FAR-RED ELONGATED HYPOCOTYL3 and FAR-RED IMPAIRED RESPONSE1 Transcription Factors Integrate Light and Abscisic Acid Signaling in Arabidopsis[1[C][W][OPEN]]

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Light and the phytohormone abscisic acid (ABA) regulate overlapping processes in plants, such as seed germination and seedling development. However, the molecular mechanism underlying the interaction between light and ABA signaling is largely unknown. Here, we show that FAR-RED ELONGATED HYPOCOTYL3 (FHY3) and FAR-RED IMPAIRED RESPONSE1 (FAR1), two key positive transcription factors in the phytochrome A pathway, directly bind to the promoter of *ABA-Insensitive5* and activate its expression in Arabidopsis (*Arabidopsis thaliana*). Disruption of *FHY3* and/or *FAR1* reduces the sensitivity to ABA-mediated inhibition of seed germination, seedling development, and primary root growth. The seed germination of the *fhy3* mutant is also less sensitive to salt and osmotic stress than that of the wild type. Constitutive expression of *ABA-Insensitive5* restores the seed germination response of *fhy3*. Furthermore, the expression of several ABA-responsive genes is decreased in the *fhy3* and/or *far1* mutants during seed imbibition. Consistently, *FHY3* and *FAR1* transcripts are up-regulated by ABA and abiotic stresses. Moreover, the *fhy3* and *far1* mutants have wider stomata, lose water faster, and are more sensitive to drought than the wild type. These findings demonstrate that FHY3 and FAR1 are positive regulators of ABA signaling and provide insight into the integration of light and ABA signaling, a process that may allow plants to better adapt to environmental stresses.

Light is an important environmental signal that affects multiple plant processes, such as seed germination and seedling growth. Plants utilize a set of photoreceptors, including phytochromes and cryptochromes, to monitor the light environment and transduce the signals to downstream mediators (Chory, 2010). Numerous intermediate regulators that play important roles in the light signaling network have been identified. Among them, FAR-RED ELONGATED HYPOCOTYL3 (FHY3) and FAR-RED IMPAIRED RESPONSE1 (FAR1) were originally identified as positive regulators of the phytochrome A (phyA) signaling pathway (Hudson et al., 1999; Wang and Deng, 2002). *FHY3* and *FAR1* encode novel transcription factors derived from ancient mutator-like transposases and belong to the FAR1-Related Sequences gene family, which is specific to plants (Hudson et al., 2003; Lin and Wang, 2004; Lin et al., 2007). These two homologous proteins act redundantly to activate the expression of *FHY1* and *FHY1-Like*, whose proteins promote the nuclear translocation of phyA, resulting in the activation of phyA signaling (Hiltbrunner et al., 2006; Lin et al., 2007). Accumulating studies report that FHY3 and FAR1 are required for regulating various aspects of plant processes, such as far-red-mediated seedling deetiolation, the circadian clock, chloroplast division, and chlorophyll biosynthesis (Hudson et al., 1999; Wang and Deng, 2002; Allen et al., 2006; Li et al., 2011; Ouyang et al., 2011; Tang et al., 2012). Molecular evidence demonstrated that these two transcription factors bind to promoter regions containing the FBS (for FHY3/FAR1-binding site) motif of downstream targets and activate their expression (Lin et al., 2007; Li et al., 2011; Ouyang et al., 2011; Tang et al., 2012). A recent genome-wide analysis suggested that FHY3 has numerous putative direct targets in Arabidopsis (*Arabidopsis thaliana*; Ouyang et al., 2011), suggesting that FHY3 might have broad functions in plant growth and development, most of which, however, are unknown.

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The phytohormone abscisic acid (ABA) regulates many plant processes that are also regulated by light, such as seed germination and seedling development. During seed maturation and under unfavorable conditions, such as drought and salinity, ABA accumulates to high levels and plays important roles, including maintaining seed dormancy, inhibiting seedling growth, and closing stomata (Finkelstein et al., 2002). Genetic studies identified a number of ABA-responsive components, such as transcription factors, protein kinases, phosphatases, and RNA metabolic proteins, that are essential for regulating these processes (Finkelstein et al., 2002, 2008; Cutler et al., 2010). Mutations in a group of ABA-insensitive (ABI) loci resulted in insensitivity to ABA during seed germination, whereas overexpression of these genes led to hypersensitivity to ABA (for review, see Leung and Giraudat, 1998; Finkelstein and Rock, 2002). *ABI3*, *ABI4*, and *ABI5* encode transcription factors, while *ABI1* and *ABI2* encode protein phosphatase 2Cs (Leung et al., 1997; Finkelstein et al., 2002). ABI5 was identified by screening for mutants with ABA insensitivity at germination or during seedling growth or with altered ABA-induced transcription (Finkelstein, 1994; Lopez-Molina and Chua, 2000; Carles et al., 2002). ABI5 is a member of a small subfamily of basic leucine zipper transcription factors and is highly expressed in mature seeds and young seedlings exposed to ABA or water deficit stresses (Finkelstein and Lynch, 2000). The loss-of-function *abi5* mutant germinates and grows well even in the presence of high concentrations of ABA (Lopez-Molina and Chua, 2000).

Although cross talk between ABA and light signaling pathways has been observed (e.g. ABA metabolism in seeds is regulated by phytochrome; Seo et al., 2006, 2009), the underlying molecular mechanism is largely unknown. In this study, we show that knockout mutants of *FHY3* and/or *FAR1* have reduced sensitivity to ABA-mediated inhibition of seed germination and seedling growth, lose water faster, and are less tolerant to drought stress than are wild-type plants. We demonstrate that FHY3 directly activates *ABI5* expression and that overexpression of *ABI5* rescues the seed germination defect of *fhy3*. *FHY3* and *FAR1* transcription is induced by ABA and abiotic stresses, and these proteins confer drought tolerance. Our study suggests that FHY3 and FAR1 are positive regulators of ABA signaling.

RESULTS

FHY3 and FAR1 Directly Activate *ABI5* Expression

Our previous chromatin immunoprecipitation (ChIP)-based sequencing study revealed that FHY3 binds to numerous downstream targets involved in various hormonal responses and that *ABI5* is one of the targets precipitated by FHY3 in dark-grown seedlings (Ouyang et al., 2011). We then focused on ABI5 and performed detailed experiments to further elucidate the relationship between FHY3 (together with its homolog FAR1) and ABI5. By analyzing the promoter sequence of *ABI5*, we found that a putative FBS (with core sequence CACGCGC) is present 1,009 bp upstream of the ATG start codon of *ABI5* (Fig. 1A). A yeast one-hybrid assay showed that AD-FAR1 (fused with the B42 activation domain) was able to bind to wild-type *ABI5* oligonucleotides containing the FBS sequence upstream of a *LacZ* reporter gene (*ABI5wt*:*LacZ*) and strongly activated *LacZ* expression, while AD-FHY3 activated the reporter gene to a lesser extent. Mutations in the FBS motif (*ABI5m*:*LacZ*; in which CACGCGC was changed into CACttGC) abolished the activation of the *LacZ* reporter (Fig. 1B). Next, we performed an electrophoretic mobility shift assay (EMSA) and showed that a FHY3 recombinant protein (N-terminal 250 amino acids of FHY3 fused with glutathione *S*-transferase [GST], GST-FHY3N; Lin et al., 2007) caused an up-shifted band with *ABI5* probes labeled with ^{32}P, and this band was abolished by excess amounts of unlabeled wild-type oligonucleotides but not by unlabeled mutants (Fig. 1C). To further investigate whether FHY3 interacts with the *ABI5* promoter in vivo, we carried out a ChIP assay using 35S:*GUS-FHY3* (GUS fused with FHY3) transgenic seedlings (Wang and Deng, 2002). The promoter fragment containing the FBS motif (b in Fig. 1A), but not fragments farther upstream in the promoter (a) and coding region (c), was drastically enriched in samples precipitated by the anti-GUS antibody but not by the serum control (Fig. 1D). Together, these results confirm that FHY3 directly binds to the *ABI5* promoter through the FBS motif, both in vitro and in vivo.

We next examined how FHY3 and FAR1 regulate *ABI5* expression using quantitative reverse transcription (qRT)-PCR. The level of *ABI5* transcript was modestly decreased in *far1-2* and was even lower in *fhy3-4* single and *fhy3far1* double mutants (Fig. 1E), suggesting that FHY3 and FAR1 up-regulate *ABI5* expression. Consistent with this, nuclear targeting of FHY3 (induced by 1 μM dexamethasone) in the *FHY3p*:*FHY3-GR* transgenic plants (Lin et al., 2007) promoted *ABI5* expression compared with mock treatment (Supplemental Fig. S1). Next, a *LUCIFERASE* (*LUC*) reporter gene under the control of the *ABI5* promoter (*ABI5p*:*LUC*) was transformed into Arabidopsis protoplasts isolated from wild-type and *fhy3* mutant seedlings. This transient expression assay showed that LUC activity was remarkably reduced in *fhy3* compared with the wild type regardless of ABA treatment (Fig. 1F), further confirming that FHY3 activates *ABI5* expression.

Disruption of *FHY3* and *FAR1* Reduces ABA Sensitivity in Seed Germination

ABI5 is a critical positive regulator of seed germination and seedling establishment in the ABA pathway (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001). Previous public data (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi;

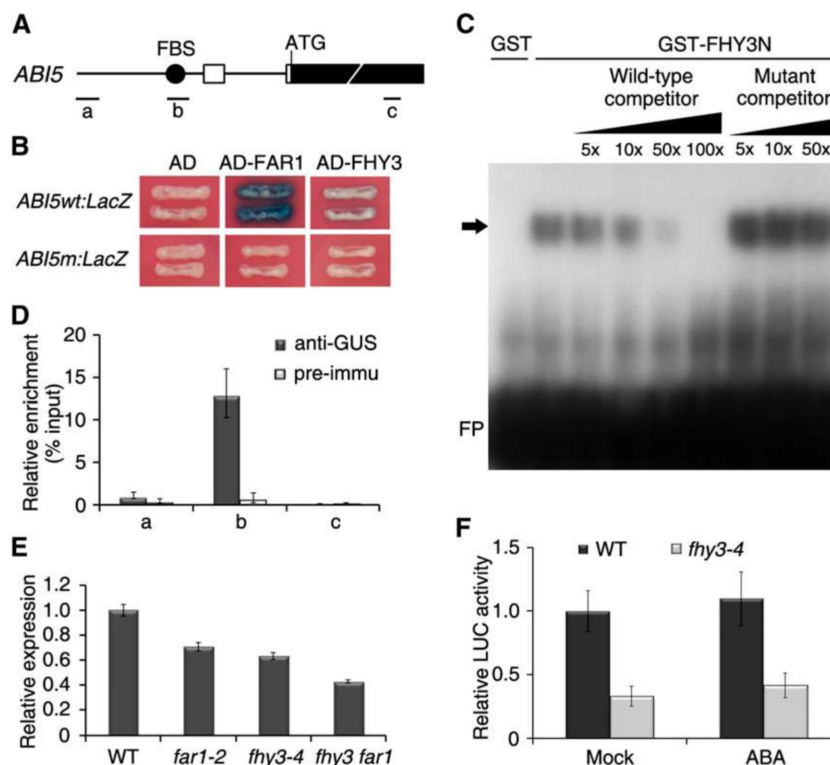

Figure 1. FHY3 directly activates *ABI5* expression. A, Schematic diagram of *ABI5*. Black rectangles represent exons, and white rectangles denote untranslated regions. The circle indicates the FBS motif (CACGCGC). a, b, and c indicate fragments used for ChIP-PCR. ATG is the ABI5 translational start codon. B, Yeast one-hybrid assay showing the activity of *LacZ* reporters driven by either wild-type (*ABI5wt:LacZ*) or mutant (*ABI5m:LacZ*) *ABI5* and activated by activation domain (AD) fusion effectors. C, EMSA showing the binding activity of GST-FHY3N or GST recombinant proteins with ^{32}P-labeled wild-type *ABI5* oligonucleotides in the presence of excess amounts of unlabeled competitors (wild-type and mutant probes). The arrow indicates shifted bands of protein-DNA complexes. FP denotes free probe. D, ChIP assay showing the specific precipitation of the *ABI5* fragment by GUS antibody in extracts from *35S:GUS-FHY3* transgenic plants. Precipitation by preimmune serum served as the negative control. ChIP DNA was quantified by real-time PCR with primers targeting fragments as shown in A. Values are means ± SD; *n* = 3. E, Relative *ABI5* expression in the seeds of various mutants and the wild type (WT) after imbibition for 12 h. Values are means ± SD; *n* = 3. F, Relative activity of the *LUC* reporter gene in protoplasts isolated from wild-type and *fhy3* mutant seedlings transformed with both *ABI5p:LUC* and *35S:GUS*. After transformation, the protoplasts were incubated without (Mock) or with 50 μM ABA in weak light for 12 h. Relative activities are expressed as the ratio of LUC to GUS (internal control). Values are means ± SD; *n* = 5. [See online article for color version of this figure.].

Winter et al., 2007) show that *FHY3* and *FAR1* transcripts accumulate in dry seeds and are down-regulated by imbibition during seed germination in a similar pattern to *ABI5* (Supplemental Fig. S2). We thus speculated that FHY3 and FAR1 might be involved in regulating seed germination. To test this possibility, we examined the germination response of *fhy3-4*, *far1-2*, and *fhy3 far1* mutants and wild-type seeds on Murashige and Skoog (MS) medium in the absence or presence of various concentrations of ABA. As shown in Figure 2, A and B, in the absence of ABA or in the presence of low ABA concentrations (less than 1 μM), the germination rate of the *fhy3-4*, *far1-2*, and *fhy3 far1* mutants was indistinguishable from that of the wild-type seeds. However, in the presence of high ABA concentrations (3, 5, and 10 μM), the germination rate of *fhy3-4* and *far1-2* was higher than that of the wild type, and the *fhy3 far1* double mutant had the highest germination rate (Fig. 2, A and B; Supplemental Fig. S3). Seedling establishment is also sensitive to ABA. We further found that, 3 weeks after seed germination on plates containing 3 μM ABA, the *fhy3-4* and *fhy3 far1* mutants were less sensitive to ABA than were the wild type and the *far1-2* mutant (Fig. 2, C and D). Therefore, similar to ABI5, FHY3 and FAR1 positively regulate ABA-mediated inhibition of seed germination and seedling greening.

The *fhy3* Mutant Is Less Sensitive to Salinity and Osmatic Stresses

Under abiotic stress, such as salt and osmotic stresses, plants often trigger the accumulation of ABA (Finkelstein et al., 2002). Therefore, we evaluated seed germination of the *fhy3* and *far1* mutants in response to these stresses. In the presence of 200 mM NaCl, approximately 81% and

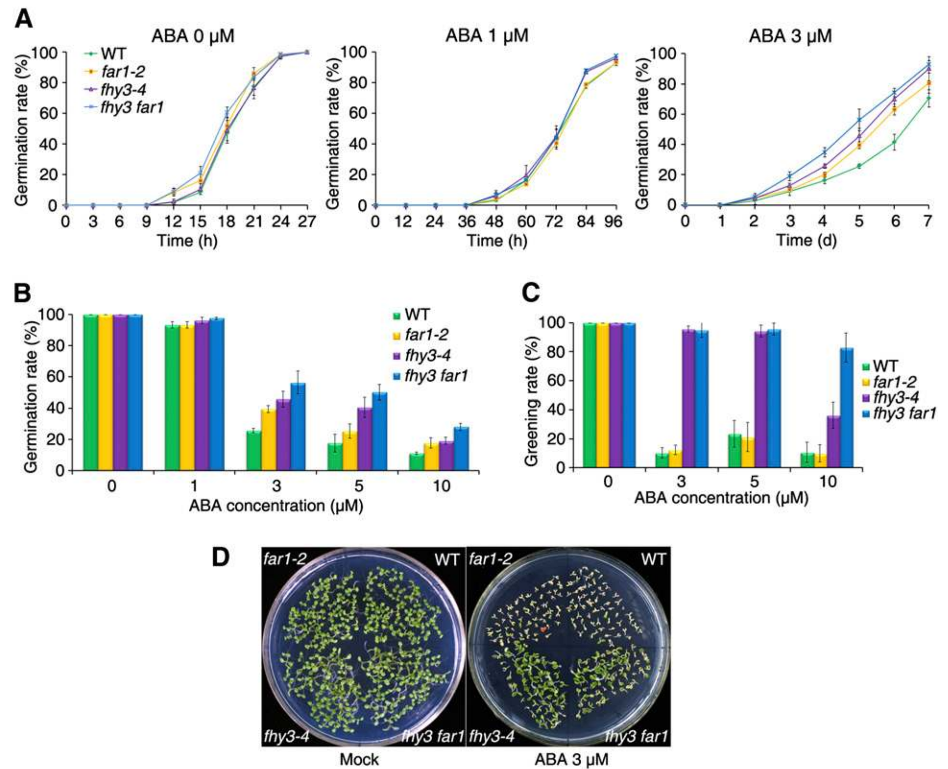

Figure 2. FHY3 and FAR1 knockout mutants are hyposensitive to ABA-mediated inhibition of seed germination and seedling greening. A, Percentage of seed germination of the NO wild type (WT) and *fhy3-4*, *far1-2*, and *fhy3 far1* mutants on medium containing various concentrations of ABA. Germination rate was monitored at the indicated time points. Values are means ± SD; $n = 3$. B, Percentage of seed germination 5 d after imbibition as shown in A and Supplemental Figure S1. Values are means ± SD; $n = 3$. C, Greening rate of seedlings grown in various concentrations of ABA for 4 weeks. Values are means ± SD; $n = 3$. D, Representative images of seedlings grown in medium without (Mock; 7 d old) or with 3 μM ABA (21 d old).

75% of *fhy3-4* and *fhy3 far1* seeds, respectively, germinated within 60 h, but only about 25% of *far1-2* and wild-type seeds germinated under these conditions (Fig. 3A). Similarly, when seeds were germinated on medium containing 400 mM mannitol for 60 h, 85% of *fhy3* and 75% of *fhy3 far1* seeds germinated, whereas only about 40% of *far1* and wild-type seeds germinated (Fig. 3B). Consistent with these results, the cotyledon greening rates of the *fhy3* and *fhy3 far1* mutants were also higher than those of *far1-2* and the wild type (Fig. 3C). Thus, disruption of *FHY3* causes hyposensitivity of seeds to high salt and osmotic stress, and FAR1 might have a slight opposite effect to FHY3.

FHY3 and FAR1 Are Required for ABA-Inducible Gene Expression during Seed Germination

To further verify the involvement of FHY3 and FAR1 in regulating ABA signaling at the molecular level, we examined the expression of a number of ABA- and stress-responsive markers, including *ABI1* (Gosti et al., 1999), *ABI2* (Leung et al., 1997), *ABF3* (Kang et al., 2002), *RAB18* (Lång and Palva, 1992), *KIN2* (Kurkela and Borg-Franck, 1992), *COR47* (Gilmour et al., 1992), *DREB2A* (Liu et al., 1998), and *RD22* (Yamaguchi-Shinozaki and Shinozaki, 1993), in the mutant and wild-type lines. Seeds of the wild type and the *fhy3*, *far*, and *fhy3 far1* mutants were imbibed for 12 h, and RNA was isolated for qRT-PCR analysis. We found that the transcripts of these genes were moderately down-regulated in the *fhy3* and *fhy3 far1* mutants compared with the wild type. The expression of *ABI1*, *KIN2*, *COR47*, and *ABF3* was also slightly reduced in the *far1* mutant seedlings (Fig. 4). These results suggest that FHY3 and FAR1 transcription factors affect the expression of these genes during seed imbibition.

Overexpression of *ABI5* Restores the *fhy3* Mutant Phenotypes

To test the genetic relationship between FHY3 and ABI5 in regulating the ABA response, we overexpressed *ABI5* (*35S*:*ABI5*; Dai et al., 2013) in the *fhy3-4* mutant background and used lines homozygous for the transgene in the following experiments. Transgenic plants overexpressing *ABI5* are hypersensitive to ABA (Lopez-Molina et al., 2001). In the presence of 3 or 5 μM ABA, the seed germination and seedling greening rates of *fhy3* were restored to near wild-type levels or even below those of the wild type by *ABI5* overexpression (Fig. 5). These data demonstrate that constitutive expression of *ABI5* rescues the ABA responsiveness of the *fhy3* mutant and that ABI5 acts downstream of FHY3.

Up-Regulation of *FHY3* and *FAR1* by ABA and Abiotic Stresses

To investigate how the endogenous signal and exogenous stress input into the pathway, we determined the effect of ABA, salt, and osmotic stresses on the expression patterns of *FHY3* and *FAR1* by qRT-PCR.

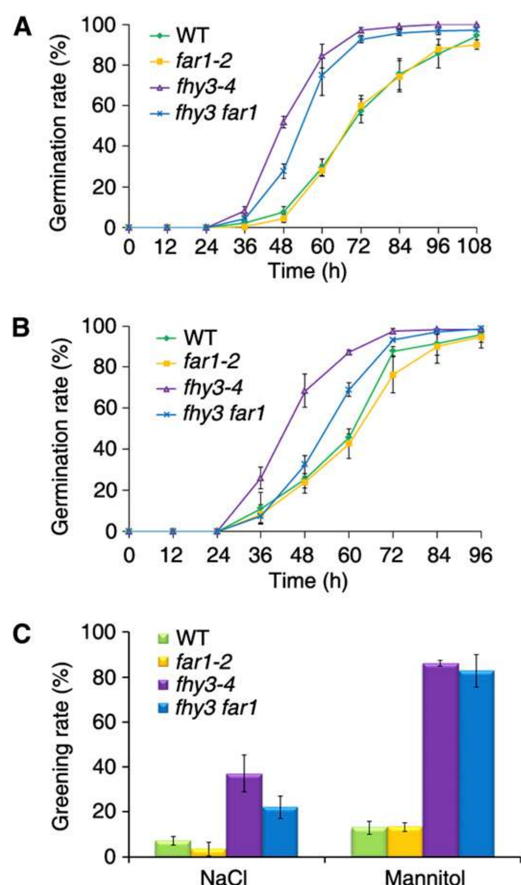

Figure 3. The *fhy3* mutant is less sensitive to salinity and osmotic stress. A and B, Kinetics of seed germination on medium containing 200 mM NaCl (A) and 400 mM mannitol (B). Germination rate was monitored at the times indicated. Values are means ± SD; $n = 3$. C, Quantification of seedlings with green cotyledons shown in A and B. The greening rate was recorded 21 and 14 d after germination for NaCl and mannitol treatment, respectively. Values are means ± SD; $n = 3$.

When 5-d-old seedlings were treated with 100 μM ABA, *FHY3* and *FAR1* transcript levels gradually increased over time, with an 8-fold induction after 9 h of treatment (Fig. 6A). Furthermore, *FHY3* or *FAR1* expression was also induced in seedlings treated with 200 mM NaCl or 400 mM mannitol, respectively (Fig. 6, B and C). In addition, when seedlings were subjected to drought treatment for up to 3 h, the mRNA levels of both *FHY3* and *FAR1* were also remarkably up-regulated (Fig. 6D). These data indicate that ABA and abiotic stresses induce *FHY3* and *FAR1* transcription, consistent with their roles in regulating the ABA response.

To test whether *FHY3* has tissue-specific expression, we used the *FHY3p:GUS* transgenic line, in which the GUS reporter gene is driven by the *FHY3* promoter (Lin and Wang, 2004). The GUS histochemical staining assay showed that *FHY3* was strongly expressed in germinating seeds, the whole seedling during establishment, the roots, and the mature leaves (Fig. 6, E–H). Most strikingly, strong GUS activity was detected in the guard cells (Fig. 6I). These expression patterns suggest that FHY3 has additional roles in roots and leaves.

FHY3 Regulates ABA-Mediated Root Elongation

Since high concentrations of ABA inhibit root growth, we grew the seedlings in MS medium for 2 d and then transferred them to medium supplemented with various concentrations of ABA. In the presence of 10 or 20 μM ABA, *far1-2* developed longer and *fhy3-4* and *fhy3 far1* exhibited much longer roots than did the wild type. The root growth of these mutant seedlings was comparable to the wild type in medium lacking ABA (Fig. 7). These observations support the notion that FHY3 also plays a role in ABA-mediated root growth. We also noticed that, without ABA treatment, the single and particularly the double mutants have more lateral roots than the wild type, indicating a reduced sensitivity of these seedlings to endogenous ABA.

FHY3 and FAR1 Regulate Stomatal Movement and Confer Drought Tolerance

Since *FHY3* is strongly expressed in guard cells and stomatal movement is regulated by ABA, we then compared the stomatal apertures of 4-week-old plants of the wild type and *fhy3-4*, *far1-2*, and *fhy3 far1* mutants. The epidermal peels from rosette leaves at the same developmental stage were observed with a microscope.

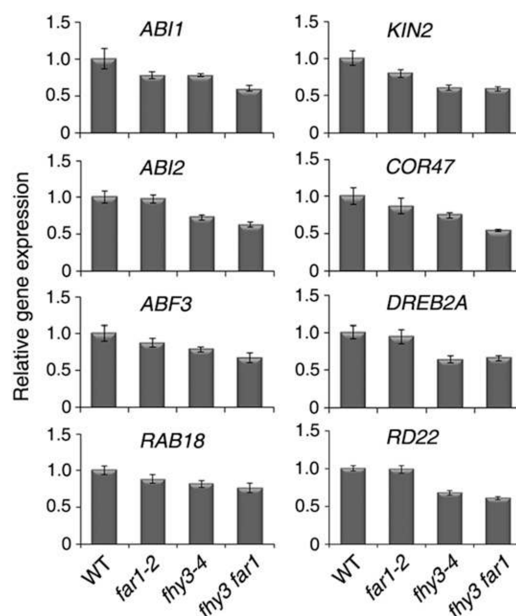

Figure 4. FHY3 and FAR1 are required for ABA-responsive gene expression. Total RNA was isolated from wild-type (WT), *fhy3-4*, *far1-2*, and *fhy3 far1* seeds after 12 h of imbibition. qRT-PCR was performed using specific primers as listed in Supplemental Table S1. Values are means ± SD; $n = 3$.

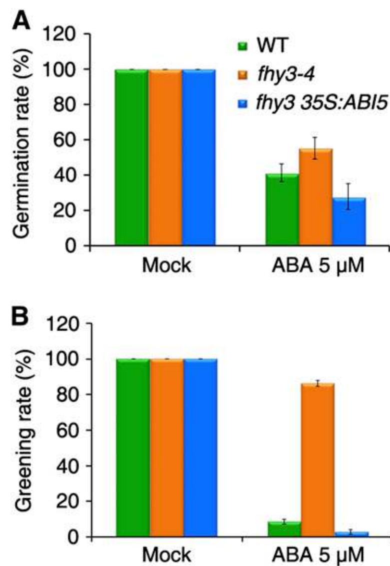

Figure 5. Overexpression of *ABI5* rescues *fhy3* mutant phenotypes. The percentage of seed germination (A) and of seedlings with green cotyledons (B) on medium in the absence (Mock) or presence of 5 μM ABA is shown. The germination rate was recorded after 5 d, and the greening rate was calculated after 4 weeks. Values are means ± SD; *n* = 3. WT, Wild type.

As shown in Figure 8A, *far1-2* displayed slightly wider stomatal apertures than did the wild type. The stomatal apertures of *fhy3-4* and especially of *fhy3 far1* were much wider than those of *far1-2* and the wild type. We also measured the stomatal apertures of *fhy3-4* and the wild type in the presence or absence of ABA. Mutants with loss of *FHY3* function were less sensitive to both ABA-promoted stomatal closure and ABA-inhibited stomatal opening (Supplemental Fig. S4).

The impaired stomatal regulation of *fhy3* and *far1* mutants prompted us to further test whether their water loss was affected. We found that the detached plants of *fhy3* and *far1* lost water more quickly than those of the wild-type, with the effect being less pronounced for *far1* (Fig. 8B). Next, when 3-week-old plants were exposed to dehydration by withholding water for 2 weeks, *fhy3* and *far1* mutant plants showed a more severe drought stress phenotype than did the wild type. After rewatering for 3 d, the majority of *fhy3* and some of the *far1* plants died, whereas all of the wild-type plants survived (Fig. 8C), indicating that FHY3 and FAR1 promote drought tolerance.

DISCUSSION

In this study, we collected molecular and genetic evidence that FHY3 and FAR1 are essential regulators of seed germination and ABA signaling that function by activating the expression of the ABI5 transcription factor. We show that *fhy3* and/or *far1* mutants are hyposensitive to ABA-mediated inhibition of seed germination and seedling greening (Fig. 2). The *fhy3* mutant phenotype can be restored to the wild type by the overexpression of *ABI5* (Fig. 5). At the molecular level, FHY3 and FAR1 physically bind to the promoter region of *ABI5* through the FBS cis-element and directly activate its gene expression (Fig. 1). Moreover, the expression of several ABA- and stress-responsive marker genes was down-regulated by mutations in *FHY3* and/or *FAR1* during seed imbibition (Fig. 4). Interestingly, *fhy3* and *far1* mutants possess an altered seed germination response in the presence of relatively high concentrations of exogenous ABA. Since *ABI5* is the direct target of FHY3 and FAR1, mutation of *ABI5* also promotes seed germination in the presence of high concentrations of ABA (Finkelstein, 1994). In addition, we found that plants deficient in *FHY3* or *FAR1* are less sensitive to ABA-mediated stomatal movement than are wild-type plants; therefore, FHY3 and FAR1 confer increased resistance to drought (Fig. 8). The drought-sensitive phenotype of *fhy3* may be partly caused by the reduced sensitivity of guard cell

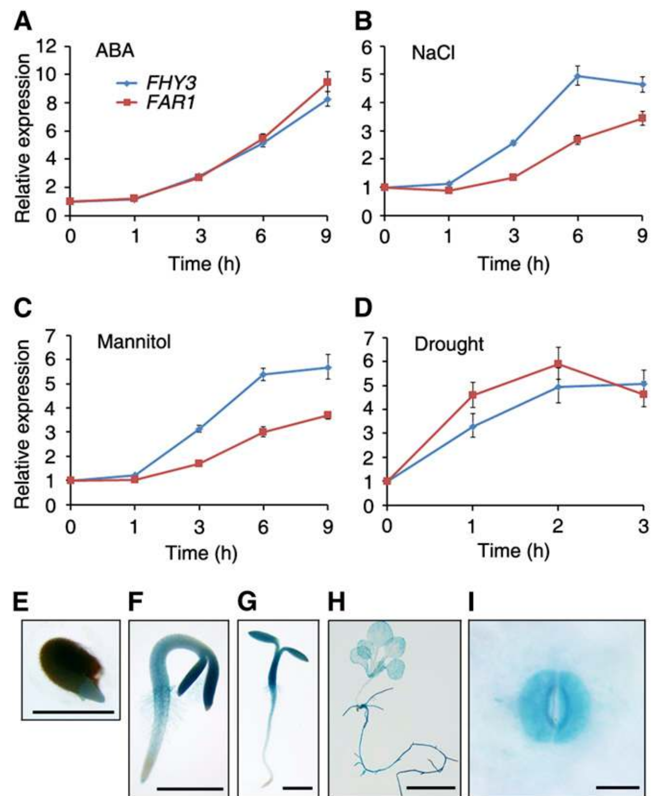

Figure 6. Expression patterns of *FHY3* and *FAR1*. A to C, Seven-day-old NO wild-type seedlings were transferred to medium containing 100 μM ABA (A), 200 mM NaCl (B), or 400 mM mannitol (C) for various periods of time. D, Seven-day-old NO wild-type seedlings were placed on filter paper under normal growth conditions for up to 3 h. The expression of *FHY3* and *FAR1* was analyzed by qRT-PCR. Relative expression levels were normalized to that of *UBQ*. Values are means ± SD; *n* = 3. E to I, GUS staining of *FHY3p:GUS* transgenic plants during seed germination (E) and of 2-d-old (F), 3-d-old (G), and 3-week-old (H) *FHY3p:GUS* plants and the guard cells of 3-week-old *FHY3p:GUS* plants (I). Bars = 0.5 mm (E), 1 mm (F and G), 5 mm (H), and 10 μm (I).

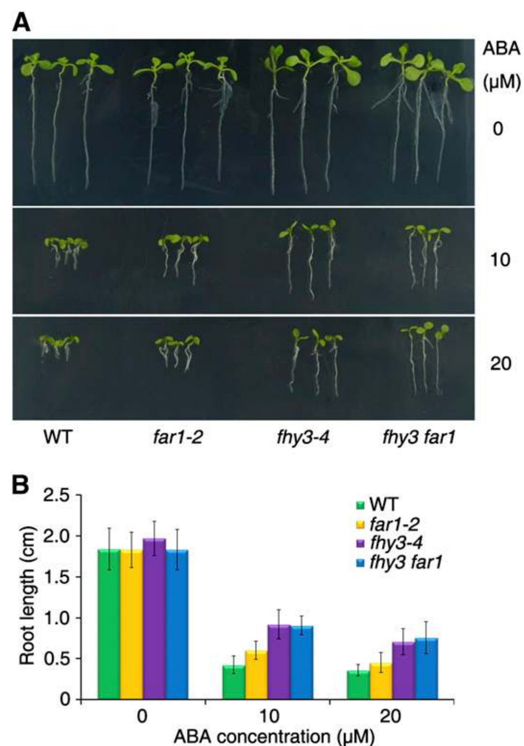

Figure 7. FHY3 and FAR1 regulate ABA-mediated root growth. A, Representative images of root growth on medium with or without ABA. Two-day-old seedlings were transferred to MS medium containing various concentrations of ABA and grown for an additional 7 d. B, Quantification of primary root length of the seedlings shown in A. Values are means ± SD; *n* = 20. WT, Wild type.

movement under drought stress conditions, which may induce the production of ABA. In agreement with this, *FHY3* is highly expressed in guard cells (Fig. 6I). We propose that other targets of FHY3/FAR1 are involved in mediating these processes at the adult stage, as *ABI5* is mainly expressed in seeds. Interestingly, *ABI5* expression is induced by drought stress, and plants that overexpress this gene retained water more efficiently than did wild-type plants (Lopez-Molina et al., 2001). Nevertheless, our study reveals that FHY3 and FAR1 are positive regulators of ABA responses.

It should be noted that FHY3 and FAR1 have redundant functions in ABA-mediated seed germination, seedling growth, and drought responses, with FHY3 playing the predominant role. However, FAR1 might have an opposite effect on FHY3 in modulating seed germination in response to salt and osmotic stresses (Fig. 2). A recent study reported that FHY3, but not FAR1, functions in the early photomorphogenic UV-B response (Huang et al., 2012). Thus, these two proteins could have divergent roles likely through protein subfunctionalization (Lin et al., 2008). The functional diversity of homologous proteins was also observed for two other light signaling components, ELONGATED HYPOCOTYL5 (HY5) and HY5 HOMOLOGY (Holm et al., 2002; Sibout et al., 2006). Since plant salt and drought stress responses involve ABA-dependent and ABA-independent pathways (Liu et al., 1998; Kizis et al., 2001), we could not exclude the possibility that the hyposensitivity of *fhy3* to salt and drought is due to ABA-independent signaling. It is worth noting that FAR1 interacts more strongly with the *ABI5* promoter than does FHY3 (Fig. 1B), whereas the *fhy3* mutant has stronger ABA-insensitive phenotypes than *far1* (Fig. 2, A–C). This is likely due to

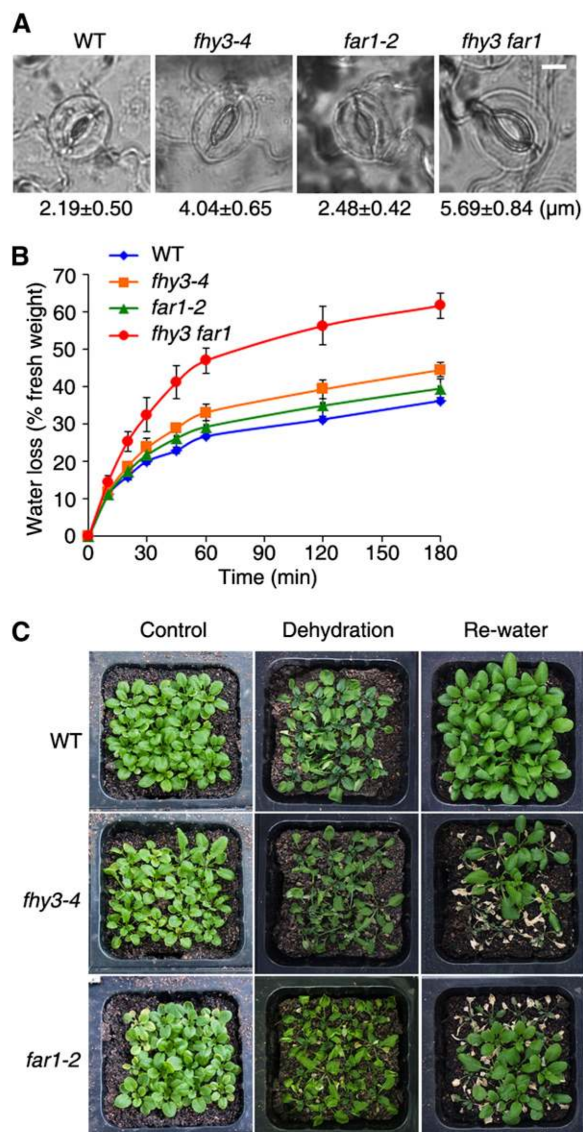

Figure 8. FHY3 and FAR1 regulate stomatal movement and confer drought tolerance. A, Representative images of stomata. Values below the images are quantification of the stomatal aperture. Values are means ± SD; *n* = 20. Bar = 10 μm. B, Water loss from detached plants. Three-week-old plants of various genotypes were measured at different periods of time. Values are means ± SD; *n* = 3. C, Reduced drought tolerance of *fhy3* and *far1* mutant plants. Three-week-old soil-grown seedlings were subjected to dehydration by withholding water for 2 weeks and then rewatered normally for 3 d. Three independent assays were performed with similar results, and representative images are shown. WT, Wild type.

a higher transcription activity of FHY3 compared with FAR1. A similar observation was also made regarding FHY3's other direct targets, including *FHY1* and *HEMB1* in photomorphogenic response and chlorophyll biosynthesis, respectively (Lin et al., 2007; Tang et al., 2012).

Whereas light is an environmental signal, phytohormones such as ABA are endogenous cues that regulate diverse plant growth and developmental processes. The existence of a regulatory loop between light and ABA signaling pathways has been proposed (Seo et al., 2009). Red light decreases whereas far-red light augments endogenous ABA levels. It has been documented that PIL5, a phytochrome-interacting factor also known as PIF1, represses phyB-mediated seed germination partly by activating the expression of ABA biosynthetic genes and repressing an ABA catabolic gene, consequently increasing ABA levels (Oh et al., 2004, 2007). In addition, PIL5 interacts with ABI3 to activate *SOMNUS* (*SOM*) expression in imbibed seeds, suggesting that the *SOM* promoter integrates ABA and light signaling to regulate seed germination (Park et al., 2011). It has been shown that disruption of *HY5* confers tolerance to the inhibitory effect of ABA on lateral root growth, seedling growth, and seed germination (Chen et al., 2008). Although interplay between light and ABA has been observed, the underlying molecular basis was hitherto largely unknown (Lau and Deng, 2010).

FHY3 and FAR1 were identified as key positive components in the phyA-mediated photomorphogenic pathway (Hudson et al., 1999; Wang and Deng, 2002). Later, they were shown to play essential roles in converting the light signal to regulate other plant growth and developmental programs, such as the circadian clock and chlorophyll synthesis (Li et al., 2011; Ouyang et al., 2011; Tang et al., 2012). Here, we demonstrate that FHY3 and FAR1 are also involved in ABA signaling. These two transcription factors thus act as a convergence point that integrates light and ABA signaling during seed germination and early seedling development. Consistent with this notion, *FHY3* and *FAR1* transcripts are up-regulated by light, ABA, and abiotic stresses (Tang et al., 2012; Fig. 6). The expression of *ABI5* is also activated by ABA and light (Lopez-Molina et al., 2001; Chen et al., 2008). We propose that, in the presence of abiotic stresses (e.g. salt, osmotic, and drought), *FHY3* and *FAR1* transcription is induced; consequently, the expression of ABA-responsive and (or) stress-related genes (e.g. *ABI5*; Ouyang et al., 2011) is promoted, which up-regulates the ABA signaling network, resulting in adaptation of the plant to various environments by shaping their growth and development. This functionality is significant, since abiotic stresses affect plant biomass and productivity.

Studies demonstrated that the levels of ABI5 protein play important roles in mediating ABA signaling and are tightly regulated (Lopez-Molina et al., 2001, 2003; Stone et al., 2006; Lee et al., 2010; Dai et al., 2013). Our results support the notion that *ABI5* is regulated at the transcriptional level and identify two transcription factors that directly bind to the *ABI5* promoter. PIL5, ABI3, and HY5 also function as essential regulators upstream of *ABI5* (Lopez-Molina et al., 2002; Chen et al., 2008; Oh et al., 2009). HY5 also directly activates *ABI5* expression, and overexpressing *ABI5* rescues ABA sensitivity in *hy5* (Chen et al., 2008). Previous studies demonstrated that FHY3/FAR1 and HY5 physically interact and thereafter either coordinately or antagonistically regulate *EARLY FLOWERING4* or *FHY1/FHY1 HOMOLOG* expression, respectively (Li et al., 2010, 2011). In these cases, the cis-elements of the FBS motif (bound by FHY3/FAR1) and the ACGT-containing element (bound by HY5) in the downstream promoters are close to each other (less than 20 bp away; Li et al., 2010, 2011). However, their respective cis-elements are more than 130 bp away in the *ABI5* promoter, suggesting that FHY3/FAR1 and HY5 might not physically interact at the promoter of *ABI5*. Rather, these transcription factors likely have independent regulatory modes in mediating the ABA response. Consistent with this notion, we observed that, compared with the wild type, *fhy3* seeds have a higher germination rate in the presence of relatively high concentrations of ABA (greater than 3 μM). Moreover, *fhy3* mutant plants display reduced sensitivity to ABA-induced stomata movement and are less tolerant to drought stress than the control plants (Fig. 8), whereas the *hy5* mutation does not have such effects (Chen et al., 2008). Therefore, FHY3 and HY5 play both overlapping and distinct roles regarding the regulation of plant growth and development in response to ABA.

Accumulating studies reveal that FHY3 and FAR1, two transposase-derived transcription factors, function broadly in the life of higher plants (Hudson et al., 1999; Wang and Deng, 2002; Allen et al., 2006; Li et al., 2011; Ouyang et al., 2011; Stirnberg et al., 2012; Tang et al., 2012; this study). FHY3 achieves these physiological responses largely through physically binding to the promoter of the corresponding target genes via the FBS motif (Ouyang et al., 2011). FHY3 might have additional effects on plant growth and development. Nevertheless, our study provides insight into the functional divergence of these transposase-derived proteins in plants during evolution.

MATERIALS AND METHODS

Plant Materials and Conditions

The *fhy3-4*, *far1-2*, and *fhy3 far1* mutants are of the Arabidopsis (*Arabidopsis thaliana*) Nossen (NO) ecotype (Lin et al., 2007). *35S:GUS-FHY3* (Wang and Deng, 2002) and *FHY3p:FHY3-GR* (Lin et al., 2007) are transgenic plants in the *fhy3-4* mutant background. *FHY3p:GUS* (Lin and Wang, 2004) and *35S:ABI5* (Dai et al., 2013) were described previously. *fhy3/35S:ABI5* was generated by genetic crossing, and a homozygous line was used. After sterilization, seeds were sown on MS medium containing 1% Suc, 0.8% agar, and various concentrations of ABA, NaCl, or mannitol as described. Seeds were incubated at 4°C in darkness for 3 d, followed by irradiation for 9 h with white light to promote uniform germination.

Seed Germination and Root Growth Assay

Seeds of different genotypes were harvested on the same day from plants grown in identical conditions. Seed germination was observed with a microscope and determined based on the appearance of radicle protrusion. The greening

rate was determined by calculating the percentage of seedlings with dark green cotyledons. For the root elongation assay, seedlings were grown on normal MS plates for 2 d and were then transferred to plates containing the indicated concentrations of ABA for an additional 7 d before measurement.

Stomatal Aperture Measurement

Epidermal peels from rosette leaves were floated in KCl-Tris solution (50 mM KCl, 10 mM MES, and using 1 M of pH 8.0 Tris-HCl to adjust the solution pH to 5.7) and exposed to light (100 μmol m^{-2} s^{-1}) or kept in the dark for 3 h to induce stomatal opening or closure, respectively. Subsequently, the peels were incubated in KCl-Tris solution with or without 50 M ABA under the light condition for an additional 3 h. Stomata were photographed with a dissecting microscope (Olympus), and stomatal aperture was measured using NIH ImageJ software (http://rsbweb.nih.gov/ij/).

Water Loss and Drought Tolerance Assays

Rosette leaves from 4-week-old long-day-grown plants were detached and weighed immediately. The leaves were then placed on a laboratory bench (50% relative humidity) and weighed at various time points. Relative water loss was expressed as the percentage of fresh weight to the initial weight of the leaves.

For drought tolerance experiments, plants were grown at similar density in pots under identical growth conditions for 3 weeks and exposed to dehydration by withholding water for an additional 2 weeks. They were then rewatered to examine the survival status.

Plasmid Construction

To produce *LacZ* reporters under the control of the *ABI5* promoter with a wild-type or mutant FBS motif, 39-bp oligonucleotides were synthesized as two complementary primers (ABI5wF and ABI5wR for the wild type and ABI5mF and ABI5mR for the mutant; for sequences, see Supplemental Table S1) with an *Eco*RI site overhang at the 5′ end and an *Xho*I site overhang at the 3′ end. The annealed DNA was ligated into the *Eco*RI-*Xho*I sites of pLacZi2μ (Lin et al., 2007), resulting in *ABI5wt*:*LacZ* and *ABI5m*:*LacZ*, respectively.

To generate the *LUC* reporter gene driven by the *ABI5* promoter, a 2.1-kb fragment upstream of the *ABI5* ATG translational start codon was PCR amplified with primers ABI5PF and ABI5PR from ecotype Columbia genomic DNA. The PCR fragment was inserted into the pGEM-T Easy (Promega) vector to produce pGEM-ABI5P and verified by sequencing. The promoter fragment was released from pGEM-ABI5P cut with *Hin*dIII and *Bam*HI and then ligated into the *Hin*dIII-*Bam*HI site of the LUC vector (Chen et al., 2013) to produce *ABI5p*:*LUC*.

The yeast vectors AD-FHY3 and AD-FAR1 and the recombinant protein construct GST-FHY3N were described previously (Lin et al., 2007).

Yeast One-Hybrid Assay

The activation domain fusion constructs (AD-FAR1 and AD-FHY3) were cotransformed with the *LacZ* reporter plasmids (*ABI5wt*:*LacZ* and *ABI5m*:*LacZ*) into yeast strain EGY48. Transformants were grown on synthetic dropout plates without tryptophan or uracil containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for color development.

ChIP Assay

Five-day-old *35S*:*GUS-FHY3* transgenic seedlings were used in the ChIP assay following a previously described procedure (Tang et al., 2012). Chromatin complexes were incubated with anti-GUS (Invitrogen) or the serum control. The precipitated DNA fragments were quantified by real-time PCR using the primers shown in Supplemental Table S1.

EMSA

EMSA was performed as described by Tang et al. (2012).

LUC Activity Assay

The *ABI5p*:*LUC* reporter plasmid and the *35S*:*GUS* internal control were cotransformed into Arabidopsis protoplasts isolated from wild-type and *fhy3-4* mutant seedlings. After overnight incubation, the activity of LUC and GUS was quantified using a Modulus Luminometer/Fluometer (Promega) as described previously (Tang et al., 2012). Relative *ABI5* expression was expressed as the ratio of LUC to GUS.

GUS Histochemical Analysis

Seeds or seedlings of the *FHY3p*:*GUS* transgenic line were subjected to GUS staining as described previously (Jing et al., 2013).

RNA Extraction and qRT-PCR

The treatment of seeds or seedlings is described in the text. Plant total RNA was extracted using an RNA extraction kit (Tiangen), and the first-strand complementary DNA was synthesized by reverse transcriptase (Invitrogen). Real-time PCR was performed using the SYBR Premix ExTaq kit (Takara) and a LightCycler 480 thermal cycler (Roche), following the manufacturers' instructions. Three biological replicates were performed for each sample, and the expression levels were normalized to those of *UBQ*. Primers are listed in Supplemental Table S1.

The Arabidopsis Genome Initiative locus numbers for the major genes discussed in this article are as follows: *FHY3* (At3g22170), *FAR1* (At4g15090), *ABI5* (At2g36270), *ABI1* (At4g26080), *ABI2* (At5g57050), *ABF3* (At4g34000), *KIN1* (At1g14370), *COR47* (At1g20440), *DREB2A* (AT5g05410), *RAB18* (At5g66400), *RD22* (At5g25610), *RD29B* (AT5g52300), and *UBQ1* (At3g52590).

Supplemental Data

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

Supplemental Figure S1. *ABI5* expression in *FHY3p*:*FHY3-GR* transgenic seedlings.

Supplemental Figure S2. Expression of *FHY3*, *FAR1*, and *ABI5* during seed germination.

Supplemental Figure S3. Kinetics of seed germination on medium containing high concentrations of ABA.

Supplemental Figure S4. Regulation of stomatal aperture in the wild type and the *fhy3* mutant by ABA.

Supplemental Table S1. List of primers used in this study.

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