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Phytochrome Control of the *Arabidopsis* Transcriptome Anticipates Seedling Exposure to Light [W]

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Phytochromes mediate a profound developmental shift when dark-grown seedlings are exposed to light. Here, we show that a subset of genes is upregulated in *phytochrome B* (*phyB*) mutants even before dark-grown *Arabidopsis thaliana* seedlings are exposed to light. Most of these genes bear the RY *cis* motif, which is a binding site of the transcription factor ABSCISIC ACID INSENSITIVE3 (ABI3), and the *phyB* mutation also enhances *ABI3* expression. These changes in transcriptome have physiological consequences, because seedlings of the *abi3* mutant showed enhanced responses to pulses of far-red light, whereas ABI3 overexpressers exhibited the opposite pattern. Seedlings of the wild type derived from seeds germinated in full darkness showed enhanced expression of genes bearing the RY *cis* motif and reduced responses to far-red light. We propose that, via changes in *ABI3* expression, light, perceived mainly by phyB in the seed, generates a downstream transdevelopmental phase signal that preconditions the seedling to its most likely environment.

INTRODUCTION

In plants, the timing of key developmental transitions is adjusted by environmental cues. In dark-grown seedlings, deetiolation is initiated by the dark-to-light transition. In response to this signal, hypocotyl growth is arrested, the cotyledons expand, unfold, and acquire photosynthetic capacity, root growth increases, and photoprotective pigments appear in specific tissues (Chen et al., 2004). A massive rearrangement of the transcriptome accompanies this shift from skotomorphogenesis to photomorphogenesis (Ma et al., 2001; Tepperman et al., 2001; Ohgishi et al., 2004). These effects are mediated by the red light (R) and far-red light (FR) photoreceptors, phytochromes (phyA through phyE), the blue–UV-A photoreceptors, cryptochromes (cry1 and cry2), and phototropins. Significant changes in the expression of transcription factors and genes involved in hormone responses can already be detected in the first hour after photoreceptor activation (Tepperman et al., 2001; Folta et al., 2003). These observations support the idea of a transcriptional network downstream of the photoreceptors involving points of convergence with hormone signaling.

Mutants deficient in specific photoreceptors show wild-type levels of expression of photosynthetic genes in darkness (D) and fail to respond normally to light (Anderson et al., 1997; Tepperman et al., 2001). The pattern followed by these end point targets can guide the search for genes whose expression responds to light as components of the network between the photoreceptors and the target developmental genes. As a complement to this supervised analysis based on previous knowledge, unsupervised analysis can show unexpected patterns and may therefore reveal new paths of the signaling network (Perelman et al., 2003). Here, we follow the latter approach and report differences in the expression of a specific subset of genes between *Arabidopsis thaliana* seedlings of the wild type and *phyB* mutants even before they are exposed to light. Based on this finding, we revealed a signaling network that preconditions seedling development according to the light environment experienced by the seed.

RESULTS AND DISCUSSION

Searching for the Patterns That Emerge from Transcriptome Data

Dark-grown seedlings of wild-type *Arabidopsis*, ecotype Landsberg *erecta* (L*er*), and of the *phyA phyB* double mutant, the *cry1*

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cry2 double mutant, and the *phyA phyB cry1 cry2* quadruple mutant were exposed to 0 (dark control), 1, or 3 h of white light (WL) before harvest, processing, and hybridization to 8 K Affymetrix chips. To summarize the information, we used correspondence analysis (Perelman et al., 2003). This procedure constructs theoretical variables (axes) that maximally account for the structure of a multidimensional cloud of points (the matrix defined by genes and light/genotype in our case), making possible the reduction of dimensionality (e.g., from the multispace to a plane [McGarigal et al., 2000]). Each axis is subject to the constraint of being uncorrelated with previous axes, to ensure that new information is expressed (Jongman et al., 1987). Because morphological responses and the expression of some genes are unaffected by photoreceptor mutations in D (Tepperman et al., 2001; Chen et al., 2004), we expected that the information provided by thousands of genes was going to group all of the genotypes in D and progressively scatter them over the plane with increasing light exposure, with the wild type exposed to 3 h of WL reaching the most distant position. However, this pattern was not found on the planes defined by axes 1 and 2 (Perelman et al., 2003) or axes 1 and 3 (data not shown). On the plane generated by axes 2 and 3, the wild type and the *cry1 cry2* mutants formed a group separate from that of the *phyA phyB* and *phyA phyB cry1 cry2* mutants (*PHY* versus *phy* in Figure 1; $P < 0.01$, multiresponse permutation procedure) (Mielke, 1984). This pattern suggests that the expression of some genes is affected by the *phyA phyB* mutations even in dark-grown seedlings.

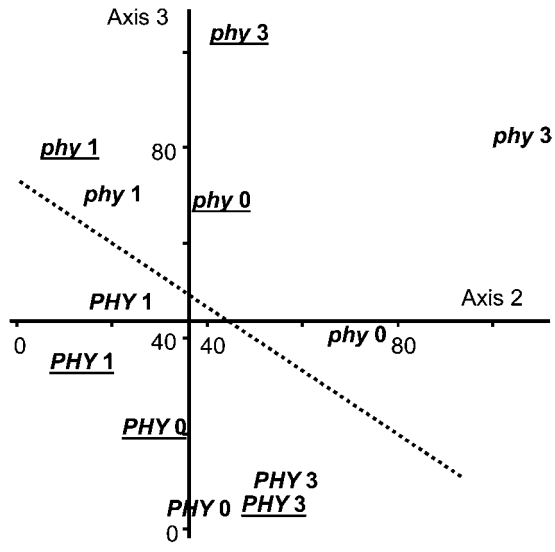

Figure 1. Unsupervised Analysis of Microarray Data.

Plot of the analysis of correspondence from the 8 K microarray experiment. The seedlings bearing the *phyA phyB* (*phy*) or *PHYA PHYB* (*PHY*) alleles occupy opposite sides of the plane, irrespective of the exposure to 0, 1, or 3 h of WL (indicated by the numbers after *phy* or *PHY*), indicating that phyA and/or phyB affect gene expression even in D. Underlined names identify samples from seedlings bearing the *cry1 cry2* mutations.

Phytochrome Control of Gene Expression in Dark-Grown Seedlings

To investigate the genes affected by phyA and/or phyB in D, we conducted two additional fully independent experiments using near full-genome *Arabidopsis* microarrays (ATH1). In the first ATH1 experiment, we included dark-grown seedlings of the wild type and of the *phyA phyB* double mutant in the Columbia (Col) background. In the second ATH1 experiment, we included the wild type and the *phyA*, *phyB*, and *phyA phyB* mutants in Col. To be considered repressed by phyB in D, a gene had to comply with all three of the following conditions: (1) significantly ($P < 0.05$) higher expression in *phyA phyB* compared with the wild type in the first experiment; (2) significant ($P < 0.05$) difference between *PHYB* (wild type and *phyA* mutant) and *phyB* (*phyB* and *phyA phyB* mutants) in the second experiment; and (3) a confidence threshold (Grant et al., 2005) of 0.8 for the analysis of pooled *phyA phyB* versus wild-type data from both experiments (confidence values estimate the probability that a gene represents a truly differentially expressed gene). Twenty-one genes passed these filters (Figures 2A and 2B). Those genes from this list that were represented and expressed in the 8 K microarray also showed enhanced expression in *phyA phyB* in the 8 K experiment (Figure 2C). Analysis of some of these genes by RT-PCR also showed enhanced expression in *phyB* compared with wild-type dark-grown seedlings (Figure 2C, inset). This finding underscores the reproducibility of the effect (although the magnitude is variable) and precludes artifacts attributable to the use of a single allele (the 8 K and RT-PCR experiments were in the L*er* background, and the two ATH1 experiments were in the Col background).

The 21 genes whose expression in D was repressed by phyB include 7 seed storage proteins, 3 oleosins, a major latex protein, and a dehydrin. Genes of these functional families are controlled by abscisic acid (Hoth et al., 2002; Suzuki et al., 2003). These genes and a Cys proteinase also affected by *phyB* are normally expressed in seeds (Gruis et al., 2002; Ruuska et al., 2002). Of note, the promoter regions (1000 bp) of 20 of these 21 selected genes (Figure 2) contain at least one core RY motif (CATGCA), which is a binding site of the B3 domain transcription factors FUSCA3 and ABSCISIC ACID INSENSITIVE3 (ABI3) (Reidt et al., 2000). This proportion (95%) is significantly greater ($P < 10^{-9}$) than expected based on the proportion of *Arabidopsis* promoters with the RY motif (8705 of 28,088; 31%). In 18 of the 21 genes, the RY motif was in the 500 bp upstream of the start codon ($P < 10^{-12}$). Randomly selected lists of 21 genes contained zero to nine (1000 bp) or zero to four (500 bp) genes with RY motifs (minimum $P = 10^{-2}$). Many of the selected 21 genes show enhanced expression in transgenic *Arabidopsis* seedlings bearing the maize (*Zea mays*) homolog of *ABI3*, *VIVIPAROUS1* (Suzuki et al., 2003).

Five of the genes selected for their response to phyB also showed significant interaction ($P < 0.05$) between phyA and phyB because the *phyA* mutant had enhanced expression compared with the wild type, whereas the *phyA phyB* and *phyB* mutants had similar levels of expression in D (At4g28520, At3g27660, At3g54940, At1g73190, and At2g16410; $P < 0.05$; Figure 2B). Therefore, the expression of these genes was inhibited primarily

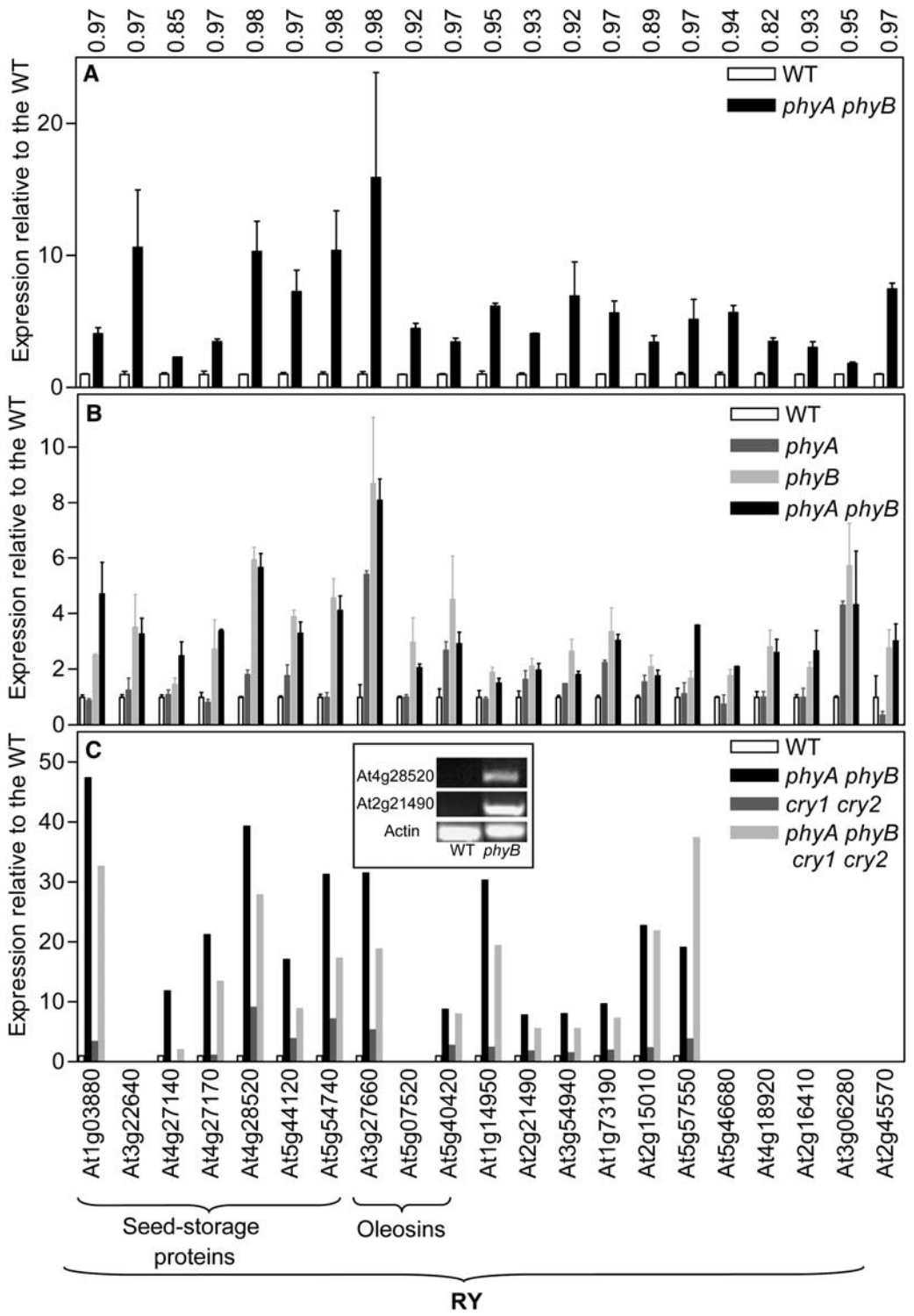

Figure 2. The *phyB* Mutation Affects Gene Expression in Dark-Grown *Arabidopsis* Seedlings.

(A) and **(B)** Expression in two independent experiments with the 22 K *Arabidopsis* microarray of genes filtered by their significant ($P < 0.05$) difference between the wild type and *phyA phyB* **(A)**, their significant difference between *PHYB* (wild type, *phyA* mutant) and *phyB* (*phyB* and *phyA phyB* mutants) **(B)**, and their confidence (1 minus the false discovery rate) > 0.8 (confidence values are indicated at top) in pooled wild-type versus *phyA phyB* data from the two experiments. Data shown are means ± SE of duplicate samples. Data were log-transformed for the analysis, but they are displayed without transformation.

by phyB and secondarily by phyA. According to criteria equivalent to those described in the previous paragraph, we identified genes inhibited by phyA alone (At5g55590, At3g27060, At4g02390, At3g14450, At1g07500, and At5g24280), promoted by phyB alone (At4g38690, At5g48180, and At1g06040), promoted by phyA and phyB (At2g19970, At2g21830, and At5g10460), or promoted by phyA alone (At1g09575 and At5g07580) (see Supplemental Figure 1 online).

The effects of phytochrome mutations on gene expression in dark-grown seedlings are very selective. As a negative control, a light-harvesting chlorophyll *a*/*b* binding protein of photosystem II (*Lhcb*) gene (At5g54270) showed a *phyA phyB*:*PHYA PHYB* ratio of 1.1 ± 0.1 in D, despite its strong sensitivity to phytochrome revealed by a ratio of 0.6 ± 0.1 after 1 h of WL, which activates other photoreceptors in addition to phyA and phyB.

The Light Environment Perceived by Seeds Affects Gene Expression in Dark-Grown Seedlings

Because the aforementioned changes in gene expression occurred in seedlings grown and harvested in full D, we investigated whether the light environment perceived during a previous developmental stage affects subsequent gene expression in D. Immediately after chilling (5 d), some seeds were exposed to 8 h of R and transferred to D. Other seeds were allowed to germinate without any exposure to light (Figure 3, top). Dark-grown seedlings were harvested for RNA extraction 3 d later. For the analysis, we selected a seed storage protein (At4g28520), a major latex protein (At1g14950), and a dehydrin (At2g21490) from the group shown in Figure 2. R given to the seeds inhibited the expression of these genes in the seedlings (Figure 3). In other words, seedlings produced by dark-germinated wild-type seeds phenocopied the *phyB* mutant.

Phytochrome Control of *ABI3* Expression

The aforementioned results prompted us to investigate whether the expression of *ABI3* is under the control of light absorbed by seed phytochrome. Chilled seeds of transgenic lines bearing the β-glucuronidase (GUS) reporter under the control of the *ABI3* promoter (Parcy et al., 1994) were either exposed to R (8 h), transferred to D, and harvested 4 d later or kept in D until harvest (Figure 4A). The seedlings produced by seeds exposed to R showed significantly less GUS activity driven by the *ABI3* promoter than the seedlings from seeds germinated in D (Figure 4A). No differences in GUS activity occurred in transgenic seedlings when the reporter was under the control of an *Lhcb* promoter, which is highly sensitive to phytochrome when the light treatments are given to the seedlings (Cerdán et al., 2000) (Figure 4A). This underscores the selectivity of the effect.

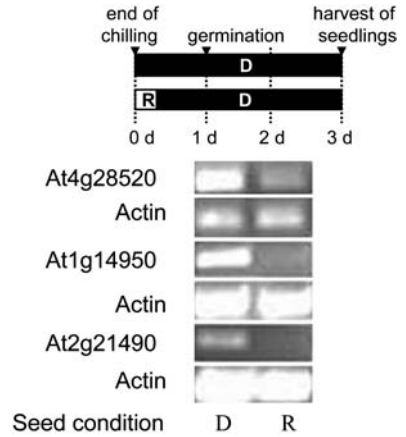

Figure 3. The Light Environment Experienced by the Seed Affects Gene Expression in Dark-Grown Seedlings.

Chilled seeds were transferred to D at 22°C with or without exposure to 8 h of R. Seedlings were harvested 3 d later.

The kinetics and localization of the *ABI3* response were investigated in further detail using the aforementioned R/D protocol (Figure 4A) in combination with different times of harvest. GUS staining was intense in the cotyledons of germinating seeds (day 1). By day 2, only 8% of the seedlings derived from R-irradiated seeds retained staining, whereas 86% of the seedlings derived from dark-incubated seeds retained staining (68 [R] and 43 [D] seedlings analyzed in three independent experiments; $P < 10^{-15}$ by χ^2 test). GUS activity was not detectable at day 3, irrespective of R or D given to the seeds. On day 4, GUS activity reappeared in the area surrounding the apex in 73% of the seedlings derived from seeds that had not been exposed to R and in only 22% of those derived from R-treated seeds (109 [D] and 160 [R] seedlings analyzed in three independent experiments; $P < 10^{-11}$ by χ^2 test) (Figure 4B).

Eight hourly R pulses (3 min) given to the seeds effectively reduced GUS staining of the seedlings at day 2, and this effect was reversed by following each one of the R pulses with a FR pulse, indicating a R/FR-reversible response typically mediated by phyB (Figure 4C). Seedlings of the *phyB* mutant grown in D after induction of seed germination by 8 h of R showed enhanced *ABI3* expression compared with wild-type seedlings (Figure 4F). Repeated pulses of FR that activate phyA (Yanovsky et al., 1997) had a small but significant effect compared with D (Figure 4C), indicating a contribution of this photoreceptor as observed in microarray experiments (Figure 2B).

A single pulse of FR fully reversed the effect of 8 h of R if given immediately after the R exposure (Figure 4D). A 16-h dark period

Figure 2. (continued).

(C) Expression of the genes selected after the 22 K experiments in the 8 K experiment. Note that some genes are not present in the 8 K microarray and that At5g07520, At1g61580, and At2g45570 had low expression values and were excluded (although their expression was higher in *phyA phyB* than in the wild type). The inset shows RT-PCR analysis for two genes in dark-grown wild-type and *phyB* seedlings in L*er* (biological samples independent from those used in the microarray experiments).

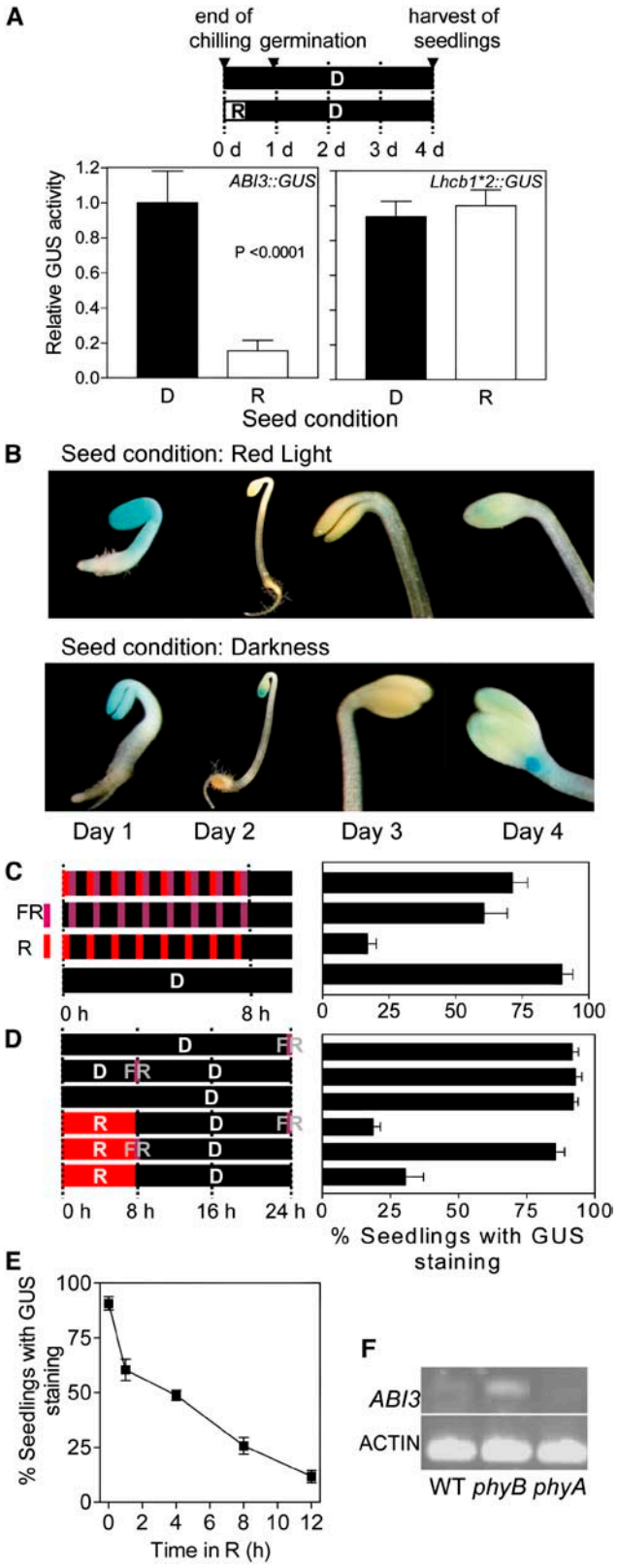

Figure 4. Phytochrome Control of *ABI3* Expression in Dark-Grown Seedlings.

(A) The activity of the *ABI3* promoter is downregulated by R given to the seeds. Chilled seeds were transferred to D at 22°C with or without exposure to R during the first 8 h. Seedlings were harvested 4 d later. Data shown are means ± SE from 10 independent samples in two experiments. Seedlings bearing the *Lhcb1*2* promoter fused to the reporter were included as controls.

(B) Time course and localization of GUS staining under the control of the *ABI3* promoter. The protocol was as in **(A)** but with different harvest times (1, 2, 3, or 4 d). Amplification was 15-fold (day 2) or 60-fold.

(C) Reversion of the effect of R by FR. After chilling (day 0, 0 h), the seeds were incubated (22°C) for 8 h in D or under hourly R pulses (3 min), hourly FR pulses (12 min), or hourly R pulses immediately followed by FR (3 min, 12 min) before transfer of all seeds to D. The proportion of seedlings with GUS staining in the cotyledons was scored on day 2 (data from 80 to 185 seedlings were pooled from three independent experiments).

(D) Escape from FR reversibility. After chilling (day 0, 0 h), the seeds were exposed to 8 h of R, 8 h of R immediately followed by a FR pulse, or 8 h of R followed by 16 h of D and then a delayed FR pulse. D, FR, and delayed FR controls were included. Then, all of the seeds were transferred to D. The proportion of seedlings with GUS staining in the cotyledons was scored on day 2.

(E) The activity of the ABI3 promoter decreases with the duration of exposure to R. Chilled seeds were transferred to D (day 0) after 0 to 12 h of R. The proportion of seedlings with GUS staining in the cotyledons was scored on day 2.

(F) Enhanced expression of *ABI3* in *phyB* mutant seedlings. Chilled seeds were transferred to D at 22°C after exposure to R during the first 8 h (day 0) and harvested on day 2.

interposed between the 8-h R treatment and the FR pulse made FR fully ineffective at reversing the effect of R (Figure 4D). In other words, the phytochrome effect escaped from reversibility during the 16 h of D.

The seeds were also exposed to R treatments of variable duration (0 to 12 h). The degree of GUS staining at day 2 decreased with the extension of the R treatment (Figure 4E). This pattern could account for the larger effect of the phytochrome mutations observed in the 8 K compared with the 22 K experiments (Figure 2), because seed germination was induced by 8 or 1 h of light, respectively. These observations are consistent with a role of ABI3 in the sequence of events between phyB perception of the light environment in the seeds and seedling gene expression.

ABI3 Modulates Phytochrome-Mediated Responses in Deetiolating Seedlings

Because phyB represses the expression of *ABI3*, we investigated phytochrome-mediated responses in the *abi3-6* mutant (Nambara et al., 1995) and two transgenic lines overexpressing the *ABI3* gene (*ABI3-OX-4* and *ABI3-OX-6*) (Parcy et al., 1994). In these experiments, we used only 15 min of R to induce seed germination (Figure 5A) without repressing *ABI3* expression with prolonged treatments (Figure 4E). Despite homogeneous germination, hypocotyl growth in D was enhanced slightly in *abi3* and reduced in the ABI3-OX lines (Figure 5B). None of the genotypes showed cotyledon unfolding in D. The inverse relationship between hypocotyl length in D and *ABI3* gene dosage is consistent with the proposed role of ABI3 in the induction of

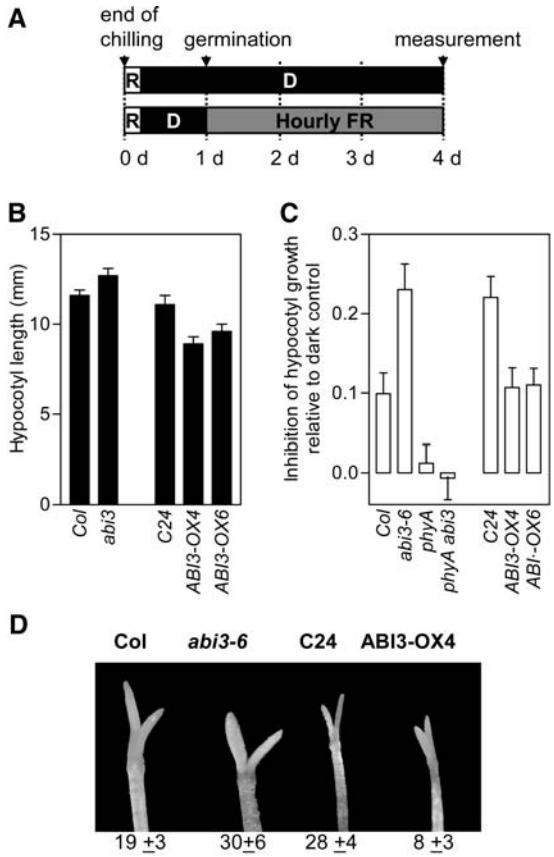

Figure 5. *ABI3* Downregulates the Response of Seedlings to Pulses of FR.

(A) Chilled seeds were given 15 min of R and transferred either to D for 4 d before measurements or to D for 1 d and then exposed for 3 d to hourly pulses (3 min) of FR before measurements.

(B) Hypocotyl length in D. Data shown in **(B)** and **(C)** are means ± SE from 10 replicate samples (a total of 100 seedlings) in three independent experiments.

(C) Inhibition of hypocotyl growth by pulses of FR relative to the dark controls ([length D – length R]/length D).

(D) Unfolding of the cotyledons by pulses of FR. Representative seedlings from one experiment and data from the same experiments shown in **(A)**. None of the genotypes unfolded the cotyledons in D (angle = 0°).

quiescence in prolonged D (Rohde et al., 1999). Expression of the *ABI3* homolog of poplar (*Populus trichocarpa*) occurs in the buds in response to short days that initiate bud set and dormancy (Rohde et al., 2002).

Hypocotyl growth and the cotyledon-unfolding response to pulses of FR compared with D are mediated by phyA (Yanovsky et al., 1997). The *abi3* mutant showed enhanced responses to pulses of FR, whereas *ABI3-OX-4* and *ABI3-OX-6* showed reduced responses (Figures 5C and 5D). The reduced hypocotyl growth inhibition is genuine and not an artifact resulting from the small differences in dark controls, because other genotypes that reduce growth in D do not necessarily show reduced inhibition by light (Luccioni et al., 2002). As expected, the *phyA* and *phyA abi3* mutants failed to respond to pulses of FR (Figure 5C).

Pulses of R activate phyB in addition to phyA (Yanovsky et al., 1997). The effect of *abi3* decreased (inhibition of hypocotyl growth relative to dark control, means ± SE: wild type = 0.33 ± 0.04; *abi3* = 0.40 ± 0.02) and the effect of *ABI3-OX* did not increase (means ± SE: wild-type C24 = 0.45 ± 0.03; *ABI3-OX-4* = 0.30 ± 0.03; *ABI3-OX-6* = 0.32 ± 0.03) under pulses of R compared with FR. In principle, these R treatments given to the seedlings could reduce *ABI3* expression in the wild type, narrowing the differences with *abi3*.

The Light Environment Perceived by Seeds Affects Phytochrome-Mediated Responses in the Seedling

PhyB in the seed affects *ABI3* expression (Figure 4), and ABI3 affects responses to light in seedlings (Figure 5). These findings suggest that the light environment of the seed could affect light responses in the seedlings. To investigate this issue, chilled seeds were exposed to 8 h of R or no light pulse, incubated in D for the rest of the first 24 h, and then either transferred to hourly pulses of FR or left in D (Figure 6A, top). In wild-type Col, hourly pulses of FR were more effective at inhibiting hypocotyl growth in seedlings derived from R-treated seeds than in seedlings originating from dark-incubated seeds (Figure 6A). Seedlings derived from dark-incubated seeds of the *abi3* mutant showed a greater response to the FR pulses than did wild-type seedlings, but the response was constitutive (i.e., not enhanced by R given to the seeds) (Figure 6A). Seedlings of the *phyA* and *phyA abi3* mutants failed to respond to pulses of FR irrespective of the exposure of the chilled seeds to R or full D. ABI3-OX showed an enhanced response to pulses of FR when the seeds where exposed to R compared with full D (Figure 6A). This finding suggests either an effect mediated by the reduction of endogenous ABI3 gene expression or an additional point of control by light (posttranscriptional or via a different player).

To investigate whether the seed light environment also affects gene expression responses, chilled seeds of *Arabidopsis* were exposed to 4 h of R, FR, or no light pulse and transferred to D. Two days later, the seedlings were either exposed to 24 h of FR or left as dark controls before harvest for the analysis of GUS activity driven by the *Lhcb1*2* promoter (Figure 6B, top). The seeds germinated homogeneously, but the seedlings from R-treated seeds showed enhanced GUS compared with those from seeds exposed to FR or D (Figure 6B). In the controls that remained without the 24-h FR treatment, GUS activity was low and unaffected by the light conditions experienced by the seeds. In the *phyB* mutant, FR given to the seeds was more effective than in the wild type (negative interference of phyB on phyA-mediated responses has been reported [Hennig et al., 2001]), but R was not more effective than FR, in agreement with the role of phyB in mediating the response to R compared with FR (Figure 6B). The effect of the *phyB* mutation compared with the wild type was confirmed for different *Lhcb1*2* transgene–*phyB* allele combinations (Figure 6B, inset). The *phyB* mutant showed reduced *Lhcb1*2* gene expression under FR, which is perceived by phyA but not by phyB (Quail et al., 1995). Although largely ignored, a similar *phyB* effect has been reported for other genes (Martinez-Hernandez et al., 2002; Yadav et al., 2002). Together,

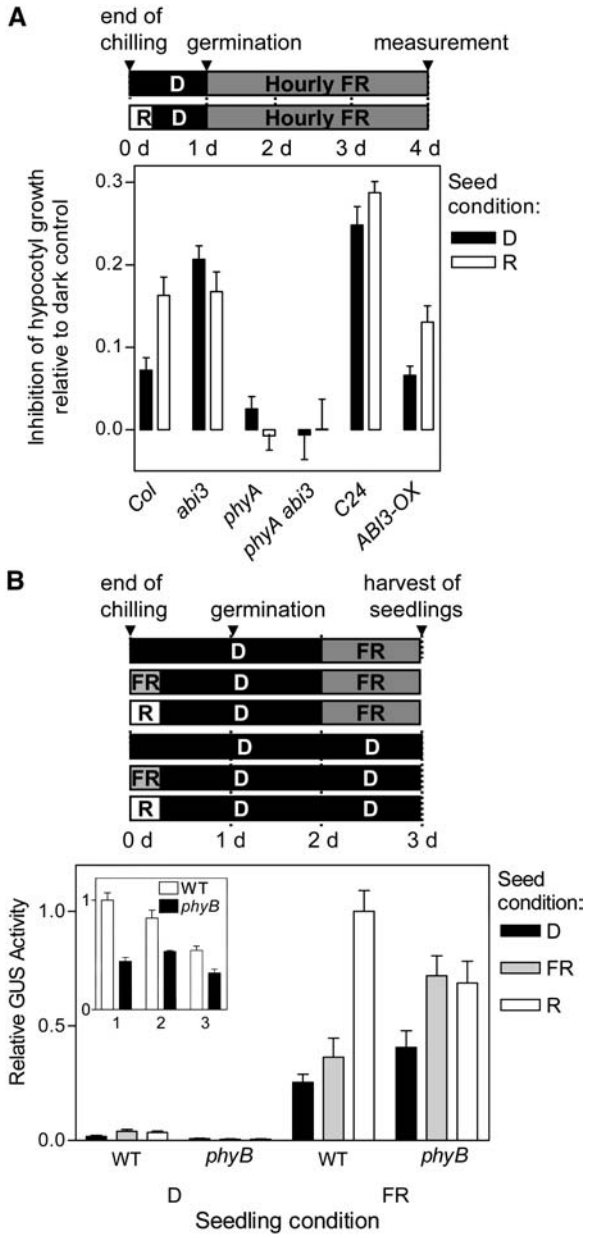

Figure 6. The Light Environment of the Seeds Affects the Subsequent Response to Light Given to the Seedlings.

(A) Chilled seeds were exposed to 8 h of R or no light pulse, incubated in D for the rest of the day, and then either left in D or transferred to hourly pulses (3 min) of FR for 3 d. Data shown are means ± SE from 15 replicate boxes in three different experiments.

(B) Chilled seeds of the wild type or the *phyB* mutant bearing the *Lhcb1*2* promoter fused to the GUS reporter were transferred to D with or without exposure to R or FR during the first 4 h. Two days later, the seedlings were exposed to 24 h of FR before harvest or remained as dark controls. Data shown are means ± SE from 10 independent samples in three different experiments. The inset shows GUS activity in wild-type and *phyB* transgenic seedlings of the L*er* background bearing the *Nicotiana plumbaginifolia Lhcb1*2* (1), of the Col background bearing the *N. plumbaginifolia Lhcb1*2* (2), or of the Col background bearing the *Arabidopsis Lhcb1*2* (3), where the seeds received R and the seedlings received FR.

these experiments indicate that light perceived by phyB in the seeds via ABI3 affects seedling responses to light treatments perceived by phyA.

The Seed-Derived Signal Is Not Active Phytochrome Itself

The earliest effects of R on GUS activity driven by the *ABI3* promoter were detected on day 2, whereas the response had already escaped from FR reversibility on day 1 (i.e., 16 h after the end of the R treatment) (Figures 4B and 4D). Because the long-wavelength FR pulse eliminates almost all active phyB, the signal carrying the information from the seed to the seedling cannot be phyB. Similarly, the different extent of response to FR of the *Lhcb1*2* promoter in seedlings derived from D- versus R-treated seeds was not eliminated by a long-wavelength FR pulse given 24 h after the beginning of R to remove virtually all phyB in its active form (GUS activity [nmol 4-methylumbelliferone·mg^{-1}·min^{-1}, means ± SE] with no light to the seeds, FR on day 1 = 1.9 ± 0.2; with R to the seeds, FR on day 1 = 3.4 ± 0.3). Therefore, a downstream signal generated by active phyB in the seed controls gene expression and responsivity to light in the subsequent dark-grown seedlings.

The expression of phaseolin, the most important seed storage protein of bean (*Phaseolus vulgaris*), is repressed by the position of a nucleosome over three-phased TATA motifs and the transcription start site (Li et al., 1998). This repressive chromatin structure is remodeled by the bean homolog of ABI3, allowing the expression of the phaseolin gene in response to abscisic acid (Li et al., 1999). Thus, it is intriguing to speculate that light signals perceived by phyB could alter chromatin structure subsequently remodeled by ABI3. The genetic interactions observed between ABI3 and DET1 (Rohde et al., 2000) favor this idea, because the DET1-DAMAGED DNA BINDING PROTEIN1 complex has been proposed to interact with chromatin, negatively regulating transcription (Benvenuto et al., 2002; Schroeder et al., 2002).

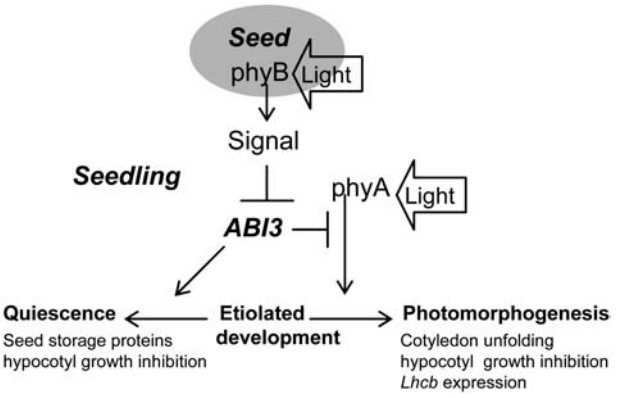

Figure 7. Proposed Model of the Control of Seedling Development by the Light Environment Experienced by the Seed.

Down arrows indicate a promotive effect, and the brackets indicate a repressive effect.

Conclusions

Compared with the wild-type, dark-grown seedlings of the *phyB* mutant show enhanced expression of a set of genes controlled by ABI3, enhanced expression of the *ABI3* gene itself, and reduced responses to FR (a light treatment perceived by phyA). Seedlings of the wild-type phenocopy these phenotypes of *phyB* when the seeds germinate without exposure to light (i.e., a condition that prevents the formation of active phyB). Hypocotyl growth in D and light responses are enhanced in the *abi3* mutant and reduced in ABI3-OX seedlings. Figure 7 integrates these findings in a model in which, via changes in *ABI3* expression, the light environment perceived mainly by phyB in the seeds regulates the developmental decision that leads either to vegetative quiescence or to photomorphogenesis in the seedlings. If a seed germinates very deep in the D of the soil, the seedling could benefit from a conservative use of reserves after an initial effort to reach sunlight. If a seed has been exposed to enough light to establish a biologically significant amount of active phyB, the seedling is unlikely to be very deep in the soil. Thus, the light environment experienced at a previous developmental stage originates a transdevelopmental phase signal that preconditions the seedling to its most likely light environment. This signal is not active phyB itself, and the involvement of chromatin remodeling provides a provocative hypothesis.

METHODS

Microarray Experiments

In the first experiment, we used the wild type of *Arabidopsis thaliana*, the *phyA-201 phyB-5* and *cry1* (*hy4*) *cry2* (*fha*) double mutants, and the *phyA phyB cry1 cry2* quadruple mutant (same alleles), all in the L*er* background. In all microarray experiments, the seeds of different genotypes were of similar age (always <1 year old). Seeds were sown on 0.8% (w/v) agar in clear plastic boxes and incubated at 6°C for 5 d. Chilled seeds were exposed to 8 h of R at 22°C to induce homogeneous seed germination. After 3 d in full D at 22°C, the seedlings were given 0 (dark control), 1, or 3 h of WL. Samples were harvested in liquid nitrogen, and total RNA was extracted using the RNEasy plant mini kit (Qiagen). cDNA and cRNA synthesis and hybridization to 8 K Affymetrix Arabidopsis Gene Chips were performed according to Affymetrix instructions.

Two additional independent experiments were conducted with 22 K (ATH1) Affymetrix Gene Chips. In one of these experiments, we used the wild type and the *phyA-211 phyB-9* double mutant in the Col background. In the other experiment, we used the wild type, the *phyA-211* and *phyB-9* single mutants, and the *phyA phyB* double mutant in Col. Seedlings were grown and harvested basically as described above, but 1 h of WL was used to induce germination. In each experiment, the scaling tab of the Affymetrix microarray suite in the mode ''all probe sets'' was used to standardize the trimmed mean signal of each array to the target signal according to the manufacturer's instructions. Raw expression data are shown in Supplemental Tables 1 to 3 online and have been deposited at www.ncbi.nlm.nih.gov/geo.

Descriptive Analysis of the Transcriptome

To investigate the main transcriptome patterns in the 8 K experiment, we first used a nonhierarchical k-groups partitioning method (McGarigal et al., 2000) to define distinctive large groups of genes. This method maximizes within-cluster homogeneity and uses a Euclidean measurement of the distance. We varied the number of groups (k) between 20 and 100 and found a group of 4730 genes with low expression values, which was consistent for the different k values. Subsequent analyses were performed with the remaining 4045 genes. Data were rank transformed to avoid distortions caused by outliers and used for correspondence analysis (Greenacre, 1984) (PROC CORRESPOND; SAS/STAT version 8.02). We used a multiresponse permutation procedure (Mielke, 1984) (PC-ORD version 4) to test the hypothesis of no difference between the groups of samples.

Significance of the Differences in Gene Expression

Expression data were log-transformed for the statistical analysis. One-way analysis of variance (ANOVA) was used for the ATH1 experiment with wild-type and *phyA phyB* seedlings (duplicate biological replicates). Factorial ANOVA with two factors (phyA and phyB) and two levels for each factor (wild-type allele and null allele) was used for the other ATH1 experiment (duplicate biological replicates). Transformed data showed homogeneity of variances. No gene is considered to have enhanced expression if any of its samples has expression values below a threshold (100 for data normalized as indicated above), because differences below that threshold were erratic and poorly reproducible. We calculated the probability that a gene is truly differentially expressed between the wild type and the *phyA phyB* double mutant using the permutation-based procedure described by Grant et al. (2005). This probability of true positives is defined as confidence and is equivalent to 1 minus the false discovery rate (Grant et al., 2005). For this purpose, we used wild-type and *phyA phyB* mutant data from both ATH1 experiments. The data were standardized to the average expression of the wild type in each experiment and log-transformed (log[$x + 1$], to avoid negative values), and the modified *t* statistics (Grant et al., 2005) were used for the analysis. We selected those genes that fulfilled each of three requisites: (1) significant ($P < 0.05$) differences between the wild type and *phyA phyB* in the first ATH1 experiment; (2) significant effects ($P < 0.05$) of phyA and/or phyB in the second ATH1 experiment; and (3) confidence of at least 0.8 for the difference between the *phyA phyB* mutant and the wild type. For comparative purposes, we also applied ANOVA to the wild-type and *phyA phyB* data pooled from both ATH1 experiments and used the P values to calculate the false discovery rate (*q*) according to Storey and Tibshirani (2003). The false discovery rates were larger with the latter procedure. For instance, At4g28520 showed a confidence (Grant et al., 2005) of 0.985 (i.e., a false discovery rate of 0.015) and a *q* value (Storey and Tibshirani, 2003) of 0.14. This difference is not surprising because procedures based on the marginal distribution of the P values without any assumption on gene expression changes (Storey and Tibshirani, 2003) are necessarily conservatively biased (Dalmasso et al., 2005). In particular, Reymond et al. (2004) have noted that high false discovery rates are estimated by the method of Storey and Tibshirani (2003) when the number of truly affected genes is relatively small, as is the case here. A direct assessment of the probability of false positives is provided by the observation of expression data in the 8 K experiment with genes selected on the basis of their expression in ATH1 experiments. All of these genes showed a two-fold difference if present in the 8 K array and expressed above the expression threshold.

RT-PCR

For RT-PCR experiments, total RNA was extracted using a guanidinium thiocyanate method (Logemann et al., 1987). Two micrograms of RNA was reverse transcribed into cDNA using the RNAimage kit (GenHunter). PCR was performed in 25 μL final volume (2 μL of template cDNA, 10× PCR buffer, 2 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate mix, 0.8 μmol of each primer, and 2.5 units of Platinum *Taq* DNA polymerase [Invitrogen]). Amplification was performed with a thermal cycler (PTC 100;

MJ Research) as follows: 5 min at 94°C; 35 cycles of 0.5 min at 94°C, 0.5 min at 55°C, and 1 min at 72°C; and a 10-min elongation at 72°C. PCR products were resolved on 1.5% agarose gels in 1× Tris acetic EDTA buffer containing 0.5 μg/mL ethidium bromide. The primers for At4g28520 were 5′-AAAACCAACTCGACCGCAA-3′ and 5′-TCCAGTGCAGTACAAGATCT-3′; primers for At1g14950 were 5′-GAGAGTCTTCGTAGCGCTT-3′ and 5′-CGACGTCAGGTACATACGT-3′; primers for At2g21490 were 5′-TTGGTCGGTAGTGGCAGGT-3′ and 5′-CATGTACCTAACCGGTGTTG-3′, primers for *ABI3* (At3g24650) were 5′-GCAGGACAAATGAGAGATCAG-3′ and 5′-TCATTTAACAGTTTGAGAAG-3′; primers for Actin8 (At1g49240) were 5′-ATGAAGATTAAGGTCGTGGCA-3′ and 5′-GTTTTTATCCGAGTTTGAAGAGGC-3′. RT-PCR analysis was performed in duplicate with RNA from two different sets of plants. Results of the two replicates were similar, and only one replicate is depicted.

Promoter Analysis

We analyzed the promoter elements of selected genes using the Statistical Motif Analysis in Promoter (The Institute for Genomic Research version 5.0, January 2004 release) tool available at the The Arabidopsis Information Resource website (www.arabidopsis.org). Promoter elements of samples of equal numbers of genes but selected randomly from the ATH1 Affymetrix array list were also analyzed for comparative purposes. The program compares the frequency of 6-mer words in a set of sequences (on both strands) with the frequency of the words in the region 500 or 1000 bp upstream of the start codon of the current 28,088 sequences of the *Arabidopsis* genome.

Activities of the *ABI3* and *Lhcb1*2* Promoters

Fifty seeds of transgenic plants of *Arabidopsis* ecotype L*er* carrying the promoter at positions −752 to +67 of the light-harvesting complex gene *Lhcb1*2* from *Nicotiana plumbaginifolia* fused to the GUS reporter gene (Cerdán et al., 1999) or the *ABI3* promoter fused to the GUS reporter gene (Rohde et al., 1999) were sown on 0.8% (w/v) agar in clear plastic boxes and incubated at 6°C for 5 to 7 d. In some experiments, the transgene was expressed in *phyB-5* L*er*, wild-type Col (Cerdán et al., 1999), or *phyB-9* Col (Cerdán et al., 2000). The *Lhcb1*2* transgene from *Arabidopsis* in the wild-type Col (Susek et al., 1993) or *phyB-9* Col (Casal et al., 1998) background was also included. Measurements of GUS activity were performed as described previously (Cerdán et al., 2000).

For in vivo GUS staining (Blázquez et al., 1997), transgenic seedlings were incubated at 37°C for 75 min in staining solution (2 mM 5-bromo-4-chloro-3-indolyl β-glucuronide, 2 mM ferrocyanide, and 50 mM sodium phosphate buffer), fixed for 30 min in 20% ethanol, transferred to 35% ethanol, and photographed immediately.

Hypocotyl Growth and Cotyledon Unfolding

Seeds of the *abi3-6* mutant (Nambara et al., 1995) and two independent lines carrying the *35S:ABI3* transgene (ABI3-OX-4 and ABI3-OX-6) (Parcy et al., 1994) were compared with their respective Col or C24 wild type. Fifteen seeds of each genotype were sown on 0.8% (w/v) agar in the clear plastic boxes and incubated at 6°C for 5 to 7 d. Unless indicated otherwise, chilled seeds were exposed to R at 22°C to induce homogeneous seed germination. Hypocotyl length was measured to the nearest 0.5 mm with a ruler on the 10 longest seedlings from each box (this eliminates defective seedlings). The angle between cotyledons was measured with a protractor using the same 10 seedlings. Seedling data were averaged per box (one replicate) and used for statistical analysis.

Light Treatments

WL (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was provided by fluorescent tubes, R (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was provided by red fluorescent tubes, and FR (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was provided by incandescent lamps in combination with a water filter and yellow, orange, red, and blue plastic filters. Long-wavelength FR (12 min, 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was provided by incandescent lamps in combination with an RG9 filter (Schott).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for genes expressed are shown in Supplemental Tables 1 and 2 online. Locus identifiers for genes listed in Figure 2 and Supplemental Figure 1 are shown in Supplemental Table 4 online.

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