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Transcriptional Programs Related to Phytochrome A Function in *Arabidopsis* Seed Germination

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ABSTRACT In *Arabidopsis* seeds, germination is promoted only by phytochromes, principally phytochrome B (phyB) and phytochrome A (phyA). Despite the abundant information concerning the molecular basis of phyB signaling downstream of PIF1/PIL5, the signaling network inducing germination by phyA is poorly known. Here, we describe the influence of phyA on the transcriptome of *Arabidopsis* seeds when germination is induced by a far-red (FR) pulse. The expression of 11% of the genome was significantly regulated by phyA. Most of the genes were up-regulated and the changes noted late (i.e. 5 h after a FR pulse), whereas changes in down-regulated genes were more abundant earlier (i.e. 0.5 h after a FR pulse). Auxin- and GA-associated elements were overrepresented in the genes that were modified by phyA. A significant number of genes whose expression was affected by phyA had not been previously reported to be dependent on PIL5. Among them, homozygotic mutant seeds of MYB66, a SAUR-like protein, PIN7, and GASA4 showed an impaired promotion of germination by phyA. Natural variation at the transcriptional level was found in early signaling and GA metabolic genes, but not in ABA metabolic and expansin genes between Columbia and Landsberg *erecta* accessions. Although phyA and phyB/PIL5 signaling pathways share some molecular components, our data suggest that phyA signaling is partially independent of PIL5 when germination is promoted by very low fluences of light.

Key words: transcriptome; germination; light; *Arabidopsis* seeds; PIF1/PIL5; phytochrome A (phyA); phytochrome B (phyB).

INTRODUCTION

Timing of seed germination is of the utmost importance for the adjustment of plant populations to a particular habitat and, in an important number of species, it depends on the fine perception of environmental cues (Finch-Savage and Leubner-Metzger, 2006). To seeds, light is one of the richest sources of information about the environmental scenario and plays a crucial role in the adjustment of plant populations to their habitat (Donohue, 2002). Promotion of germination by light is exclusively mediated by phytochromes (Bae and Choi, 2008). Phytochrome B (phyB) and other stable phytochromes sense the red (R, 600–700 nm) to far-red (FR, 700–800 nm) ratio of incoming light and promote germination through the Low-Fluence Response (LFR). Experimentally, the LFR is maximally induced by a saturating pulse of R and is inhibited by a FR pulse establishing low levels of active form of the phytochrome, namely Pfr (Shinomura et al., 1994; Botto et al., 1995). In natural environments, the LFR is promoted when vegetation soil cover decreases due to the increase in the R/FR ratio of the light reaching the soil (Vázquez-Yañes and Smith, 1982; Deregibus et al., 1994; Insausti et al., 1995). On the other hand, the light response induced by phytochrome A (phyA) is usually saturated with extremely low amounts of Pfr, lower than those established by FR filters, and consequently this response is not R/FR reversible (i.e. Very-Low-Fluence Response (VLFR); Botto et al., 1996; Shinomura et al., 1996). The VLFR may be elicited by small amounts of photons equivalent to tenths of seconds of sunlight exposure (Scopel et al., 1991; Botto et al., 1998a). The VLFR participates in the promotion of germination by soil disturbances that briefly expose the seeds to sunlight, like those caused by tilling operations (Botto et al., 1998b, 2000).

Although the ecological consequences of germination responses promoted by phyB and other stable phytochromes (hereafter, phyB) and phyA are well established, the knowledge available on the molecular mechanisms underlying both light responses is quite different. While the information

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concerning the molecular basis of phyB signaling is abundant (Penfield et al., 2005; Oh et al., 2006, 2007; Kim et al., 2008; Oh et al., 2009; Gabriele et al., 2010; Park et al., 2011), that concerning phyA signaling inducing germination is poorly known. One central actor in phyB signaling is PIF1/PIL5 (hereafter, PIL5), which represses germination either directly or indirectly through DELLAs (RGA and GAI), SOMNUS, and other regulatory inhibitory elements (Oh et al., 2007; Kim et al., 2008; Gabriele et al., 2010; Park et al., 2011). Very recently, Cho et al. (2012) demonstrated that *JMJ20* and *JMJ22* are positive elements acting on PIL5 signaling directly repressed by SOMNUS. After phyB activation, *JMJ20* and *JMJ22* are de-repressed and then histone arginine methylations are removed at the promoters of *GA3ox1* and *GA3ox2*, which, in turn, promote germination.

Global gene expression analysis carried out in wild-type and *pil5* mutant seeds displaying a R/FR reversible response has shown that the transcriptome of *pil5* seeds irradiated with a FR pulse is similar to that of wild-type seeds irradiated with a R pulse (Oh et al., 2009). This suggests that most, if not all, phyB–Pfr actions are mediated through their effects on PIL5 stability. Similarly, the fact that phyA can bind PIL5 and induce its degradation opens the possibility that phyA promotion of germination could be mainly or exclusively related to PIL5-controlled genes (Seo et al., 2009). However, at least two pieces of experimental evidence suggest that other factors independent of PIL5 must be present to induce germination by light; the increase in *pil5* and *pil5phyB* germination with a FR pulse suggests that factors other than PIL5 also regulate phyA-dependent germination (Oh et al., 2004). Moreover, Penfield et al. (2010) reported that low dormant *pil5* seeds responded differentially to a FR pulse compared with germination in darkness, reinforcing the idea that phyA-dependent germination is at least partially independent of PIL5. This physiological evidence led us to test the possibility that phyA signaling promoting germination involves molecular components independently of PIL5. For that purpose, we analyzed the phyA-dependent transcriptome induced after a FR pulse in germinating seeds of *Arabidopsis*. Besides, we identified phyA signaling elements not affected by PIL5 when comparing the phyA-dependent transcriptome with that reported by Oh et al. (2009) as PIL5-dependent. By using a mutagenesis approach, we confirmed the involvement of some molecular components in phyA signaling inducing seed germination. Finally, we described natural variation at the transcriptional level dependent on phyA action.

RESULTS

Identification of phyA-Regulated Genes in a Temporal Expression Pattern

We performed a microarray expression analysis to identify phyA-dependent genes associated with the induction of germination by a FR pulse. We compared the light germination response between L*er* wild-type and *phyA* seeds. L*er* and *phyA* seeds were incubated at 25°C for 24 h and then irradiated with a FR or R pulse to induce germination. Compared with darkness, approximately 50% of L*er* seeds germinated with a FR pulse (Pfr/P = 0.03) and 100% of the seeds germinated with a R pulse (Pfr/P = 0.87). As expected, *phyA* seeds did not germinate after a FR pulse but their germination increased to 100% after a R pulse (Supplemental Figure 1). We used these seeds to define the transcriptome involved in the promotion of germination by phyA. We carried out a global expression analysis using ATH1 Affymetrix microarrays with L*er* and *phyA* biological samples extracted at three time intervals after the FR pulse (0, 0.5, and 5 h; Figure 1A). From 22 746 genes on the ATH1 microarrays, we selected 12 745 genes which had appeared in the two samples of at least one treatment. We used this list of genes for all the statistics generated in this study. The analysis identified a total of 1388 genes regulated by phyA in a statistical significant fashion (i.e. 11% of the expressed transcriptome; Supplemental Figure 2 and Supplemental Table 1). Most of them (1032 genes) were induced, whereas the remaining ones (356 genes) were repressed by a FR pulse. The genes were classified into four clusters depending on whether their expression was induced or inhibited by a FR pulse at 0.5 or 5 h (early- and late-regulated genes, respectively). Only 153 phyA-dependent genes showed differential early expression, most of them (82%) down-regulated by a FR pulse (Figure 1B and Supplemental Table 1). In contrast, 1235 phyA-dependent genes were late-regulated, most of them (81%) up-regulated by a FR pulse (Figure 1B and Supplemental Table 1). Down-regulated genes were overrepresented in response to stress, transcription, and signal transduction gene ontology signatures, whereas up-regulated genes were overrepresented in DNA–RNA metabolism and cell elongation and biogenesis signatures (Figure 1C). We validated the results of the ATH1 microarray by qRT–PCR. This analysis confirmed the expression pattern of a dormancy transcription factor (At1g09250), *ATHB2*/*HAT4*, *PIF6*/*PIL2*, and *GA20OX3* (Supplemental Figure 3).

A Diversity of Transcription Factors Are Regulated by phyA in Germinating Seeds

In regard to molecular function, we studied whether our list of 1388 phyA-dependent genes was enriched in transcription factors. We found that 101 genes (i.e. 7.3% of the phyA-dependent transcriptome) encoded transcription factors. This proportion is slightly but not significantly higher than the genes in the whole *Arabidopsis* genome (Representation Factor; RF: 1.1; $P < 0.142$). However, a more detailed analysis of phyA-dependent genes showed that the list of late-regulated genes is enriched in transcription factors (RF: 1.9; $P < 5.462\ e^{-04}$). phyA-dependent transcription factors are represented by AP2-EREBP, GRAS, bHLH, ATHB, and CH3 family types, among others. Transcription factors

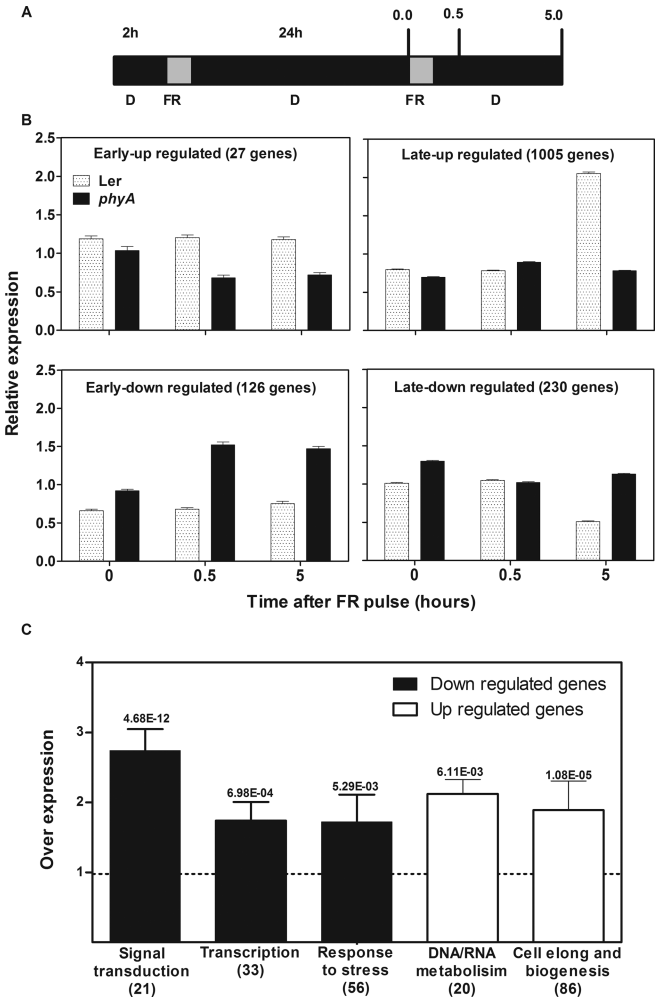

Figure 1. Global Expression Pattern of phyA-Dependent Genes.
(A) Experimental protocol for RNA extraction for ATH1 microarray analysis. Samples were extracted at 0, 0.5, and 5 h after a FR pulse.
(B) Temporal classification of 1388 phyA-dependent genes whose expression was statistically affected by one-way ANOVA (*q*-value < 0.01) and L*er*/phyA ratio > 1.5. The four clusters were defined by up- and down-regulated genes whose expression was affected early or late after a FR pulse (0.5 and 5 h, respectively).
(C) Gene Ontology (GO) assignments for phyA-dependent genes. The graph shows the functional categories of biological processes overrepresented for down- and up-regulated genes. Only signatures overrepresented with a ratio higher than 1.5 are shown. Data are means ± SE. The *P*-value is shown for each signature.

include light signaling genes (*PIL2*, *HFR1*, *HAT4*), ABA signaling genes (*FUS3*, *ABI4*, *ABI1*), auxin signaling genes (*AXR1*, *AXR4*, *ARF18*), a brassinosteroid signaling gene (*BEH1*), cytokinin signaling genes (*CRF1*, *CRF2*, *CRF3*), GA signaling genes (*RGL3*, *RGL1*, *SCL3*, *SCL14*, *SCL8*), and a jasmonic acid (JA) signaling gene (*MYC3*; Table 1). Interestingly, genes down-regulated by phyA are enriched significantly in seven ABA- and one light-motif-promoter sequence (Supplemental Table 2). These results are coherent with the notion that phyA regulates the expression of transcription factors, which modulate the transcription of other genes at the upper hierarchy of the light and hormonal transcriptional cascades in imbibed seeds.

Signaling and Metabolic Hormone Genes Related to phyA-Dependent Germination

phyA-dependent germination was associated with the up-regulation of genes involved in signaling, transport, and metabolism of auxin (Table 1 and Supplemental Figure 4). At 5 h after the FR pulse, phyA up-regulated two auxin resistance proteins (*AXR1* and *AXR4*), a transcription factor (*ATMYB34*), three auxin transport proteins (*PIN1*, *PIN2*, *PIN7*), and the *SUR1* gene involved in the auxin metabolism (Table 1). Besides, other metabolic genes, such as *CYP79B2*, which is involved in tryptophan metabolism, and a nitrilase (*NIT3*), which catalyzes the hydrolysis of indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA), were down-regulated by the action of phyA (Supplemental Figure 4). Except for these two genes, auxin-related genes showed an increase in their expression, suggesting that the promotion of germination by light involves the participation of auxin signaling elements.

Among GA genes, *XERICO*, *SOMNUS*, a GA receptor gene (*GID1A*), two SCARECROW-LIKE genes (*SCL14*, *SCL3*), a GA responsive gene (*GASA4*), and a GA metabolic gene (*GA20ox3*) were down-regulated by phyA. Other GA signaling genes, such as *SCL8* and *GASA6*, were up-regulated by phyA (Table 1). In addition, the up-regulation of two DELLA genes (*RGL1* and *RGL3*) was unexpected. This suggests that the promotion of germination by a FR pulse is independent of the transcription regulation of some DELLAs. The regulation of ABA genes by a FR pulse inducing germination indicates that phyA–Pfr blocks the expression of signaling and metabolic ABA genes. Several ABA genes, such as a transcription factor (*FUS3*), an ABA receptor gene (*PYL7*), the RARE-COLD INDUCIBLE 2A gene (*RCI2A*), and an ABA catabolic gene (*CYP707A3*), were down-regulated by phyA. Also, other ABA genes, such as transcription factor (*ABI4*), a catabolic ABA gene (*CYP707A2*), and an ABA biosynthesis gene (*NCED5*), were up-regulated by phyA (Table 1).

Brassinosteroid (BR) and ethylene signaling are known to promote germination (Kucera et al., 2005). Some BR and ethylene genes were significantly expressed by the action of phyA. The *BEH1* gene was up-regulated by phyA, inducing BR signaling and metabolic gene expression. The BEH1 transcription factor is a homolog of BES1/BZR1, known to bind and activate BR target gene promoters (Yin et al., 2005). The positive regulation of BEH1 induces *DWF4* expression, which encodes an enzyme known to catalyze a rate-limiting step of BR biosynthesis in *Arabidopsis* (Kim et al., 2006). Also, an ethylene response transcription factor gene (*ERF104*) was down-regulated by phyA, while an ethylene-associated gene (*EGYP3*) was up-regulated by phyA, as expected for the positive effects of ethylene on seed germination. *EGYP1*, a homolog of *EGYP3* encoding a membrane-associated and

Table 1. List of Signaling and Metabolic Hormone Transcripts Expressed in a phyA- and PIL5-Dependent Manner.

Hormone	Class	AGI	Name	Ler/*phyA*	Col/*pil5*
ABA	Metabolic	At1g30100	NCED5	2.82	Absent
		At1g54100	ALDH7B4	–1.86	1.76
		At2g29090	CYP707A2	4.55	–2.2
		At3g51810	AT3/AtEM1	–3.25	3.54
		At5g45340	CYP707A3	–2.3	Absent
	Signaling	At2g40220	ABI4	6.42	–3.1
		At3g05880	RCI2A	–3.27	Absent
		At3g26790	FUS3	–3.67	Absent
		At3g51920	ATCML9	–4.6	2.41
		At3g63210	MARD1	2.33	Absent
		At4g01026	PYL7	–4	Absent
		At4g26080	ABI1	1.98	–1.51
	Transporter	At1g59870	ABCG36	–3.55	Absent
Auxin	Metabolic	At2g20610	SUR1	3.11	–2.64
		At3g44320	N IT3	–1.67	2.25
		At4g39950	CYP79B2	–2.63	Absent
	Signaling	At1g05180	AXR1	1.5	Absent
		At1g54990	RGR1/AXR4	1.77	Absent
		At1g54990	AXR4	1.77	Absent
		At3g07390	AIR12	5.66	Absent
		At3g61830	ARF18	1.6	1.64
		At4g31500	RED1	1.97	Absent
		At4g31500	CYP83B1	1.97	Absent
		At5g54510	DFL1/GH3.6	4.21	–3.41
		At5g60890	AtMYB34	19.02	Absent
	Transporter	At1g23080	PIN7	8.17	Absent
		At1g73590	PIN1	4.49	–3.06
		At5g57090	PIN2	1.99	–3.07
BR	Metabolic	At3g50660	CYP90B1	1.71	–3.32
	Signaling	At3g50750	BEH1	17.41	–2.83
Citokinin	Metabolic	At1g75450	CKX5/CKX6	4.38	Absent
	Signaling	At4g11140	CRF1	4.93	–2.37
		At4g23750	CRF2	6.57	–3.18
		At5g53290	CRF3	5.25	–4.49
Ethylene	Signaling	At1g17870	EGYP3	3.28	–1.61
		At5g61600	ERF104	–2.6	1.81
GA	Metabolic	At5g07200	20OX3	–5.88	3.69
	Signaling	At1g03790	SOM	–1.98	2.93
		At1g07530	SCL14	–2.08	1.91
		At1g66350	RGL1	3.53	–2.84
		At1g74670	GASA6	11.92	Absent
		At2g04240	XERICO	–8.4	5.37
		At3g05120	GID1A	–2.84	3.45
		At4g11140	CRF1	4.93	–2.37
		At4g23750	CRF2	6.57	–3.18
		At5g07200	GA20OX3	–5.88	3.69
		At5g14750	MYB66	5.42	Absent
		At5g15230	GASA4	–7	Absent
		At5g17490	RGL3	2.37	Absent

(Continued)

Table 1. (*Continued*)

Hormone	Class	AGI	Name	Ler/*phyA*	Col/*pil5*
		At5g52510	SCL8	2.34	Absent
JA	Signaling	At3g17860	JAZ3	1.9	Absent
		At5g20900	JAZ12	–2.92	2.03
		At5g46760	MYC3	–4.09	1.82
Light	Signaling	At1g02340	HFR1	1.89	Absent
		At1g09570	PHYA	1.54	2.2
		At3g62090	PIL2/PIF6	–3.47	34.53
		At4g16780	ATHB2/HAT4	–3.37	13.91

Fold change expression is shown between L*er*/phyA and Col/*pil5*. The PIL5-dependent genes were obtained from Oh et al. (2009). Positive and negative values indicate up- and down-regulated genes by phyA, respectively. The inverse corresponds for PIL5.

ATP-independent metalloprotease required for chloroplast development, is positively regulated by ethylene and light (Park et al., 2004).

Jasmonic acid is known to inhibit germination (Kucera et al., 2005). In agreement with this, two JA signaling genes were down-regulated by phyA (*JAZ12* and *MYC3*). The former gene is known to encode a transcription factor that acts together with MYC2 and MYC4 to activate JA responses (Fernández-Calvo et al., 2011). *JAZ3* expression was up-regulated in germinating seeds after a FR pulse, as expected for the repressor function of JAZ3 in JA signaling. JAZ3 is involved in binding JAZ proteins to the F-box protein COI1 and their posterior ubiquitination and degradation by the 26S-proteosome (Brose, 2009). Finally, three genes encoding cytokinin regulator factors (*CRF1*, *CRF2*, *CRF3*) and a catabolic cytokinin gene (*CKX5*) were up-regulated by phyA (Table 1).

phyA-Dependent Genes Are Overrepresented in Auxin and Gibberellin Signatures

To evaluate the possibility that phyA influences the hormone-dependent genes differentially, we carried out an overrepresentation analysis using hormonal signatures related to dormancy and germination processes (Ogawa et al., 2003; Carrera et al., 2007). We used the TAGGIT classification approach designed for visualization of seed developmental signatures to define the reference list of genes (Carrera et al., 2007). We compared our list of 1388 phyA-dependent genes with the TAGGIT list of genes associated with hormonal signatures. We found that only auxin signaling elements were overrepresented in the phyA-dependent transcriptome and that all of them corresponded to late up-regulated genes (RF: 2.9, $P < 0.007$). To have a broader insight into the influence of GA-dependent genes on the regulation of germination by phyA, we compared our data with a GA-dependent transcriptome described by Ogawa et al. (2003) comparing the germination of *ga1-3* with GA addition. Interestingly, we found an overrepresentation of GA-dependent genes associated with the induction of germination by phyA (RF: 2.2, $P < 3.75\ e^{-12}$). These data indicate that the phyA-dependent transcriptome is enriched in auxin and GA genes and suggest that both hormones may be involved directly or indirectly in the phyA signaling.

FR-Induced Germination through phyA Signaling Partially Overlaps with PIL5 Signaling

We compared the transcriptome related to PIL5 (Oh et al., 2009) with our phyA-dependent transcriptome. The phyA-dependent genes were compared with 2555 genes whose expression was PIL5-dependent (Oh et al., 2009). We found 618 genes shared by both phyA- and PIL5-dependent signaling pathways (i.e. 45% of phyA-dependent genes) and 770 genes linked to phyA signaling that had not been reported to be affected by PIL5 (i.e. 55%, Figure 2A and Supplemental Table 3). Gene ontology (GO) enrichment analysis was performed to select biological processes overrepresented in either phyA- or PIL5-dependent transcriptomes. Some signatures, such as protein metabolism, developmental processes, and response to abiotic/biotic stimuli, were represented significantly in both microarrays (Figure 2B). DNA/RNA metabolism and cell organization and biogenesis signatures were overrepresented in the phyA-dependent transcriptome, whereas signal transduction and transport-related genes were overrepresented in the PIL5-dependent transcriptome (Figure 2B). We found a positive but low correlation between the expression of genes common to both phyA- and PIL5-dependent transcriptomes ($R^2 = 0.43$). The low correlation between both microarray data was due to differences in the expression of some genes such as *EXP1*, *EXP10*, *PIL2*, and *ATHB2/HAT4*. By removing these four genes, the correlation index improved ($R^2 = 0.65$; Figure 2C). The results indicate that a common set of genes are involved in the promotion of germination by phyA and in the inhibition of germination by PIL5. Among them, a common group of cell-wall-modifying enzyme genes were up-regulated by phyA and down-regulated by PIL5, confirming that the photocontrol of germination by a FR or R pulse involves the regulation of a common set of cell-wall enzymes (Figure 2D). The exception to this observation was *EXP2*, whose expression showed phyA dependence but not PIL5 dependence. Unexpectedly, we found seven genes that

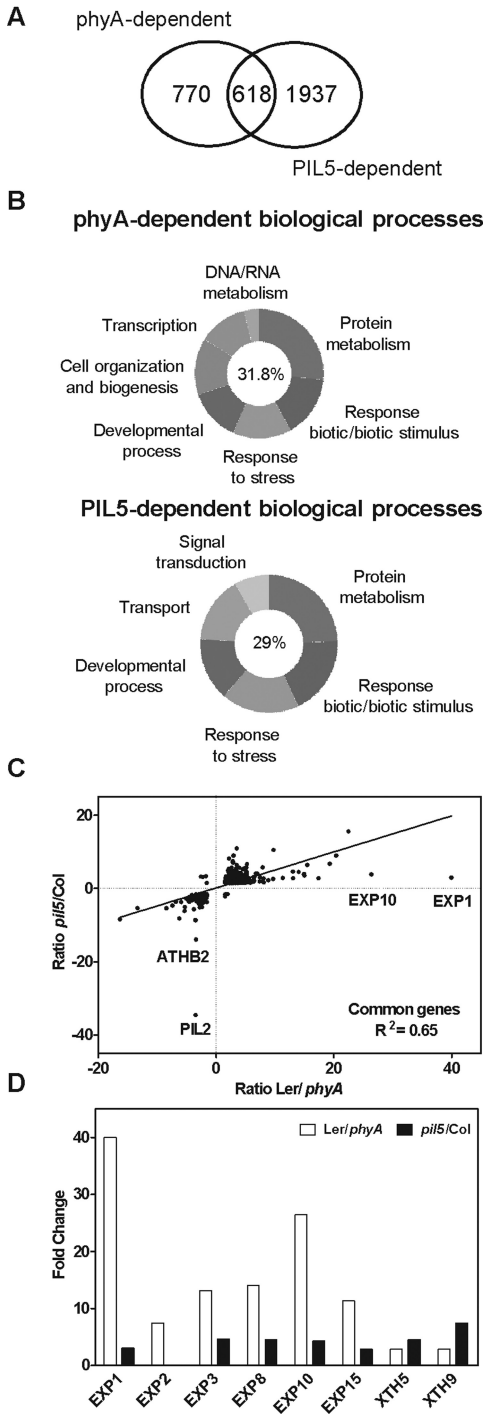

Figure 2. phyA- and PIL5-Dependent Transcriptomes Overlap Partially.
(A) Venn diagram of phyA- and PIL5-dependent genes.
(B) Proportion of significantly overrepresented biological processes of phyA- and PIL5-dependent genes. The graph shows common and specific biological processes related to phyA- and PIL5-dependent germination. The numbers inside the ring represent the percentage of genes belonging to the signatures indicated in the graph (1388 genes for phyA-dependent germination and 2555 genes for PIL5-dependent germination).
(C) Correlation of expression ratio between pil5/Col and Ler/phyA for common genes expressed in phyA- and PIL5-induced germination.
(D) Fold change ratio for wall-related genes promoted by phyA or PIL5.

were regulated in the same direction by phyA–Pfr and PIL5. Some of them were *PHYA*, *ARF18* (an auxin regulated factor), *SAG29* (a senescence-associated gene), and a glycosyl hydrolase with α-mannosidase activity (*At3g26720*) involved in metabolic activities in the elongation structures of the embryo.

Germination of T-DNA Mutants Confirms Gene Function in phyA-Dependent Germination

We confirmed the function of some phyA-dependent genes in the promotion of germination by a FR pulse by analyzing the light response of homozygotic T-DNA mutant seeds. The germination of wild-type seeds was 20% in darkness and higher than 75% after a FR pulse, indicating that 60% of the population germinate by phyA action. Another fraction of the seed population showed a R/FR reversible response mediated by phyB (Figure 3). Germination of mutant seeds of MYB66, *PIN7*, and a SAUR-like auxin-responsive protein (At4g34750) showed a reduced or null response to a FR pulse but a full response to a R pulse, which confirmed the positive function of these proteins in the regulation of germination by phyA (Figure 3). Also, *pil2* and *gasa4* seeds showed an impaired germination to a FR pulse (Figure 3). The light germination response of *pil2* and *gasa4* seeds is apparently contradictory with the output of our wide genome analysis (Supplemental Table 1). One possible reason for the discrepancy might be genetic background differences used as control in both experiments (L*er* for the transcriptome analysis and Col for the T-DNA mutant experiment). Also, some of these differences could be explained by posttranscriptional mechanisms,

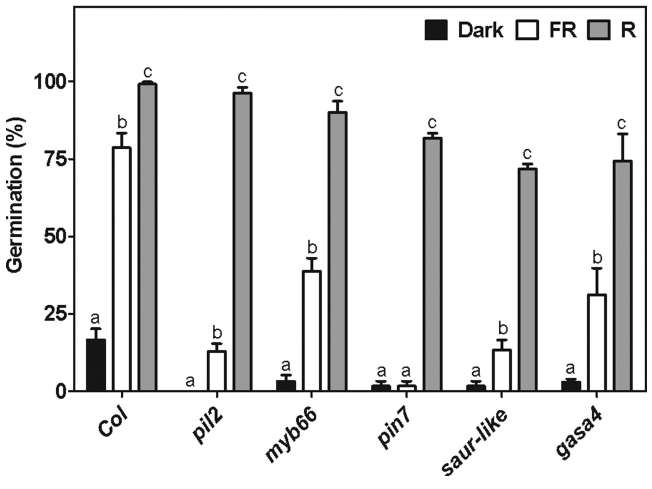

Figure 3. Germination Induced by Light in T-DNA Mutant Seeds.
Col and T-DNA mutant seeds of some phyA-dependent genes were incubated at 5°C for 24 h in darkness and then transferred at 25°C for 24 h before irradiation with a FR pulse, R pulse, or maintained in darkness until the evaluation of germination. Data are means ± SE of at least three independent experiments. Different letters indicate significant effects between the light treatments in each genotype ($P < 0.05$).

as has been previously documented for *PIL2* regulation during seed germination (Penfield et al., 2010).

phyA Down-Regulates *DELLA* Gene Expression

Although the most conspicuous DELLAs (i.e. *GAI*, *RGA*, and *RGL2*) did not shown significant differences in the microarray study (Supplemental Table 1), previous reports have shown that these DELLAs are central regulators of seed germination (Cao et al., 2006; Oh et al., 2007; Piskurewicz et al., 2008). DELLAs are early components of GA signaling. When GAs are low, DELLAs block the transcription of GA-regulated genes by direct interaction with their promoters (Cao et al., 2006). Also, GAI and RGA can act as integrators of GA and light signals mediated by phyB (Oh et al., 2007). Considering the importance of DELLAs in the control of seed germination, we evaluated by qRT–PCR the expression of the three DELLAs at 0.5 and 5 h after the promotion of germination by a FR pulse (Figure 4A). We found that *GAI*, *RGA*, and *RGL2* were all down-regulated by the action of phyA but with different timing (Figure 4B and 4C). *RGL2* was down-regulated rapidly after 0.5 h of the FR pulse and the effect persisted until 5 h later. In contrast, *GAI* was down-regulated early; meanwhile, *RGA* was regulated later after a FR pulse. We conclude that phyA reduces the expression of DELLAs as a pre-requisite to promote germination by a FR pulse. In view of the relevance of RGL2 for ABI5 action in seed germination (Piskurewicz et al., 2008), we studied the importance of the RGL2 interaction with ABI5 when germination was promoted by phyA. For this purpose, we employed the transgenic seeds containing the constructions 35S::HA–ABI5 and 35S::HA–ABI5 in *rgl2* background. We demonstrated that the overexpression of ABI5 decreases the germination induced by a FR pulse only if RGL2 is present (Figure 5). Moreover, similar results were found for seeds previously imbibed at 5°C for 24 h, suggesting that the phyA–RGL2–ABI5 signaling pathway induced by a FR pulse operates independently of chilling in our experimental conditions (Supplemental Figure 5).

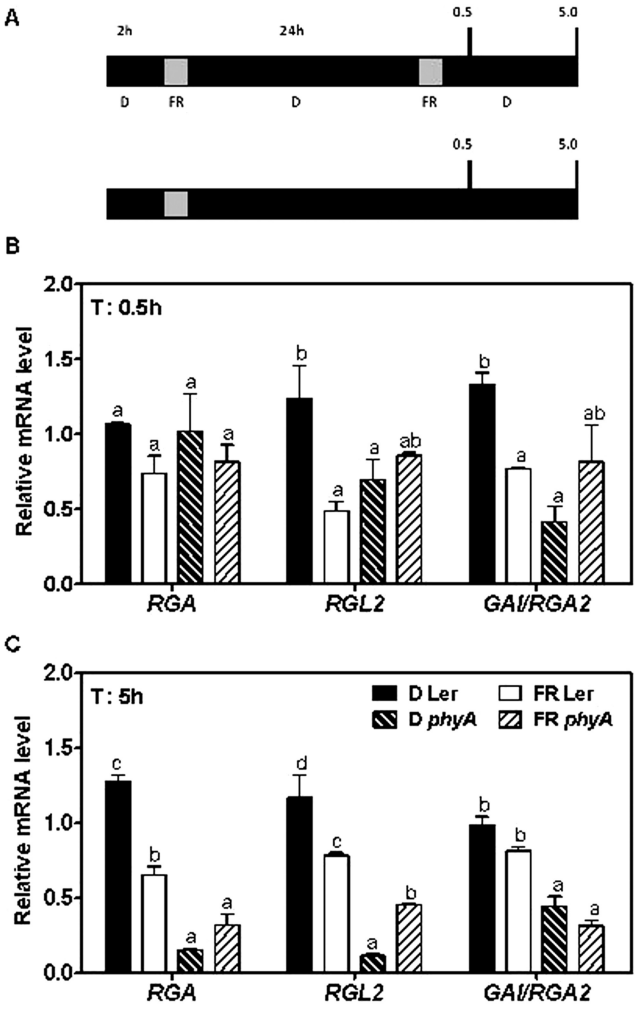

Figure 4. Function of DELLAs in phyA-Dependent Germination.
(A) Experimental protocol for the extraction of RNA samples. Gene expression was evaluated by qRT–PCR at 0.5 h **(B)** and 5 h **(C)** after a FR pulse or maintained in darkness for L*er* and *phyA* seeds. Data are means ± SE, $n = 2$. Data were normalized using *PP2A* housekeeping. Different letters indicate significant effects for each gene expression ($P < 0.05$).

Expression of ABA, GA, and Expansin Genes Depends on the Genetic Background when Germination Is Induced by phyA

Taking into account the genetic variation in the control of seed germination by light (Laserna et al., 2008), we studied the effects of the genetic background on the expression of GA, ABA, and cell-wall genes in germinating seeds by a FR pulse. We evaluated the expression of 11 genes in wild-type (i.e. L*er* and Col) and *phyA* seeds that displayed near 50% of germination after a FR pulse (Supplemental Figure 1). Among GA signaling genes, *GASA4* and *GASA6* were inhibited by phyA action in Col seeds, whereas only *GASA6* was repressed by phyA in L*er* seeds (Figure 6A and 6B). Among GA metabolic genes, *GA20ox3* was inhibited by phyA in Col seeds, whereas *GA3ox1* was up-regulated by phyA in L*er* and Col seeds (Figure 6A and 6B). Among ABA genes, we evaluated the expression of *ABI5*, an ABA anabolic gene (*NCED9*), and of *CYP707A2*, an ABA catabolic gene. *ABI5* was repressed in L*er* but not in Col seeds and both ABA metabolic genes were down-regulated independently of the genetic background by phyA action (Figure 6C and 6D). Then, we focused our attention in late expressed genes like expansins, key regulators of wall expansion in germinating seeds modulated by light (Li et al., 2003; Mella et al., 2004), and found that a FR pulse strongly induced the expression of *EXP1*, *EXP2*, and *EXP10* in both genetic backgrounds and that the lack of phyA blocked the expression of all these genes (Figure 6E and 6F). In addition, a glycosyl hydrolase gene that is involved in the mannose metabolic process (At3g26720) showed an increase in its expression in L*er* and Col seeds (Figure 6E and 6F).

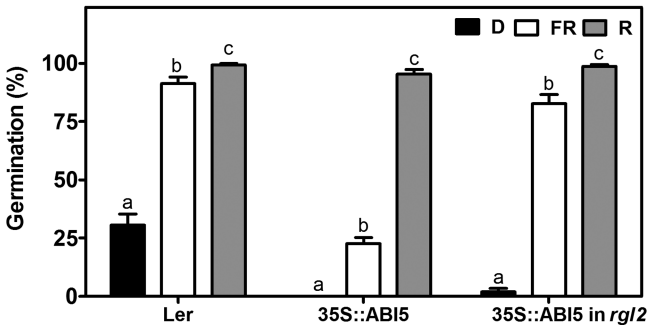

Figure 5. Germination Induced by Light in ABI5-Related Genotypes.
Germination of L*er*, 35S::ABI5 and 35S::ABI5 in *rgl2* background seeds after a R pulse, FR pulse, or maintained in darkness (D) until the evaluation of germination. Seeds were incubated at 25°C for 24 h before irradiation. Data are means ± SE of at least three independent experiments. Different letters indicate significant effects between the light treatments in each genotype ($P < 0.05$).

DISCUSSION

Light perceived by phyA is a cue for the promotion of germination in natural environments. Seeds of a large number of species germinate in response to soil perturbations like those produced by agricultural operations due to the perception of milliseconds of full sunlight (Scopel et al., 1991). The sole photoreceptor involved in the perception of that environmental signal is phyA through a VLFR (Botto et al., 1996). This light response is found in many species, including *Arabidopsis*, and shows huge genetic variation among natural populations (Laserna et al., 2008). It has been suggested that VLFR-dependent components play a more significant role than previously suggested, allowing plants to optimize the vegetative growth and flowering time (Kneissl et al., 2009).

Here, we demonstrated that the germination promoted by a VLFR through phyA action induces changes in 11% of the expressed genome in the L*er* genotype. Most phyA-dependent genes (90%) increased their expression at 5 h after a FR pulse, whereas most early phyA-dependent genes were down-regulated (i.e. 0.5 h; Figure 1B). As observed in relation to germination induced by phyB (Oh et al., 2009), the list of phyA-dependent genes includes various genes coding for proteins important for hormonal signaling during seed germination (Table 1). Briefly, seeds irradiated with a FR pulse increase the phyA–Pfr expected to interact with PIF/PIL transcription factors (Leivar and Quail, 2010). Then, hormone signaling genes, together with their indirectly regulated hormone metabolic genes, are expressed harmonically to promote seed germination. Interestingly, expression variation among *Arabidopsis* accessions was found in GA and ABA signaling genes and a more uniform expression was observed for expansins involved in the final process of radicle emergence after the induction of germination by a FR pulse (Figure 6). This result suggests that the gene expression could be a source of genetic variation in germination responses to light among seeds of different origins.

Comparing phyA-dependent and PIL5-dependent transcriptomes (Oh et al., 2009), we found that some genes controlled by phyA are also under the influence of PIL5. However, we identified that 55% of the phyA-dependent regulated genes are absent in the list of genes known to be regulated by PIL5 (Figure 2). Some of these differences may be due to different genotypes being used to investigate PIL5 and phyA transcriptomes. However, the fact that some phyA-dependent homozygotic mutant seeds have an impaired germination to a FR pulse but a normal germination to a R pulse (Figure 3) clearly supports the idea that they are in a PIL5-independent path. The existence of a way independent of PIL5 was already suggested by Oh et al. (2004) and here we provide evidence of some genes that may be part of that pathway.

Auxin-associated elements are overrepresented in phyA-dependent germination. This suggests a novel and unexpected function of auxin in the regulation of phyA-dependent germination. Several transport (*PIN1*, *PIN2*, *PIN7*), signaling (*RED1*, *AXR4*, *AXR1*, *Saur-like*, *ARF18*, *GH3.6*), and metabolic (*NIT3*, *SUR1*, *CYP79B2*) auxin genes are expressed differentially under the influence of phyA. Although most of them are up-regulated, two biosynthetic auxin genes, NIT1 and CYP79B2, are down-regulated by phyA (Table 1 and Supplemental Figure 4). Interestingly, Ogawa et al. (2003) found that several transport and metabolic auxin genes, including *CYP79B2*, were up-regulated early after imbibition of the seeds with gibberellins. However, the hormonal regulation after seed germination seems to be quite different. López-Molina's group demonstrated that ABA potentiates auxin signaling by inhibiting the embryo axis growth after radicle emergence (Belin et al., 2009). This process requires auxin transport to the peripheral elongation zone via AUX1, an auxin influx carrier, and PIN2, an auxin efflux carrier.

DELLAs are an important group of GA-related transcription factors involved in the control of germination (Cao et al., 2005, 2006). When seeds are inhibited to germinate by a FR pulse through the action of phyB, low GA levels allow an overaccumulation of GAI, RGA, and RGL2 that repress testa rupture and promote higher endogenous ABA levels (Piskurewicz et al., 2009). Using seeds germinating with a FR pulse, we demonstrated that DELLA expression is down-regulated by phyA–Pfr (Figure 4). Besides, the overexpression of ABI5 inhibits the promotion of germination by a FR pulse when RGL2 is present (Figure 5). Piskurewicz et al. (2008) demonstrated that the ABI5-mediated inhibition of germination requires the action of RGL2, which is stabilized by ABA through the XERICO signaling pathway. We suggest that the phyA–Pfr formed in imbibed seeds after a FR pulse inhibits *RGL2* expression and reduces the ABI5 repression on germination.

PIL2 expression is down-regulated by phyA (Supplemental Table 1 and Supplemental Figure 3). In agreement with our results, Oh et al. (2009) showed that *PIL2* is up-regulated by PIL5-dependent germination. After 12 h of R pulse, *PIL2* is expressed 34-fold higher in Col than in *pil5* seeds, the

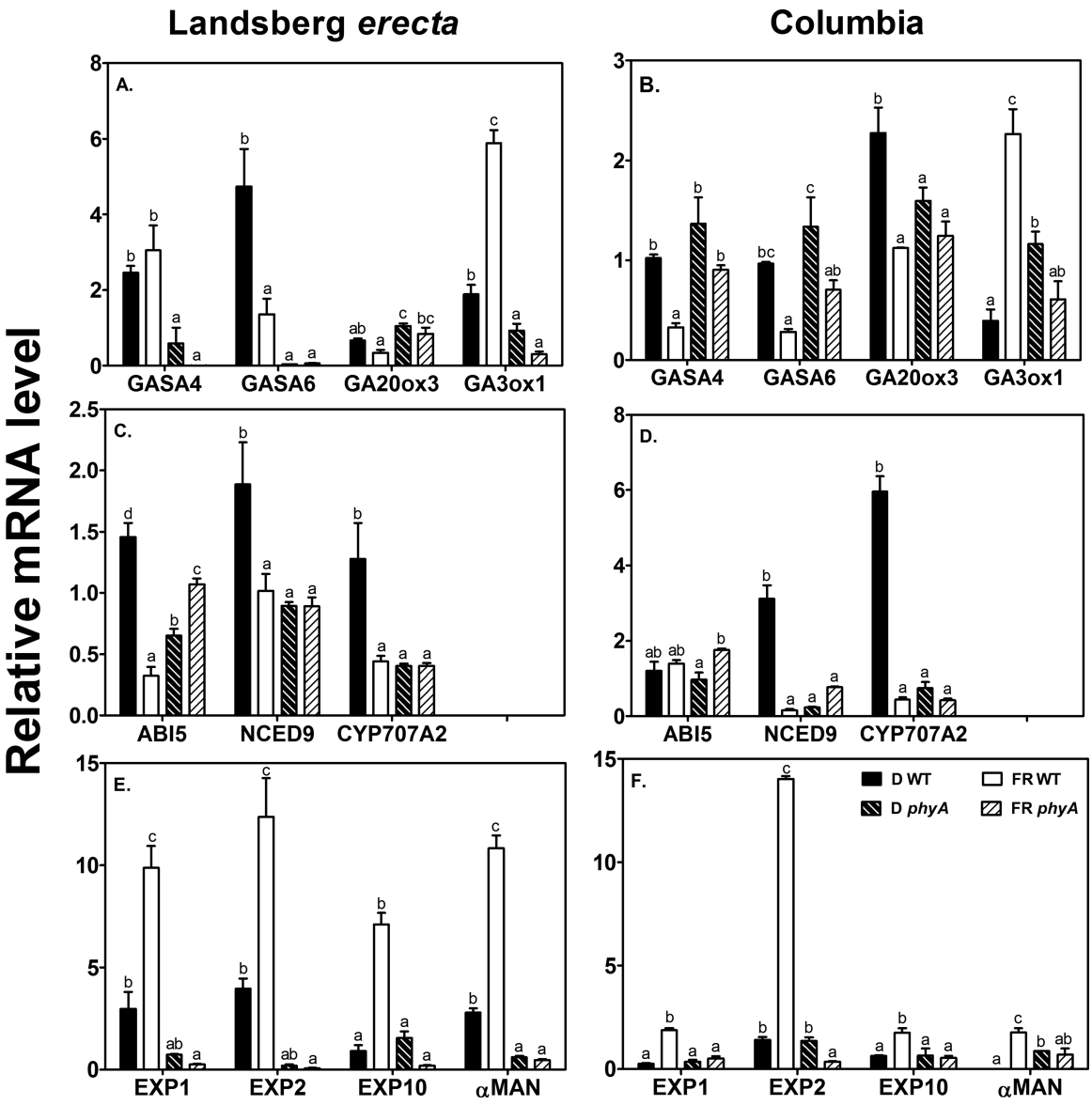

Figure 6. Natural Variation in the Expression of GA, ABA, and Cell-Wall-Related Genes in Germinating Seeds after a FR Pulse.
Gene expression was evaluated by qRT–PCR 5 h after a FR pulse or maintained in darkness (D) for L*er* **(A, C, E)** and Col **(B, D, F)** seeds. Data are means ± SE, $n = 2$. Data were normalized using *UBC* housekeeping. Different letters indicate significant effects for each gene expression ($P < 0.05$).

promoter of *PIL2* a direct target of PIL5 binding. Strikingly, the germination of *pil2* seeds showed that PIL2 contributes positively to the induction of germination by phyA signaling (Figure 3). In accordance with these results, an impaired germination response has been previously documented for *pil2* seeds imbibed in weak dormancy-breaking regimes of temperatures between 17 and 22°C but not at more extreme temperatures of 12 or 27°C (Penfield et al., 2010). Alternative splicing is the mechanism suggested to be involved in the action of PIL2, being the β-form of the protein, but not the α-form, responsible for inducing germination when dormancy is weak (Penfield et al., 2010). How to reconcile expression and mutant physiological results requires more information about the splicing mechanisms involved in the regulation of germination.

Phytochromes induce different modes of action (Casal et al., 1998; Bae and Choi, 2008). *Arabidopsis* seeds, when properly sensitized to light, germinate after irradiation with very low fluences of light (VLFR) through phyA signaling (Botto et al., 1996), whereas seeds less sensitive to light require higher photon fluence (LFR) to germinate through phyB, and secondarily phyD and phyE signaling (Botto et al., 1995; Hennig et al., 2002). On the other hand, the inhibition of germination, in seeds of several species but not in *Arabidopsis*, through a high irradiance response (HIR) is also mediated by the action of phyA (Shichijo et al., 2001;

Auge et al., 2009). In *Datura ferox* seeds, the HIR inhibits the expression of *DfGA3ox*, a gene promoted by the VLFR (Arana et al., 2007). In tomato seeds, other genes connected to the HIR branch of phyA signaling have been described. Auge et al. (2009) found that *ELIP* and *GIGANTEA* genes increase their expression when the germination of tomato seeds is inhibited by continuous FR. The antagonism between VLFR and HIR also indicates that it is very likely that the phyA–Pfr association with PIL5 is just one part of the mechanism underlying the actions of phyA on seeds. Further work, not only with *Arabidopsis*, but also with seeds of other species, is needed to better understand how phyA modulates downstream signaling events and how it interacts with other phytochromes and other signaling components to appropriately adjust germination to certain environmental circumstances.

METHODS

Growth Conditions and Plant Materials

Arabidopsis plants were grown in winter under a short photoperiod (10–11 h per day) and average temperature of 21 ± 2°C in a greenhouse with natural radiation (PAR = 350 μmol m^{-2} s^{-1}). Plants were grown together and their mature seeds were harvested on the same day. Seeds of each genotype were harvested as a single bulk sample of four or five plants and stored in open tubes inside a closed box in darkness with silica gel at 25°C for 3 months. Then, the seeds were stocked at 5°C until used for experiments. *phyA-T* (salk_014575C), *pil2* (salk_147579C), *myb66* (salk_114008C), *gasa4* (salk_042431C), *saur-like* (salk_134586C), and *pin7* (salk_044687C) T-DNA mutants in Col background were obtained from the ABRC (www.arabopsis.org). Seeds of 35S::HA–ABI5 and 35S::HA–ABI5 in *rgl2* were a gift from Dr López-Molina and described by Piskurewicz et al. (2008). 35S::HA–ABI5, 35S::HA–ABI5, and *phyA-201* mutant genotypes are in L*er* background.

Experimental Germination Conditions and Light Treatments

Samples of 20 seeds per genotype were sown in clear plastic boxes, each containing 5 ml of 0.8% (w/v) agar in de-mineralized water. To establish a minimum and equal photoequilibrium inside the seeds, seeds imbibed for 2 h were irradiated with a saturated FR pulse. Then, the seeds were incubated at 25°C in darkness for 24 h to increase the synthesis of phyA before irradiation with a saturated FR or R pulse. Because the dormancy of Col seeds is higher than that of L*er* seeds, Col seeds were incubated at 5°C for 24 h before the incubation at 25°C. The FR pulse to induce phyA-dependent germination was performed by irradiating the seeds with 20 min of a FR pulse with a RG9 filter (i.e. Pfr/P = 0.03; 42 mmol m^{-2} s^{-1}). The R treatment to induce phyB-dependent germination was carried out by irradiating the seeds with 20 min of a R pulse (i.e. Pfr/P = 0.87; 35 mmol m^{-2} s^{-1}), as indicated previously by Botto et al. (2009). After light treatments, the boxes containing seeds were wrapped in black plastic bags and incubated at 25°C for 4 d before germination was determined. The criterion for germination was the emergence of the radicle.

Microarray Experiments

Samples of dry seeds (28 mg) were sown in clear plastic boxes, each containing 3 ml of 0.8% (w/v) agar in de-mineralized water. L*er* and *phyA* seeds were incubated as indicated above and then irradiated with a FR pulse or kept in darkness. Samples were harvested at 0, 0.5, and 5 h after the FR pulse and stored at –70°C. Two biological replicates were performed for each treatment (12 microarrays). Total RNA from seeds was extracted using a RNeasy Plant Mini Kit (Quiagen, Hilden, Germany). Polyvinylpyrrolidone (PVP) at 4% was added in the extraction buffer (Rodríguez et al., 2009). The extraction was subjected to a DNAse treatment with RQ1 RNAse-Free DNAse (Promega, Madison, USA). cDNA, cRNA synthesis, and hybridization to ATH1 Affymetrix Arabidopsis Gene Chips were performed in accordance with Affymetrix instructions (Affymetrix, Santa Clara, USA).

Microarray Data Analysis

Expression data were normalized to the sum of each microarray (Clarke and Zhu, 2006). To select the list of genes for later statistical analysis, we used a restricted criterion that consisted of having the gene as 'present' in both replicates in at least one treatment. This criterion was reached by 12 745 genes from an original list of 22 746 genes. To identify genes differentially regulated by phyA, one-way ANOVA was performed with normalized data. We considered a gene to be differentially regulated by phyA if (1) the ANOVA gave a p-value ≤ 0.05 and a q-value ≤ 0.1 (Storey and Tibshirani, 2003; Tsai et al., 2003), and (2) there was at least a 1.5-fold change difference between L*er* and *phyA* gene expression at 0.5 or 5 h after the FR pulse. Clusters were generated using a DNA Chip Analyzer (www.dchip.org) described by Li and Wong (2003). Gene ontology and Venn diagram tools were used to organize and compare significant signatures of genes (http://bar.utoronto.ca/). Statistical significance of the overlap between two groups of genes was obtained by calculating the RF, which is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups (http://nemates.org/MA/progs/overlap_stats.html). Transcription factors were analyzed using the information described by Palaniswamy et al. (2006) and AGRIS at the AtTFDB–Arabidopsis transcription factor database (http://Arabidopsis.med.ohio-state.edu/AtTFDB/). Promoter sequences of phyA-dependent genes were analyzed by *Athena* (www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/analysis_select.pl).

Gene Expression Analysis by Quantitative RT–PCR

Samples of dry seeds (28 mg) were sown in clear plastic boxes, each containing 3 ml of 0.8% (w/v) agar in de-mineralized water. Two samples per genotype were harvested and stored at –70°C. cDNA derived from the extracted RNA was synthesized

using M-MLV Reverse Transcriptase (Promega, Madison, USA) and oligo-dT primers. The synthesized cDNAs were amplified with FastStart Universal SYBR Green Master (Roche, Madison, USA) using the 7500 Real Time PCR System cycler (Applied Biosystems, Foster City, CA, USA). *PP2A* and *UBC1* genes were used as normalization control (Czechowski et al., 2005). The specific primers used are described in Supplemental Table 4.

Statistical Analysis

One- or two-way ANOVA model followed by least significant difference test at $P = 0.05$ was performed to assess differences between means. Germination data were transformed with arcsine function previous to statistical analysis. Analyses were carried out with InfoStat statistical package (www.infostat.com.ar).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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