABRE binding site motif				YACGTGGC
ATHB2 binding site motif				TAATMATTA
L1-box promoter motif				TAAATGYA
Gap-box Motif				CAAATGAA
ABFs binding site motif				CACGTGGC
EveningElement promoter motif				AAAATATCT
ACGTABREMOTIFA2OSEM				ACGTGKC
GBOXLERBCS				MCACGTGGC
RY-repeat promoter motif				CATGCATG
ATHB6 binding site motif				CAATTATTA
CArG promoter motif				CCWWWWWWGG
ATHB5ATCORE				CAATNATTG
GADOWNAT				ACGTGTC
MYB1 binding site motif				MTCCWACC
LEAFYATAG				CCAATGT
MRE motif in CHS				TCTAACCTACCA
ACE promoter motif				GACACGTAGA
HY5AT				TGACACGTGGCA

P value: < 10⁻⁵ to > 10⁻¹

growth, the hypocotyls of *hfr1-4/sics1-1 phyB-9* seedlings are significantly longer than those of *phyB-9* (Supplemental Fig. S3B). Furthermore, several genes early induced by low R/FR (Carabelli et al., 1996; Devlin et al., 2003; Salter et al., 2003; Sessa et al., 2005; Roig-Villanova et al., 2006) are upregulated in *hfr1-4/sics1-1 phyB-9* relative to *phyB-9* in high R/FR (Supplemental Fig. S3C).

Several findings have indicated the involvement of phyA in attenuating the elongation response to low R/FR (Johnson et al., 1994; Devlin et al., 2003; Wang et al., 2011). Consistently, *phyA-211* mutants display an exaggerated elongation response upon 1 and 2 d of exposure to our simulated shade environment relative to the wild type (Supplemental Fig. S4).

To verify that the elongated phenotype of *hfr1-4/sics1-1* and *phyA-211* under simulated shade (Sessa et al., 2005; Supplemental Fig. S4) does indeed depend on low R/FR, control experiments were performed lowering PAR without changing the ratio between R and FR. Col-0, *hfr1-4/sics1-1*, and *phyA-211* seedlings

were grown for 4 d in a L/D cycle in high R/FR$_{high\ PAR}$ and then either maintained in the same regimen or exposed to high R/FR$_{low\ PAR}$ for 1 and 2 d. No significant difference was observed in the hypocotyl elongation of *hfr1-4/sics1-1* and *phyA-211* relative to the wild type upon exposure to high R/FR$_{low\ PAR}$, indicating that the increase in elongation rate observed upon exposure to low R/FR$_{low\ PAR}$ is induced by the change in the ratio of R to FR (Supplemental Fig. S4). Interestingly, whereas the growth response to low R/FR is reduced over time in the wild type, that of both *hfr1-4/sics1-1* and *phyA-211* occurs with little variation between days 1 and 2 (Supplemental Fig. S4).

To gain further genome-wide insights into the dynamics of shade avoidance, we analyzed global expression profiles of *hfr1-4/sics1-1* (Sessa et al., 2005; Supplemental Table S10) and *phyA-211* (Supplemental Table S11) mutants upon prolonged exposure to low R/FR (1 d) by means of the DAVID functional classification and selected the enriched GO terms corresponding

to the ones present in the most enriched functional annotation clusters in the wild type upon 1 h, 4 h, and 1 d of low R/FR (top 10 ranked with EASE score greater than 1; Supplemental Tables S4–S6). We then used a heat map to evaluate the enrichment of each GO descriptor in the wild type at different times during shade avoidance and in the mutants upon prolonged exposure to low R/FR (Fig. 6). The "shade avoidance" functional class is enriched in both *hfr1-4/sics1-1* and *phyA-211* upon prolonged exposure to FR-rich light, similar to the wild type upon brief low R/FR treatment. Furthermore, transcription factors ("transcription regulator activity") and auxin ("response to hormone stimulus" and "response to auxin stimulus") classes, which are the ones most enriched in wild-type seedlings briefly exposed to FR-rich light, are overrepresented in both *hfr1-4/sics1-1* and *phyA-211* mutants upon prolonged exposure to low R/FR (Fig. 6).
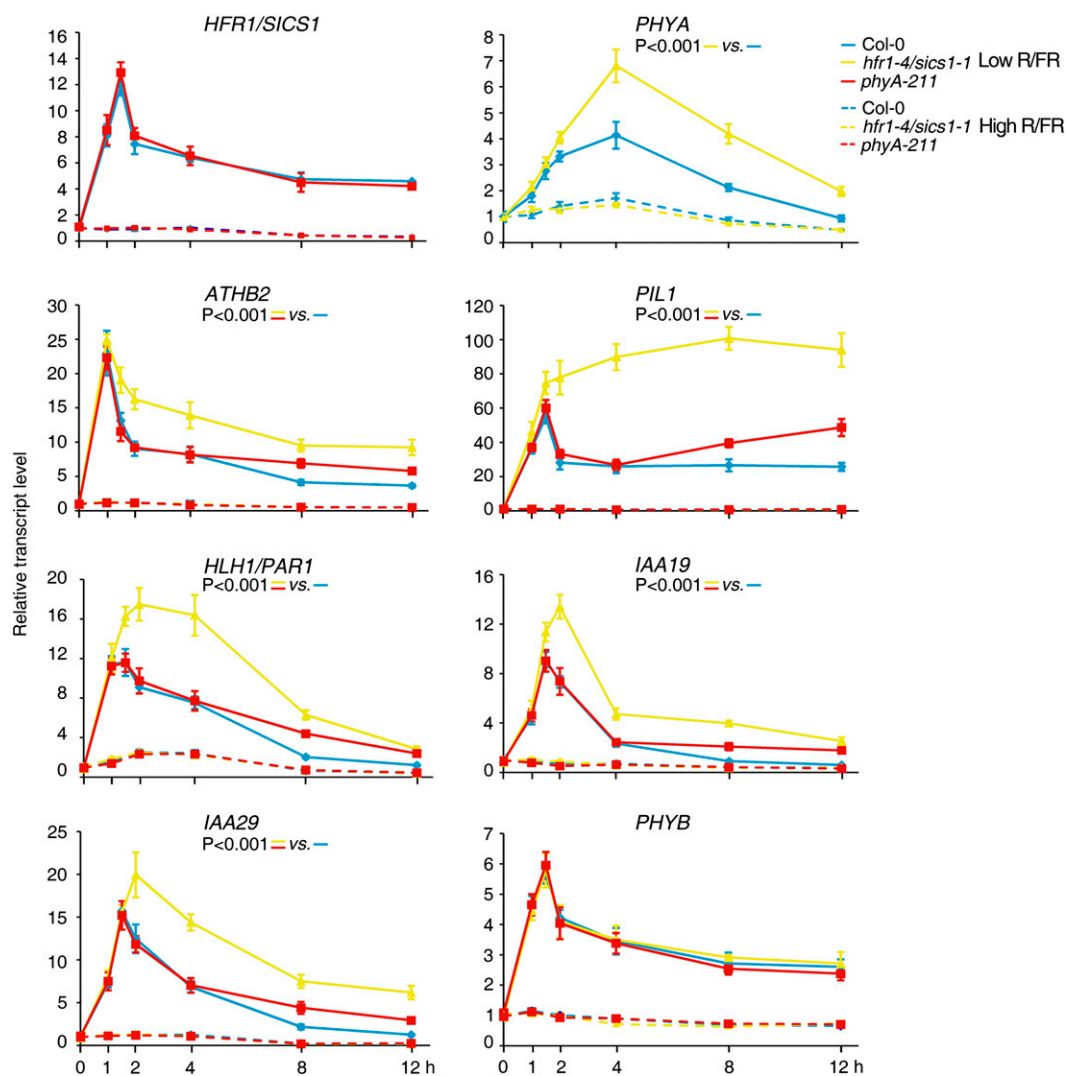
To understand the temporal dynamics of gene expression changes in seedlings lacking functional HFR1/SICS1 and PHYA proteins with respect to the wild type upon exposure to the simulated shade environment, we performed low-R/FR kinetics experiments in Col-0, *hfr1-4/sics1-1*, and *phyA-211* and analyzed the expression of key regulators of plant responses to FR-rich light. No significant difference was observed in the induction of several genes early regulated by low R/FR in *hfr1-4/sics1-1* and *phyA-211* relative to the wild type. By contrast, the transcript

levels of all of these genes are significantly higher in both *hfr1-4/sics1-1* and *phyA-211* mutants than the wild type at later times of exposure to low R/FR (Fig. 7; Sessa et al., 2005). Interestingly, the expression profile of genes early induced by light quality changes is different in *hfr1-4/sics1-1* and *phyA-211* mutants. Several transcription factor genes, such as *ATHB2* and *PIL1*, functionally implicated in the shade-avoidance response (Steindler et al., 1999; Salter et al., 2003), as well as *Aux/IAA* genes, rapidly induced by FR-rich light, are significantly up-regulated upon 2 and 8 h of low R/FR exposure in *hfr1-4/sics1-1* and *phyA-211* mutant seedlings, respectively (Fig. 7). At the later times (8 and 12 h), transcript levels of genes rapidly regulated by low R/FR are significantly higher in both mutants (Fig. 7). Consistent with *HFR1/SICS1* being largely regulated through phyB in photoautotrophic seedlings (Supplemental Fig. S3), no significant difference was observed in the *HFR1/SICS1* transcript levels in *phyA-211* seedlings relative to the wild type upon exposure to low R/FR (Fig. 7).

To investigate whether phyA indeed acts independently of HFR1/SICS1 in low R/FR, we constructed the double mutant *hfr1-4/sics1-1 phyA-211* and analyzed its phenotype. No major difference was observed in *hfr1-4/sics1-1 phyA-211* relative to *hfr1-4/sics1-1* and *phyA-211* in high R/FR (Supplemental Table S12). By contrast, the phenotype of the double mutant is extremely more severe than those of *hfr1-4/sics1-1*

Figure 6. Heat map of GO descriptor enrichment in Col-0 and mutants upon exposure to low R/FR. The heat map displays the *P* values of enriched GO descriptors identified by means of DAVID in the wild type upon low-R/FR exposure for different times (10 leading ranks with EASE score greater than 1 for each time; Supplemental Tables S4–S6). The *P* values of the same GO descriptors in *hfr1-4/sics1-1* and *phyA-211* seedlings upon prolonged exposure to low R/FR are also shown.

| GO descriptor	Col-0 1 h Low R/FR	Col-0 4 h Low R/FR	Col-0 1 d Low R/FR	hfr1-4/ sics1-1 1 d Low R/FR	phyA-211 1 d Low R/FR	P value
Transcription regulator activity						< 10⁻¹⁰
Developmental growth involved in morphogenesis						
Tropism						
Phototropism						
Phototransduction						
Response to red or far red light						> 10⁻¹
Shade avoidance						
Response to hormone stimulus						
Response to auxin stimulus						
Auxin mediated signaling pathway						
Brassinosteroid metabolic process						
Regulation of ethylene mediated signaling pathway						
Sulfate assimilation						
Adenylyl-sulfate reductase activity						
Sulfur compound biosynthetic process						
Glycosinolate biosynthetic process						
Regulation of glycosinolate biosynthetic process						
Protein histidine kinase activity						
Lipid transport						
Circadian rhythm						
Sulfotransferase activity						
Cellular homeostasis						
Hyperosmotic response						

Figure 7. phyA is required to down-regulate genes early induced by low R/FR. Col-0, *hfr1-4/sics1-1*, and *phyA-211* seedlings were grown for 7 d in a L/D cycle (16/8 h) in high R/FR and then maintained in high R/FR or transferred to low R/FR for different times. The graphs show the relative expression levels in high (dashed lines) and low (solid lines) R/FR of *HFR1/SICS1*, *PHYA*, *ATHB2*, *PIL1*, *HLH1/PAR1*, *IAA19*, and *IAA29* in the different genotypes. Each value is the mean ± SD of three biological replicates normalized to *EF1α* expression. *PHYB* is a light-regulated gene not differentially expressed in mutant seedlings relative to the wild type. Statistical significances between different backgrounds were assessed by means of two-way ANOVA followed by the Bonferroni posthoc test.

and *phyA-211* single mutants in low R/FR (Fig. 8A; Supplemental Fig. S5). Particularly striking is the appearance of elongated internodes such that under low R/FR, *hfr1-4/sics1-1 phyA-211* plants no longer display a rosette habit (Supplemental Fig. S6). Consistent with the extremely exaggerated phenotype of the double mutant in low R/FR, the expression of several genes rapidly and transiently induced by low R/FR is significantly higher in *hfr1-4/sics1-1 phyA-211* relative to both *hfr1-4/sics1-1* and *phyA-211* upon prolonged exposure to FR-rich light (Fig. 8, B and C).

Previous work has shown that *hfr1/sics1* mutants display an early-flowering phenotype in low R/FR (Sessa et al., 2005; Supplemental Fig. S7, A and B).

Consistent with this, it has also been shown that the expression of *FT*, encoding a key integrator of different floral induction signals including low R/FR (Cerdán and Chory, 2003; Casal et al., 2004; Kim et al., 2008; Wollenberg et al., 2008; Adams et al., 2009), was substantially higher in *hfr1/sics1* relative to the wild type (Sessa et al., 2005; Supplemental Fig. S7C). Recent work has demonstrated that the HFR1/SICS1 protein interacts with PIF transcription factors forming non-DNA-binding heterodimers, thus limiting PIF-mediated gene expression (Hornitschek et al., 2009). Furthermore, it has been reported that PIF4 directly regulates *FT* at high temperature (Franklin et al., 2011; Kumar et al., 2012). It seems likely, therefore, that the higher *FT*
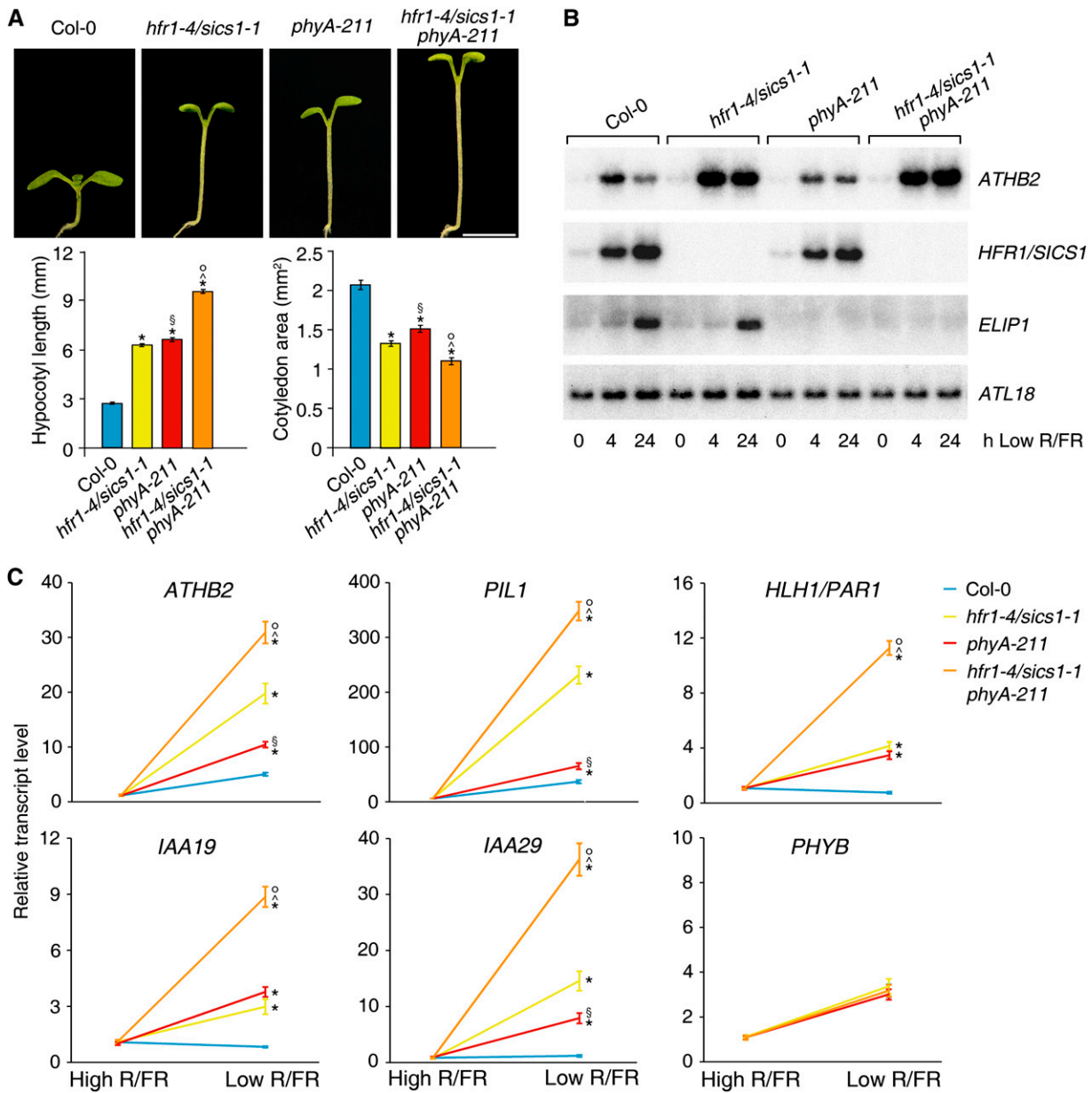
Figure 8. HFR1/SICS1 and phyA act largely independently in the regulation of the shade-avoidance response. A, Col-0, *hfr1-4/sics1-1*, *phyA-211*, and *hfr1-4/sics1-1 phyA-211* seedlings grown for 4 d in a L/D cycle (16/8 h) in high R/FR and then transferred to low R/FR for 4 d under the same L/D regimen. The histograms depict the mean ± SE hypocotyl lengths (left) and the mean ± SE cotyledon areas (right) of seedlings grown as described. At least 30 seedlings were analyzed for each line. Statistical significance was assessed by means of one-way ANOVA followed by Tukey's test. *$P < 0.01$ for *hfr1-4/sics1-1*, *phyA-211*, and *hfr1-4/sics1-1 phyA-211* versus Col-0; §$P < 0.01$ for *phyA-211* versus *hfr1-4/sics1-1*; ^$P < 0.01$ for *hfr1-4/sics1-1 phyA-211* versus *hfr1-4/sics1-1*; °$P < 0.01$ for *hfr1-4/sics1-1 phyA-211* versus *phyA-211*. Bar = 3 mm. B, Northern-blot analyses of *ATHB2* and *HFR1/SICS1* in Col-0, *hfr1-4/sics1-1*, *phyA-211*, and *hfr1-4/sics1-1 phyA-211*. Plants were grown for 7 d in a L/D cycle (16/8 h) in high R/FR (0) and then transferred to low R/FR under the same L/D regimen. Plant transfer to low R/FR was performed 4 h after the beginning of the light period, and seedlings were harvested 4 and 24 h later for northern-blot analyses. *ELIP1* is a gene not differentially regulated by low R/FR in *hfr1/sics1* mutants relative to the wild type. *ATL18* was used to monitor equal loading. C, RT-qPCR analyses of *ATHB2*, *PIL1*, *HLH1/PAR1*, *IAA19*, and *IAA29* in Col-0, *hfr1-4/sics1-1*, *phyA-211*, and *hfr1-4/sics1-1 phyA-211*. Plants were grown for 7 d in a L/D cycle (16/8 h) in high R/FR and then either maintained in high R/FR or transferred to low R/FR under the same L/D regimen for 1 d. Plant transfer to low R/FR was performed 4 h after the beginning of the light period. The graphs show the relative expression levels in high and low R/FR of *ATHB2*, *PIL1*, *HLH1/PAR1*, *IAA19*, and *IAA29* in the different genotypes. *PHYB* is a gene not differentially regulated by low R/FR in mutant seedlings relative to the wild type. Each value is the mean ± SD of five biological replicates normalized to *EF1α* expression. Statistical significance was assessed by means of one-way ANOVA followed by Tukey's test. *$P < 0.001$ for *hfr1-4/sics1-1*, *phyA-211*, and *hfr1-4/sics1-1 phyA-211* versus Col-0; §$P < 0.001$ for *phyA-211* versus *hfr1-4/sics1-1*; ^$P < 0.001$ for *hfr1-4/sics1-1 phyA-211* versus *hfr1-4/sics1-1*; °$P < 0.001$ for *hfr1-4/sics1-1 phyA-211* versus *phyA-211*.

transcript levels observed in *hfr1/sics1* in low R/FR might result from the increased PIF protein activity in this mutant. By contrast, *phyA* mutants are late flowering in low R/FR (Johnson et al., 1994; Supplemental Fig. S7), and a role of phyA in enhancing the stability of CONSTANS (CO), a positive regulator of *FT* expression, has been demonstrated (Valverde et al., 2004). Consistent with HFR1/SICS1 and phyA acting largely independently of one another, *hfr1/sics1 phyA-211* plants flower significantly earlier than *phyA-211* under low R/FR (Supplemental Fig. S7).

FARO of *hfr1/sics1* and *phyA* Mutants upon Prolonged Exposure to Low R/FR

FARO analyses of *hfr1-1/sics1-4* and *phyA-211* showed that both mutants still have a strong association with IAA profiles upon prolonged exposure to low R/FR (Fig. 9, IAA; Supplemental Tables S13 and S14), further supporting the functional relevance of auxin-related pathway enrichment highlighted by DAVID during early phases of the shade-avoidance response, followed later by the attenuation of this process through the action of HFR1/SICS1 and phyA (Fig. 6). FARO results also revealed a highly significant overlap of *hfr1-1/sics1-4* and *phyA-211* mutants exposed to low R/FR for a prolonged time with deetiolation experiments (Fig. 9). However, strikingly, the direction of the response was dissimilar, as observed in the wild type at the early stages of shade avoidance (Figs. 2 and 9).

To examine further the effects of the *hfr1-1/sics1-4* and *phyA-211* mutations, a visual inspection of gene expression profiles during the shade-avoidance response in wild-type and mutant seedlings was conducted by means of a heat map (Fig. 10). This analysis indicated that the great majority of early shade-induced genes (Supplemental Tables S1 and S2), which FARO depicts as auxin responsive (Fig. 9), were still up-regulated upon prolonged low R/FR in both *hfr1-4/sics1-1* and *phyA-211* mutants (Fig. 10A). By contrast, genes induced by simulated shade (Supplemental Tables S1–S3), which FARO identifies as regulated during deetiolation (Fig. 9), displayed a different expression profile between the two mutants (Fig. 10B). The genes rapidly and transiently induced by light quality changes in wild-type seedlings (e.g. *ATHB2*) were still up-regulated in both mutants upon prolonged exposure to low R/FR, whereas those late induced by low R/FR were mostly up-regulated in *hfr1/sics1* but not in *phyA-211* (Fig. 10B).

Light pathway genes regulated in the wild type and not in *phyA-211* upon prolonged exposure to low R/FR, extracted from the cluster that emerged from heat map analysis (Fig. 10B), were utilized to infer gene networks by means of GeneMANIA. Network analysis identified a subnetwork involving *HY5* (Fig. 11), thus suggesting that phyA might play a major role in the regulation of this transcription factor gene during the shade-avoidance response. Reverse transcription (RT)-quantitative PCR (qPCR) analyses demonstrated that

HY5 is indeed significantly induced by low R/FR in a phyA-dependent manner (Fig. 12A). The *PHYA* gene is itself induced by low R/FR (Devlin et al., 2003; Fig. 12A), and its up-regulation precedes that of *HY5* during shade avoidance (Fig. 12A). Moreover, no significant changes in *HY5* transcript levels were observed in low R/FR relative to high R/FR in *phyA-211* seedlings, thus demonstrating that *HY5* induction by light quality changes does depend on the action of phyA (Fig. 12A). Consistent with HFR1/SICS1 and phyA acting largely independently one from the other, no significant difference was observed in *HY5* induction by low R/FR in *hfr1-4/sics1-1* with respect to the wild type (Supplemental Fig. S8). In addition to *HY5*, among the genes generating the congruence between shade avoidance and deetiolation are also *HY5 HOMOLOG* (*HYH*) and *UNFERTILIZED EMBRYO SAC10* (*UNE10*; Fig. 11). There is evidence that HYH, a close homolog of HY5, has a role in the inhibition of hypocotyl elongation (Holm et al., 2002). The phenotype of the *hyh* mutant is evident only in blue light; however, increased levels of HYH can suppress the elongated phenotype of *hy5* in white light (Holm et al., 2002; Sibout et al., 2006). Furthermore, *hy5 hyh* double mutants have longer hypocotyls than *hy5* in white light (Sibout et al., 2006). *hy5 hyh* seedlings also display shoot phenotypes that are absent from the single mutants, including delayed leaf development and reduced vasculature (Sibout et al., 2006). Interestingly, based on both morphological and molecular phenotypes of *hy5* and *hy5 hyh* seedlings, it was proposed that HY5 and HYH act as negative regulators of auxin signaling (Sibout et al., 2006; Lau and Deng, 2010). *UNE10* encodes a bHLH transcription factor similar to PIF7 not yet characterized at the functional level (Leivar et al., 2008a). However, *UNE10* has been described as a gene rapidly induced in response to continuous FR during seedling deetiolation (Tepperman et al., 2004). Moreover, *UNE10* was found to be up-regulated in *phyB* but not in *phyA phyB* seedlings upon prolonged exposure to low R/FR (Devlin et al., 2003). RT-qPCR analyses demonstrated that *HYH* and *UNE10* are both significantly induced by low R/FR in a phyA-dependent manner (Fig. 12B). Furthermore, *HY5*, *HYH*, and *UNE10* are all up-regulated upon prolonged exposure to low R/FR (1 d) compared with high R/FR. This up-regulation occurs in Col-0 and *hfr1-4/sics1-1* and not in *phyA-211* (Fig. 12C).

To exclude that the observed effects were caused by the higher level of FR under our simulated shade conditions (see "Simulated Shade Environment"), phenotype and gene expression experiments were performed keeping FR constant and reducing R (low R/FR*; see "Materials and Methods"). *phyA-211* mutant seedlings display an exaggerated elongation response upon 1 and 2 d of exposure to low R/FR* relative to the wild type (Supplemental Fig. S9A). The *PHYA* gene is induced by low R/FR* (Supplemental Fig. S9B). *HY5*, *HYH*, and *UNE10* are all significantly up-regulated by low R/FR* in Col-0 and not in *phyA-211* (Supplemental Fig. S9C).
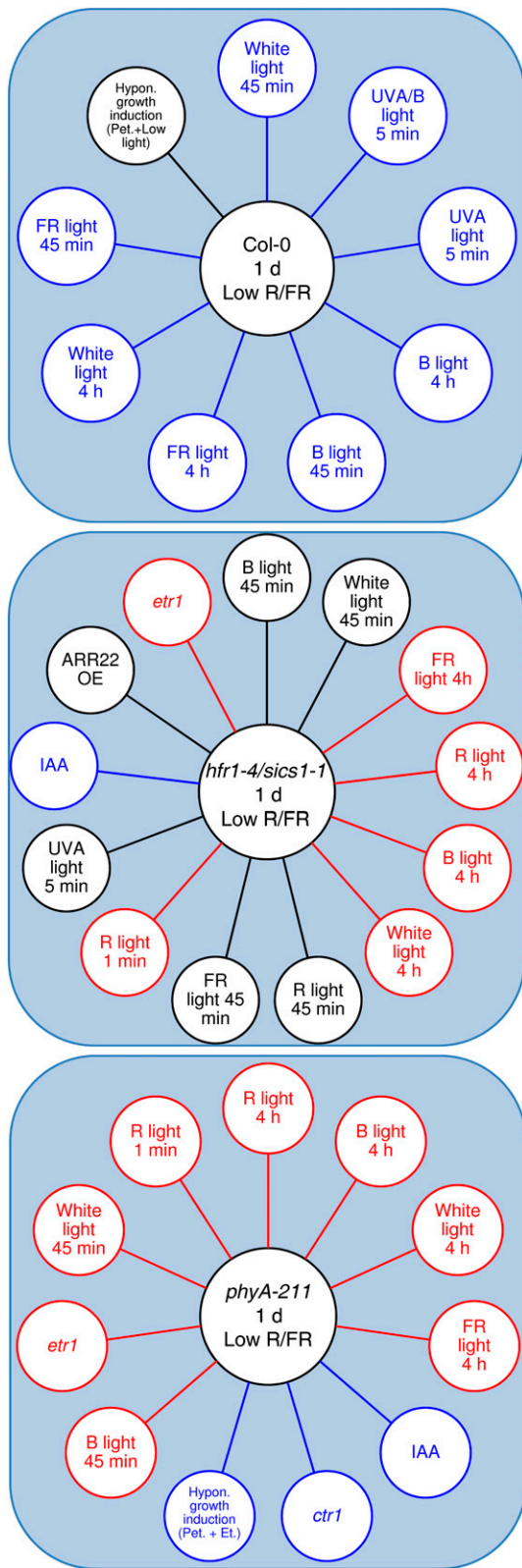
Figure 9. FARO of *hfr1-4/sics1-1* and *phyA-211* in low R/FR. Low-R/FR-regulated genes involved in light and hormone pathways are visualized by means of the FARO Web tool. The large majority of top-ranked associated factors by FARO to *hfr1-4/sics1-1* and *phyA-211*

Interestingly, *HYH* and *UNE10* are among the genes known to be bound in vivo by HY5 (Lee et al., 2007). To assess whether HY5 does regulate *HYH* and *UNE10* expression under shade conditions, we performed low R/FR kinetics experiments in the loss-of-function *hy5-215* mutant (Oyama et al., 1997). The up-regulation of *HYH* and *UNE10* expression by low R/FR depends on the action of HY5, since it does not occur in the *hy5* mutant (Fig. 13). No significant difference was observed in *HYH* and *UNE10* induction by low R/FR in *hfr1-4/sics1-1* with respect to the wild type (Fig. 13).
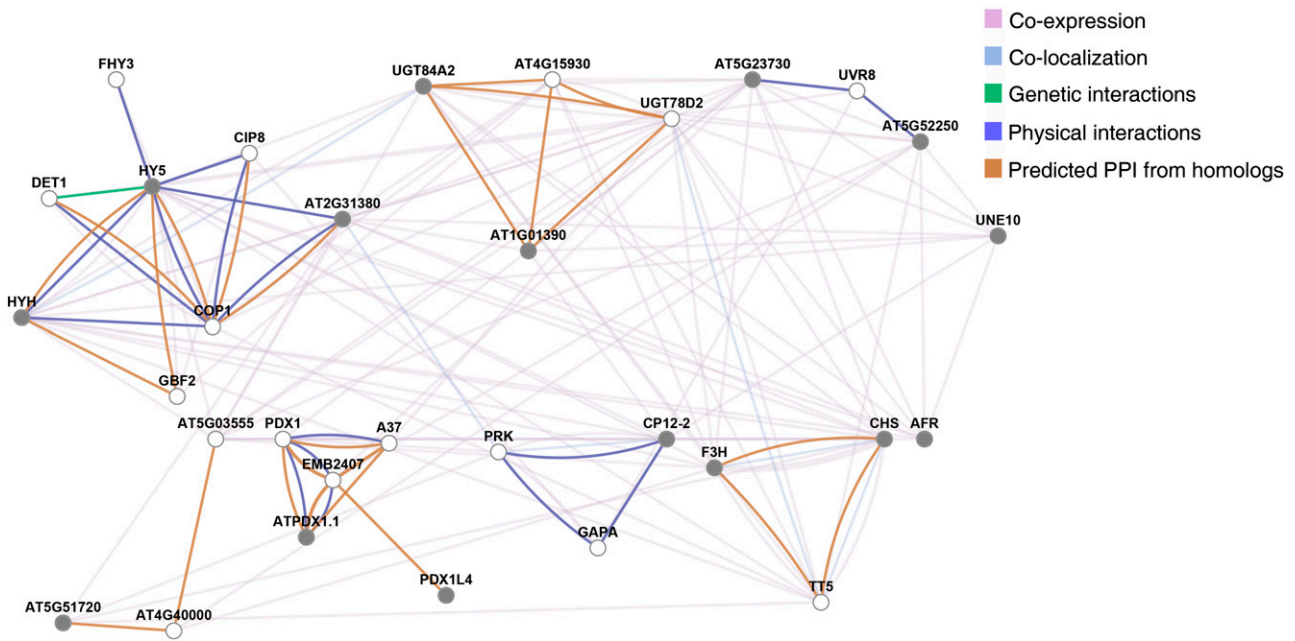
DISCUSSION

Plant responses to light quality changes are regulated by a balance of positive (PIFs) and negative (HFR1/SICS1) regulators of gene expression, which ensures a fast reshaping of the plant body toward an environment optimal for growth while at the same time avoiding an exaggerated reaction to low R/FR (Sessa et al., 2005; Lorrain et al., 2008; Hornitschek et al., 2009). Here, by combining genome-wide expression profiling and computational analyses, we show highly significant overlap between shade avoidance and deetiolation transcript profiles. Strikingly, the direction of the response is dissimilar at the early stages of shade avoidance and congruent at the late ones. Down-regulation of genes early induced by light quality changes upon prolonged exposure to low R/FR depends not only on HFR1/SICS1 (Sessa et al., 2005), which interacts with PIF transcription factors forming non-DNA-binding heterodimers, thus limiting PIF-mediated gene expression (Hornitschek et al., 2009), but also on phyA. By contrast, phyA and not HFR1/SICS1 is required for the up-regulation of genes late induced by low R/FR. Remarkably, among them is the HY5 transcription factor gene, known to act as the master regulator of seedling deetiolation (Lau and Deng, 2010).

Light Signaling Genes Are Dynamically Regulated during Shade Avoidance

Signaling downstream of the photoreceptors involves two main pathways: CONSTITUTIVE PHOTOMOR-PHOGENIC1 (COP1)-HY5 and PIFs. COP/DEETIOLATED (DET)/FUSCA (FUS) are central repressors of photomorphogenesis, which, in the dark, function in concert to target positive regulators of photomorphogenesis

upon prolonged exposure to low R/FR were composed of genes involved in light and hormone pathways, which were consequently selected for functional analysis. Genes differentially regulated in Col-0, *hfr1-4/sics1-1*, and *phyA-211* upon 1 d of low-R/FR treatment are displayed in the central circles. In the outer circles, experimental factors with strong associations with the wild type and the mutants exposed to low R/FR are shown. The factors showing overlap with the opposite direction (i.e. congruence less than 30%) with respect to our experiments are depicted in red; those showing the highest congruence (greater than 70%) are depicted in blue.

Figure 10. Expression profiles of auxin and light pathway genes in Col-0, *hfr1-4/sics1-1*, and *phyA-211* exposed to low R/FR. Genes significantly up-regulated by brief or prolonged exposure to low R/FR in wild-type seedlings, and indicated by FARO as involved in auxin (A) and light (B) pathways, are visualized by means of heat maps. The expression profiles of the same genes in *hfr1/sics1* and *phyA-211* exposed to low R/FR for a prolonged time are also shown. Red and green depict up- and down-regulation relative to control (high R/FR), respectively. r, Gene expression ratio.

(e.g. HY5) for degradation through the 26S proteasome, thus preventing deetiolation. In daylight, the activity of COP/DET/FUS proteins is reduced, resulting in the accumulation of transcription factors required for photomorphogenesis. COP1, one of the COP/DET/FUS proteins, is an E3 ligase that interacts with several transcription factors and promotes their ubiquitination together with the SUPPRESSOR OF PHYA105 proteins (Lau and Deng, 2010). By contrast, a *pifq* mutant displays a *cop*-like phenotype in darkness, demonstrating that these PIF transcription factors function in the dark to promote skotomorphogenesis (Leivar et al., 2008b; Shin et al., 2009). Upon light exposure, photoactivated phytochromes interact with PIFs and promote their

degradation via the ubiquitin-proteasome pathway (Leivar and Quail, 2011). The rapid light-induced degradation of PIF molecules does not lead to their disappearance; rather, it results in a lower steady-state level of these proteins in daylight (Leivar and Quail, 2011).

Genome-wide expression profiling of wild-type etiolated seedlings briefly exposed to light and dark-grown *pifq* mutants identified a set of genes that are potential direct targets of these PIF molecules (Leivar et al., 2009). More recently, by comparing these transcriptome profiles with those of wild-type and *pifq* seedlings briefly exposed to low R/FR, Leivar and coauthors (2012) identified a number of genes rapidly repressed and induced by light and shade signals,

Figure 11. Network analysis of light pathway genes regulated in Col-0 but not in *phyA-211* upon prolonged exposure to low R/FR. Light pathway genes regulated in the wild type but not in *phyA-211* upon prolonged exposure to low R/FR (1 d), extracted from the cluster that emerged in the heat map (Fig. 10B), were utilized to infer gene networks by means of the GeneMANIA server using available genomics and proteomics data. Networks are represented as graphs, in which the nodes correspond to genes and the edges correspond to interactions between them. Nodes colored with gray show significant differential gene expression with respect to the control in our array data (Supplemental Tables S3 and S11; for details, see "Materials and Methods"). PPI, Protein-protein interactions.

respectively. Among them are *ATHB2*, previously shown to be down-regulated in etiolated seedlings briefly exposed to R and FR and strongly and rapidly induced in photoautotrophic plants exposed to low R/FR (Carabelli et al., 1996), *IAA19*, and *IAA29* (Leivar et al., 2012). Here, by taking advantage of the FARO Web tool, we extended and broadened these results, showing an opposite regulation of genes regulated in deetiolation experiments with respect to early shade-regulated genes and consistent regulation for late-regulated ones. Consistent with this, whereas at the very early stages of shade avoidance, gene network analysis highlights the connections among phy-PIF signaling, HD-Zip transcription factors, and auxin, later, HY5 seems to gain a central role in light signal transduction.

**HFR1/SICS1 and phyA Function Largely Independently in Negatively Regulating the Shade-Avoidance Response**

phyA mutants elongate more than the wild type in low R/FR (Johnson et al., 1994; Devlin et al., 2003; Wang et al., 2011; this work). Our analysis of seedlings lacking a functional phyA demonstrates a major role of this phytochrome in regulating other aspects of the shade-avoidance response as well. phyA function is complex: it negatively regulates the growth responses of the hypocotyl, cotyledons, and leaves induced by light quality changes, thus preventing an exaggerated

plant reaction to low R/FR, and positively regulates flowering, shortening the generation time and, therefore, ensuring species survival in an unfavorable environment. A significant number of genes rapidly and transiently induced by light quality changes are upregulated in *phyA* with respect to the wild type upon prolonged exposure to low R/FR. Interestingly, several of these genes are also up-regulated under the same conditions in seedlings lacking a functional HFR1/SICS1 (Sessa et al., 2005; Fig. 7), a negative regulator of shade avoidance (Sessa et al., 2005; Hornitschek et al., 2009), also described as a downstream component of phyA signaling during deetiolation (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000; Lorrain et al., 2009). However, *hfr1/sics1 phyA* seedlings display a phenotype more severe than those of *hfr1/sics1* and *phyA* single mutants in the hypocotyl, cotyledons, and leaves in low R/FR. Moreover, the expression of several genes early induced by low R/FR is significantly higher in the double mutant relative to both *hfr1/sics1* and *phyA* upon prolonged exposure to FR-rich light. In agreement with HFR1/SICS1 and phyA acting largely independently from one another, we found that HFR1/SICS1 is regulated through phyB in photoautotrophic seedlings. Finally, we also found that *hfr1/sics1 phyA* plants flower significantly earlier than *phyA* but later than *hfr1/sics1* under low R/FR.
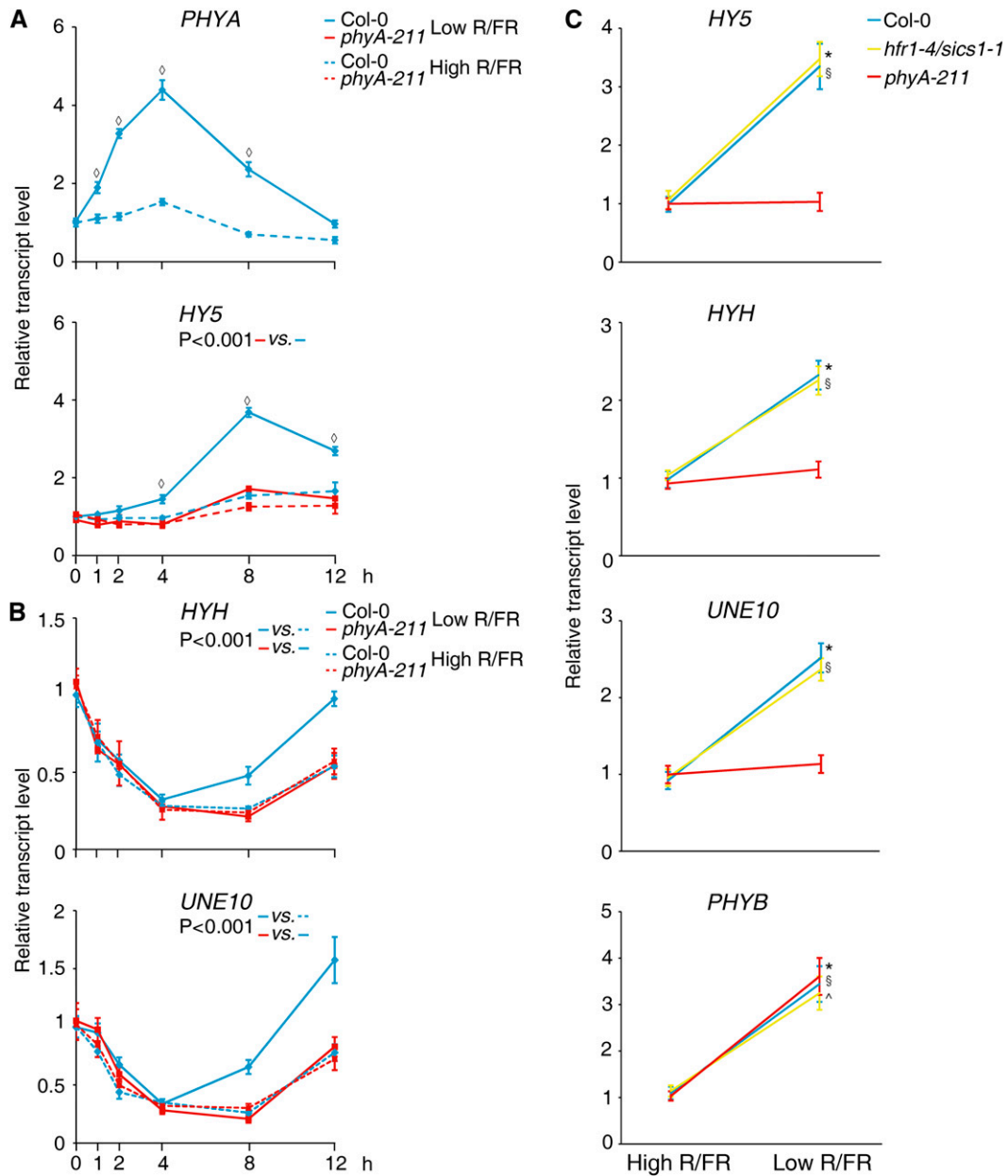
Figure 12. *HY5*, *HYH*, and *UNE10* are late induced by low R/FR largely through the action of phyA. A, RT-qPCR analyses of *PHYA* in Col-0 and *HY5* in Col-0 and *phyA-211* upon exposure to low R/FR for different times. Col-0 and *phyA-211* seedlings were grown for 7 d in a L/D cycle (16/8 h) in high R/FR (control) and then maintained in high R/FR or transferred to low R/FR for different times. The graphs show the relative expression levels in high (dashed lines) and low (solid lines) R/FR of *PHYA* in Col-0 and *HY5* in Col-0 and *phyA-211*. Each value is the mean ± sᴅ of three biological replicates normalized to *EF1α* expression. Statistical significance was assessed by means of one-way ANOVA followed by Dunnett's test. °$P < 0.001$ for low R/FR versus control (0) in Col-0. Statistical significance between different backgrounds was assessed by means of two-way ANOVA followed by the Bonferroni posthoc test. B, RT-qPCR analyses of *HYH* and *UNE10* in Col-0 and *phyA-211* upon exposure to low R/FR for different times. Col-0 and *phyA-211* seedlings were grown and treated as described in A. The graphs show the relative expression levels in high (dashed lines) and low (solid lines) R/FR of *HYH* and *UNE10* in Col-0 and *phyA-211*. Each value is the mean ± sᴅ of three biological replicates normalized to *EF1α* expression. Statistical significance between different treatments and backgrounds was assessed by means of two-way ANOVA followed by the Bonferroni posthoc test. C, RT-qPCR analyses of *HY5*, *HYH*, and *UNE10* upon prolonged exposure to low R/FR in Col-0, *hfr1-4/sics1-1*, and *phyA-211*. Col-0, *hfr1-4/sics1-1*, and *phyA-211* seedlings were grown for 7 d in a L/D cycle (16/8 h) in high R/FR and then either maintained in high R/FR or transferred to low R/FR under the same L/D regimen for 1 d. Plant transfer to low R/FR was performed 4 h after the beginning of the light period. The graphs show the relative expression levels in high and low R/FR of *HY5*, *HYH*, and *UNE10* in the different genotypes. *PHYB* is a gene not differentially regulated by low R/FR in mutant seedlings relative to the wild type. Each value is
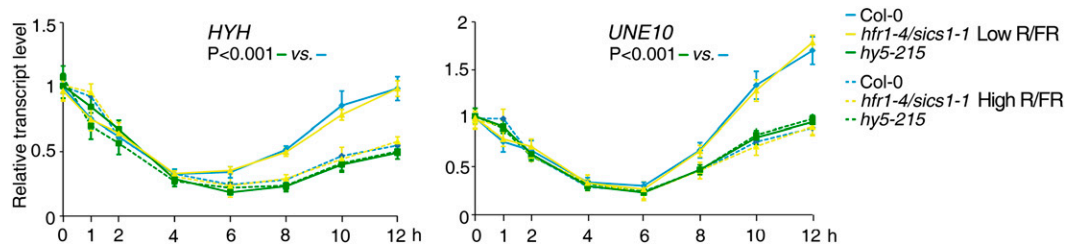
Figure 13. HY5 is required to up-regulate the *HYH* and *UNE10* transcription factor genes in low R/FR. Col-0, *hfr1-4/sics1-1*, and *hy5-215* seedlings were grown for 7 d in a L/D cycle (16/8 h) in high R/FR and then maintained in high R/FR or transferred to low R/FR for different times. The graphs show the relative expression levels in high (dashed lines) and low (solid lines) R/FR of *HYH* and *UNE10* in the different genotypes. Each value is the mean ± SD of three biological replicates normalized to *EF1α* expression. Statistical significance between different backgrounds was assessed by means of two-way ANOVA followed by the Bonferroni posthoc test.

Consistent with HFR1/SICS1 and phyA acting in the negative control of shade avoidance, FARO analyses of *hfr1/sics1* and *phyA* upon prolonged exposure to low R/FR revealed, in both mutants, a response dissimilar to that of deetiolation as observed in the wild type at the early stages of shade avoidance. However, differences emerged with respect to gene expression profiles between *hfr1/sics1* and *phyA*. In fact, whereas the great majority of early shade-induced genes that FARO depicts as regulated during deetiolation were still up-regulated in both mutants upon prolonged exposure to low R/FR, those late induced by low R/FR were mostly up-regulated in *hfr1/sics1* but not in *phyA-211*.

Taken together, the data indicate that phyA largely signals through transcription factors other than HFR1/SICS1 in low R/FR.

phyA Is Required for Late Induction by Low R/FR of the HY5 Transcription Factor Gene

Network analysis of the genes late induced by low R/FR in the wild type and not in the *phyA* mutant identified a subnetwork involving the HY5 transcription factor gene. Low-R/FR kinetics experiments demonstrated that *HY5* up-regulation is preceded by the rapid induction of *PHYA*, and it is indeed impaired in *phyA* mutant seedlings. Furthermore, network analysis also highlighted a direct connection between *HY5* and *HYH*, a gene functionally implicated in the inhibition of hypocotyl elongation (Holm et al., 2002) and known to be a direct target of the HY5 transcription factor (Lee et al., 2007). Expression studies in wild-type and mutant seedlings exposed to FR-rich light for different times revealed that the *HYH* gene is indeed late induced by

low R/FR, and its up-regulation does depend on the action of HY5, since it does not occur in the *hy5* mutant.

The role of HY5 has been most extensively studied at the early stages of seedling development. Originally identified as a negative regulator of cell elongation acting downstream of multiple families of the photoreceptors (Oyama et al., 1997; Osterlund et al., 2000), it has been shown subsequently to function as a key controller of the transcriptional cascades promoting seedling photomorphogenesis (Lau and Deng, 2010). More recently, HY5 has also been implicated in the inhibition of hypocotyl elongation induced in shaded plants by brief exposure to direct sunlight perceived primarily by phyB (Sellaro et al., 2011). HY5 is 15 to 20 times more abundant in seedlings grown in the light than in the dark (Osterlund et al., 2000). Consistent with its prominent role in the commitment to photomorphogenic development, HY5 abundance exhibits its highest level 2 to 3 d after germination and then drastically decreases at later times of seedling development (Hardtke et al., 2000). Multiple photoreceptors are involved in regulating the abundance of HY5. phyB and phyA are primarily responsible for HY5 accumulation in continuous R and FR, respectively. CRY1 and CRY2 are largely redundant and involved in the accumulation of HY5 in continuous B light. In all cases, the abundance of HY5 directly correlates with the degree of photomorphogenic development (Osterlund et al., 2000). Thus, it seems likely that increased *HY5* expression upon prolonged exposure to low R/FR may be a mechanism through which phyA exerts its regulatory role in the shade-avoidance response.

A recent study combining genome-wide analysis of HY5 binding sites under continuous white light and during the light-to-dark transition and gene expression profiling of wild-type and *hy5* seedlings identified more

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Figure 12. (*Continued.*)
the mean ± SD of five biological replicates normalized to *EF1α* expression. Statistical significance was assessed by means of one-way ANOVA followed by Tukey's test. *$P < 0.001$ for Col-0 low R/FR versus Col-0 high R/FR; §$P < 0.001$ for *hfr1-4/sics1-1* low R/FR versus *hfr1-4/sics1-1* high R/FR; ^$P < 0.001$ for *phyA-211* low R/FR versus *phyA-211* high R/FR.

than 1,000 target genes positively or negatively regulated by HY5 (Zhang et al., 2011). The positive and negative functions of this transcription factor in regulating gene expression have been further strengthened by the finding that HY5 acts as a repressor of *FAR-RED ELONGATED HYPOCOTYL1* (*FHY1*) and *FHY1-LIKE* (*FHL*) expression by modulating the transcriptional activities of FHY3 and its homolog FAR-RED IMPAIRED RESPONSE1 (FAR1), two crucial components in phyA signaling (Li et al., 2010), whereas it acts as a transcriptional activator of *EARLY FLOWERING4* (*ELF4*), a key player of the central oscillator of the circadian clock, in concert with FHY3/FAR1 (Li et al., 2011). Interestingly, the cis-elements of HY5 and FHY3/FAR1 are very close to each other in the *FHY1/FHL* promoters, whereas they are around 20 bp away in the *ELF4* promoter (Li et al., 2010, 2011). Comparison of FHY3 (Ouyang et al., 2011) and HY5 direct targets (Lee et al., 2007; Zhang et al., 2011) identified a subset of genes coregulated by these two transcription factors, including *FHY1* and *ELF4*, suggesting that HY5 is likely to work with other transcription factors to coregulate the expression of genes other than those targets of FHY3/HY5 (Ouyang et al., 2011). Low-R/FR kinetics experiments indicated that phyA is also involved in the negative regulation of genes early induced by light quality changes upon prolonged exposure to low R/FR. Remarkably, among them is *ATHB2*, functionally involved in the shade-avoidance response and identified as a gene directly repressed by HY5 (Zhang et al., 2011). *IAA19*, previously implicated in the regulation of cell elongation in the phototropic response (Tatematsu et al., 2004), and *HLH1/PAR1*, both identified as genes recognized in vivo by HY5 (Lee et al., 2007; Zhang et al., 2011), are also significantly up-regulated upon prolonged low-R/FR treatment in seedlings lacking phyA. Therefore, it is tempting to speculate that phyA through HY5 not only positively regulates photomorphogenesis-promoting genes upon prolonged exposure to low R/FR but also down-regulates genes early induced by light quality changes. Future work will be needed to identify the transcription factor(s) that may work in concert with HY5 to both activate and repress gene expression in low R/FR and to understand their regulatory mode of action on promoters of common target genes.

MATERIALS AND METHODS

Plant Lines and Growth Conditions

The wild-type strain used was Arabidopsis (*Arabidopsis thaliana*) Col-0. Other lines used were *hfr1-4/sics1-1* (Sessa et al., 2005; SALK_037727), *phyB-9* (Reed et al., 1993; NASC#N6217), *phyA-211* (Reed et al., 1994; NASC#N6223), and *hy5-215* (Oyama et al., 1997). The lines *hfr1-4/sics1-1 phyB-9* and *hfr1-4/sics1-1 phyA-211* were generated by crossing. The F2 progeny of the cross *hfr1-4/sics1-1 × phyB-9* was grown for 7 d in high R/FR together with *phyB-9*. Seedlings displaying a hypocotyl similar to or longer than that shown by *phyB-9* mutants were further analyzed for the presence of the *hfr1-4/sics1-1* mutation by PCR using the primers detailed below. The F2 progeny of the cross *hfr1-4/sics1-1 × phyA-211* was grown for 4 d in continuous FR (41.2 μmol m^{-2} s^{-1}) together with *phyA-211* and *hfr1-4/sics1-1*. Under these conditions, *phyA-211* is extremely elongated whereas *hfr1-4/sics1-1* displays

only a mild phenotype (Fairchild et al., 2000). Seedlings displaying an elongated phenotype, analogous to that shown by *phyA-211* mutants, were then analyzed for the presence of the *hfr1-4/sics1-1* mutation by PCR. The primers used for *hfr1-4/sics1-1* genotyping were as follows: HFR1/SICS15', 5'-TGGA-ATTGGGATGGAGAAACGAC-3'; HFR1/SICS13', 5'-CGAGAACCGAAAC-CTTGTCCGT-3'; LBb1, 5'-GCGTGGACCGCTTGCTGCAACT-3'.

Plants were grown as described previously (Sessa et al., 2005) in a light-emitting diode growth chamber (E30-LED; Percival Scientific) at 21°C temperature, 75% humidity, 16-h-light/8-h-dark cycles. Light outputs in high R/FR$_{high\ PAR}$ were as follows: 670 nm (R), 96 μmol m^{-2} s^{-1}; 735 nm (FR), 21 μmol m^{-2} s^{-1}; 470 nm (blue light), 15 μmol m^{-2} s^{-1}. Light outputs in high R/FR$_{low\ PAR}$ were as follows: 670 nm, 12 μmol m^{-2} s^{-1}; 735 nm, 2 μmol m^{-2} s^{-1}; 470 nm, 15 μmol m^{-2} s^{-1}. Light outputs in low R/FR$_{low\ PAR}$ were as follows: 670 nm, 12 μmol m^{-2} s^{-1}; 735 nm, 105 μmol m^{-2} s^{-1}; 470 nm, 15 μmol m^{-2} s^{-1}. Light outputs in low R/FR* were as follows: 670 nm, 12 μmol m^{-2} s^{-1}; 735 nm, 21 μmol m^{-2} s^{-1}; 470 nm, 15 μmol m^{-2} s^{-1}.

Phenotypic Analyses

Images of whole seedlings and green leaves were taken with an MZ 12 binocular microscope (Leica) using ProgRes C10 plus (Jenoptik) or captured with a Coolpix 990 digital camera (Nikon). For hypocotyl, cotyledon, leaf, and internode measurements, images were taken with the same devices and subsequently analyzed with NIH Image analysis software (http://rsb.info.nih.gov/ij), as described previously (Sessa et al., 2005; Carabelli et al., 2007). Statistical significance was assessed by means of one-way ANOVA followed by Tukey's or Games-Howell tests (Prism 5 [GraphPad Software] and PASW Statistics 18 [SPSS]). The 95% confidence intervals for the ratio of two means were computed by the method of Fieller (1940). The strength of the hypocotyl response during growth in different light regimes was measured by means of standardized effect size as described by Hedges and Olkin (1985).

Microarray Data Analyses

For gene expression analyses, 8-d-old seedlings were harvested after the designated treatments for the indicated periods of time. Affymetrix Arabidopsis Genome GeneChip array (ATH1) experiments were performed as described by Sessa et al. (2005). Two biological replicates were performed for each time point and each line. Pixel-level files from scanned arrays (.dat files) were analyzed with Affymetrix MAS 5.0 software to obtain text format files with intensity values for perfect match and mismatch features (.cel files) and absent/present calls (.chp files). All data were submitted to the ArrayExpress database (accession nos. E-MEXP-443, E-MEXP-444, E-MEXP-3266, and E-MEXP-3267) in compliance with minimum information about a microarray experiment guidelines. All .cel files were imported into GeneSpring 7.1 (Agilent Technologies) and normalized in two steps. First, the robust multiarray average algorithm (Irizarry et al., 2003) was used to convert the probe-level expression data into probe-set (or gene-level) expression data and to ensure that the distribution of the expression values was comparable across the different chips or samples; second, to account for the difference in detection efficiency between spots and to compare the relative change in gene expression levels, the expression value for one gene across the different conditions was centered on 1 by dividing the expression value by the median value of the expression values for that gene across the conditions. To identify low-R/FR-regulated genes, a rank-product analysis was carried out by means of R software, imposing false discovery rate < 0.05 (Breitling et al., 2005; Supplemental Tables S1–S3, S10, and S11). Heat maps obtained by means of FiRe 2.2 (Garcion et al., 2006) were used to compare gene expression patterns in multiple microarray experiments: fold change values were computed after robust multiarray average normalization of all the experiments together using GeneSpring 7.1 (Agilent Technologies).

DAVID Analyses

We used DAVID (version 6.7; http://david.abcc.ncifcrf.gov/home.jsp) functional annotation clustering to cluster the genes differentially regulated by low R/FR in the wild type (1 h, 4 h, or 1 d) and *hfr1-4/sics1-1* and *phyA-211* mutants (1 d) into different functional groups. DAVID uses a fuzzy clustering concept by measuring relationships among the annotation terms on the basis of the degree of their coassociation with genes within the user's list in order to cluster somewhat heterogenous, yet highly similar, annotation into functional annotation groups (Huang et al., 2009). For each condition, we identified the

significantly enriched functional groups as the top 10 ranked with EASE score greater than 1. Heat maps were used to compare P values of GO descriptors obtained from each DAVID analysis in the different genotypes.

Network Analyses

DAVID analyses of the genes differentially expressed in the wild type after exposure to low R/FR for 1 h, 4 h, and 1 d identified 29 significantly enriched functional clusters. For each cluster, the genes associated with corresponding functional annotations were extracted to feed them into the GeneMANIA server to infer network topology (version 2.1; http://www.genemania.org) using available genomics and proteomics data (protein, physical, and genetic interactions, coexpression, and colocalization data). For each cluster of genes, we ran the network analysis by weighting every individual data source equally ("equal-by-network" option) and displaying a fixed number of related genes as computed by the GeneMANIA algorithm: 10 genes in Figures 3 and 4 and Supplemental Figure S2 and 20 genes in Figure 11. After this step, we manually removed genes that were not structured in well-connected networks (e.g. composed of less than three nodes) and used the remaining ones to run the GeneMANIA Web tool on a single time point level (utilizing again the equal-by-network option but displaying no other related genes) to obtain global networks (Figs. 3, 4, and 11; Supplemental Fig. S2).

Promoter Analyses

Putative promoter regions upstream of low-R/FR-regulated genes were analyzed to identify overrepresented cis-regulatory elements by means of the Athena Web tool (http://www.bioinformatics2.wsu.edu/Athena; O'Connor et al., 2005), inspecting −2,000 bp from the 5′ untranslated region, if known (otherwise from ATG), and truncating promoter sequences that overlap with upstream genes. For each single time point, significantly overrepresented cis-elements were defined as the top-ranked 30 motifs with $P < 0.05$, calculated using a hypergeometric probability distribution.

FARO Analyses

FARO analyses were performed as described by Nielsen et al. (2007) by means of the appropriate Web tool (http://www.cbs.dtu.dk/services/faro/). The top-ranked 15 "associated factors" with more than 25% overlap were chosen for subsequent analysis. Following manual inspection, only factors related to light/hormone pathways were selected for functional analyses.

Northern-Blot Analyses

Northern-blot experiments were performed using 10 μg of total RNA as described previously (Carabelli et al., 1996). The *ATHB2, RIBOSOMAL PROTEIN L18* (*ATL18*), *EARLY LIGHT-INDUCIBLE PROTEIN1* (*ELIP1*), and *HFR1/SICS1* probes were described previously (Carabelli et al., 1993, 1996; Sessa et al., 2005).

RT-qPCR

For RT-qPCR experiments, total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions except for step 6, for which the incubation time was extended to 1 h. qPCR was performed with the LightCycler 480 instrument (Roche) using LightCycler 480 Probes Master (Roche) and Universal Probe-Library (UPL) probes (Roche), 5′ labeled with fluorescein and 3′ labeled with a dark quencher dye, according to the manufacturer's instructions, as described previously (Ciarbelli et al., 2008). Quantification of target gene expression was expressed in comparison with the reference gene *TRANSLATION ELONGATION FACTOR1α* (*EF1α*; Sibout et al., 2006), and relative expression ratio was calculated based on the qPCR efficiency for each gene and the crossing point deviation of target genes versus control (Pfaffl, 2001). Specific UPL probes and primers for RT-qPCR analyses are listed in Supplemental Table S15. Statistical analyses were performed on log-transformed relative expression ratio values as described by Rieu and Powers (2009). Relative transcript abundance of each gene was normalized to Col-0 level in high R/FR. Subsequent to data standardization (Willems et al., 2008), one-way ANOVA followed by Tukey's posthoc test was used to assess differences among means (Prism 5; GraphPad Software). Time-course experiments were analyzed by mean of one-way

ANOVA followed by Dunnett's posthoc test; experiments with different backgrounds were evaluated by mean of two-way ANOVA followed by Bonferroni posthoc tests (Prism 5; GraphPad Software).

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ATHB2* (At4g16780), *ATL18* (At3g05590), *CO* (At5g15840), *EF1α* (At1g18070.3), *ELIP1* (At3g22840), *FT* (At1g65480), *HFR1/SICS1* (At1g02340), *HLH1/PAR1* (At2g42870), *HY5* (At5g11260), *HYH* (At3g17609), *IAA19* (At3g15540), *IAA29* (At4g32280), *PHYA* (At1g09570), *PHYB* (At2g18790), *PIL1* (At2g46970), and *UNE10* (At4g00050). All array data were submitted to the ArrayExpress database (accession nos. E-MEXP-3266 and E-MEXP-3267) in compliance with minimum information about a microarray experiment guidelines.

Supplemental Data

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

Supplemental Figure S1. Arabidopsis seedling responses to low R/FR$_{low\ PAR}$ versus high R/FR$_{low\ PAR}$.

Supplemental Figure S2. Network analysis of genes regulated early in the shade-avoidance response.

Supplemental Figure S3. HFR1/SICS1 is regulated through phyB in photoautotrophic seedlings.

Supplemental Figure S4. Hypocotyl elongation of Col-0, *hfr1-4/sics1-1*, and *phyA-211* seedlings in high R/FR$_{high\ PAR}$, high R/FR$_{low\ PAR}$, and low R/FR$_{low\ PAR}$.

Supplemental Figure S5. Leaf phenotype of *hfr1-4/sics1-1 phyA-211* in low R/FR.

Supplemental Figure S6. *hfr1-4/sics1-1 phyA-211* plants exhibit internode elongation in low R/FR.

Supplemental Figure S7. *hfr1-4/sics1-1 phyA-211* plants are early flowering relative to *phyA-211* in low R/FR.

Supplemental Figure S8. Kinetics of *HY5* induction by low R/FR in Col-0 and *hfr1-4/sics1-1* seedlings.

Supplemental Figure S9. Arabidopsis seedling responses to low R/FR*.

Supplemental Table S1. Genes regulated in Col-0 after 1 h of exposure to low R/FR.

Supplemental Table S2. Genes regulated in Col-0 after 4 h of exposure to low R/FR.

Supplemental Table S3. Genes regulated in Col-0 after 1 d of exposure to low R/FR.

Supplemental Table S4. Significantly enriched cluster descriptors in Col-0 after 1 h of exposure to low R/FR.

Supplemental Table S5. Significantly enriched cluster descriptors in Col-0 after 4 h of exposure to low R/FR.

Supplemental Table S6. Significantly enriched cluster descriptors in Col-0 after 1 d of exposure to low R/FR.

Supplemental Table S7. List of experiments functionally associated with Col-0 at 1 h of low R/FR according to FARO.

Supplemental Table S8. List of experiments functionally associated with Col-0 at 4 h of low R/FR according to FARO.

Supplemental Table S9. List of experiments functionally associated with Col-0 at 1 d of low R/FR according to FARO.

Supplemental Table S10. Genes regulated in *hfr1-4/sics1-1* upon 1 d of exposure to low R/FR.

Supplemental Table S11. Genes regulated in *phyA-211* upon 1 d of exposure to low R/FR.

Supplemental Table S12. Phenotypes of Col-0, *hfr1-4/sics1-1, phyA-211*, and *hfr1-4/sics1-1 phyA-211* seedlings in high R/FR.

Supplemental Table S13. List of experiments functionally associated with *hfr1-4/sics1-1* at 1 d of low R/FR according to FARO.

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