Running head: FHY3/FAR1 integrate light and ABA signaling

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One-sentence summary: Two transcription factors link light and abscisic acid networks to regulate plant growth and development.
FHY3 and FAR1 Transcription Factors Integrate Light and Abscisic Acid Signaling in Arabidopsis

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ABSTRACT

Light and 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 ABI5 and activate its expression. Disruption of FHY3 and/or FAR1 reduces sensitivity to ABA-mediated inhibition of seed germination, seedling development, and primary root growth. Seed germination of the fhy3 mutant is also less sensitive to salt and osmotic stress than that of the wild type. Constitutive expression of ABI5 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.
INTRODUCTION

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 HYOCOTYLS3 (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 FRS gene family, which is specific to plants (Lin et al., 2007; Hudson et al., 2003; Lin and Wang, 2004). These two homologous proteins act redundantly to activate the expression of FHY1 and FHL whose proteins promote the nuclear translocation of phyA, resulting in the activation of phyA signaling (Lin et al., 2007, Hiltbrunner et al., 2006). Accumulating studies report that FHY3 and FAR1 are required for regulating various aspects of plant processes, e.g., far-red-mediated seedling de-etiolation, 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 (FHY3/FAR1-binding site) motif of downstream targets and activate their expression (Lin et al., 2007; Ouyang, et al., 2011; Li et al., 2011; Tang et al., 2012). A recent genome-wide analysis suggested that FHY3 has numerous putative direct targets in Arabidopsis (Ouyang, et al., 2011), suggesting that FHY3 might have broad functions in plant growth and development, most of which, however, are unknown.

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 (Koornneef et al., 1984; for reviews, see Leung and Giraudat, 1998 and Finkelstein and Rock, 2002). ABI3, ABI4, and ABI5 encode transcription factors, while ABI1 and ABI2 encode protein phosphatase 2Cs (Finkelstein et al., 2002; Leung et al., 1997). 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 (bZIP) 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 crosstalk 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 knock-out 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-based sequencing (ChIP-seq) 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 FHY3/FAR1 binding motif (FBS, core sequence CACGCGC) is present 1009 base pairs upstream of the ATG start codon of ABI5 (Figure 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 (Figure 1B). Next, we performed an electrophoretic mobility shift assay (EMSA) and showed that an FHY3 recombinant protein (N-terminal 250 amino acids of FHY3 fused with glutathione S-transferase, 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 (Figure 1C). To further investigate whether FHY3 interacts with the ABI5 promoter in vivo, we carried out a ChIP assay using 35S:GUS-FHY3 (β-glucuronidase fused with FHY3) transgenic seedlings (Wang and Deng, 2002). The promoter fragment containing the FBS motif (b in Figure 1A), but not fragments further 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 (Figure 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 polymerase chain reaction with reverse transcription (qRT-PCR) assay. The level of ABI5 transcript was modestly decreased in far1-2, and even lower in fhy3-4 single and fhy3far1 double mutants (Figure 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 Figure 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 (Figure 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 (Lopez-Molina and Chua 2000, Finkelstein and Lynch 2000, Lopez-Molina et al., 2001). Previous public data (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi, 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 as ABI5 (Supplemental Figure 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 fhy3far1 mutants and wild-type seeds on MS medium in the absence or presence of various concentrations of ABA. As shown in Figure 2A and 2B, in the absence of ABA or in the presence of low ABA concentrations (< 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 μM, 5μM and 10μM), 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 (Figure 2A, B, Supplemental Figure S3). Seedling establishment is also sensitive to ABA. We further found that, three 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 far1-2 mutant (Figure 2C and 2D). Therefore, similar to ABI5, FHY3 and FAR1 positively regulate ABA-mediated inhibition of seed germination and seedling greening.

**fhy3 mutant is less sensitive to salinity and osmotic stresses**

Under abiotic stresses, such as salt and osmotic stresses, plants often trigger the accumulation of ABA (Finkelstein et al., 2002). We therefore evaluated seed germination of the fhy3 and far1 mutants in response to these stresses. In the presence of 200 mM NaCl, approximately 81% and 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 (Figure 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 (Figure 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 (Figure 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 (Lang 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 to the wild type. The expression of *ABI1*, *KIN2*, *COR47*, and *ABF3* was also slightly reduced in the *far1* mutant seedlings (Figure 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 μM or 5μM 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 (Figure 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 stress on the expression pattern of *FHY3* and *FAR1* by qRT-PCR. When five-day-old seedlings were treated with 100 μM ABA, *FHY3* and *FAR1* transcript levels gradually increased over time, with an eight-fold induction after 9h of treatment (Figure 6A). Furthermore, *FHY3* and *FAR1* expression was also induced in seedlings treated with 200 mM NaCl or 400 mM mannitol, respectively (Figure 6B, 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 upregulated (Figure 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 (Figure 6E-H). Most strikingly, strong GUS activity was detected in the guard cells (Figure 6I). These expression patterns suggest that \( FHY3 \) has additional roles in roots and leaves.

\textbf{FHY3 regulates ABA-mediated root elongation}

Since high concentrations of ABA inhibit root growth, we grew the seedlings in MS medium for 2 days and then transferred them to media supplemented with various concentrations of ABA. In the presence of 10 \( \mu \)M or 20 \( \mu \)M 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 (Figure 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.

\textbf{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 under a microscope. 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 aperture 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 Figure 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 leaves of fhy3 and far1 lost water more quickly than those of the wild-type, with the effect being less pronounced for far1 (Figure 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 re-watering for 3 d, the majority of fhy3 and some of the far1 plants died, whereas all of the wild-type plants survived (Figure 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 (Figure 2). The *fhy3* mutant phenotype can be restored to wild type by the overexpression of *ABI5* (Figure 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 (Figure 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 (Figure 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, and FHY3 and FAR1 therefore confer increased resistance to drought (Figure 8). The drought-sensitive phenotype of *fhy3* may be partly caused by the reduced sensitivity of guard-cell movement under drought stress conditions, which may induce the production of ABA. In agreement with this, *FHY3* is highly expressed in guard cells (Figure 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 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 (Figure 2). A recent study reported that FHY3, but not FAR1, functions in 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 other two light signaling components, ELONGATED HYCOTYL5 (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 ABI5 promoter than does FHY3 (Figure 1B), whereas the fhy3 mutant has stronger ABA-insensitive phenotypes than far1 (Figure 2A-C). This is likely due to a higher transcription activity of FHY3 compared with FAR1. Similar observation was also made towards 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 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, and consequently increasing ABA levels (Oh et al., 2004, 2007). In addition, PIL5 interacts with ABI3 to activate SOMNUS (SOM) expression in imibed 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 (Ouyang et al., 2011, Li 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, Figure 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, and 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 (Oh et al., 2009, Lope-Molina et al., 2002, Chen et al., 2008). 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 FY1/FHY1 HOMOLOG expression, respectively (Li et al., 2011, 2010). In these cases, the cis-elements of FBS motif (bound by FHY3/FAR1) and ACE element (bound by HY5) in the
downstream promoters are close to each other (< 20 bp away) (Li et al., 2011, 2010). 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 (>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 (Figure 8), whereas the hy5 mutation does not have such effects (Chen et al., 2008). Therefore, FHY3 and HY5 play both overlap 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 (this study, Wang and Deng, 2002, Hudson et al., 1999, Tang et al., 2012, Ouyang et al., 2011, Stirnberg et al., 2012, Allen et al., 2006, Li et al., 2011). 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 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 Murashige and Skoog (MS) medium containing 1% sucrose, 0.8% agar, and various concentrations of ABA, NaCl, or mannitol as described in the text. 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 under a microscope and determined based on the appearance of radicle protrusion. 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 (KCl 50 mM, MES 10 mM, Tris-HCl, pH 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 under a dissecting
microscope (Olympus) and stomatal aperture was measured using NIH Image J software (http://rsbweb.nih.gov/ij/).

Water loss and drought tolerance assay
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 re-watered 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 mutant , see sequences in Supplemental Table 1) with an EcoRI site overhang at the 5’ end and an XhoI site overhang at the 3’ end, respectively. The annealed DNA was ligated into the EcoRI-XhoI sites of pLacZi2µ (Lin et al., 2007), resulting in ABI5wt:LacZ and ABI5m:LacZ, respectively.

To generate the luciferase (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 Col 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 HindIII and BamHI, and ligated into the HindIII-BamHI 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 AD fusion constructs (AD, AD-FAR1 or AD-FHY3) were co-transformed with the LacZ reporter plasmids (ABI5wt:LacZ or ABI5m:LacZ) into yeast strain EGY48. Transformants were grown on SD/-Trp-Ura dropout plates 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, respectively. The precipitated DNA fragments were quantified by real-time PCR using primers shown in Supplemental Table S1.

**Electrophoretic mobility shift assay (EMSA)**

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

**LUC Activity Assay**

The ABI5p:LUC reporter plasmid and 35S:GUS internal control were co-transformed into Arabidopsis protoplasts isolated from the 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/GUS.

**GUS histochemical analysis**

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

**RNA Extraction and Quantitative RT-PCR**
The treatment of seeds or seedlings is described in the text. Plant total RNA was extracted using RNA extraction kit (Tiangen) and the first strand cDNA 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 1 online.

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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signaling component directly interacting with its homologous partner FAR1.

**EMBO J** 21: 1339-1349


**Figure Legends**

**Figure 1. FHY3 directly activates ABI5 expression.**

(A) A schematic diagram of ABI5. Black rectangles represent exons and unfilled rectangles denote untranslated regions. The circle indicates the FBS motif (CACGCGC). a, b, and c indicate fragments used for ChIP-PCR. ATG, 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 AD-fusion effectors.

(C) EMSA assay showing binding activity of GST-FHY3N or GST recombinant proteins with 32P-labeled wt ABI5 oligonucleotides in the presence of excess amounts of unlabeled competitors (wild-type and mutant probes). 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 pre-immune serum served as the negative control. ChIP DNA was quantified by real-time PCR with primers targeting fragments as shown in (A). Mean ± SD, n=3.

(E) Relative *ABI5* expression in the seeds of various mutants and the wild type after

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imbibition for 12 h. Mean ± 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). Mean ± SD, n=5.

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

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

**Figure 4. FHY3 and FAR1 are required for ABA-responsive gene expression.**
Total RNA was isolated from wild type, *fhy3-4*, *far1-2*, and *fhy3 far1* seeds after 12 h of imbibition. Quantitative RT-PCR was performed using specific primers as listed in
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**Figure 5. Overexpression of ABI5 rescues fhy3 mutant phenotypes.**
Percentage of seed germination (A) and of seedlings with green cotyledons (B) on medium in the absence (Mock) or presence of 5 μM ABA. The germination rate was recorded after five days and the greening rate was calculated after 4 weeks. Data are mean ± SD, n = 3.

**Figure 6. Expression pattern of FHY3 and FAR1.**
(A-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 condition for up to 3 h. The expression of FHY3 and FAR1 was analyzed by quantitative RT-PCR. Relative expression levels are normalized to that of UBQ. Data are mean ± SD, n = 3.
(E-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 denote 0.5 mm (E), 1 mm (F, G), 5 mm (H), or 10 μm (I), respectively.

**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). Mean ± SD, n = 20.

**Figure 8. FHY3 and FAR1 regulate stomatal movement and confer drought tolerance.**
(A) Representative images of stomata. Values beneath the images are quantification of the stomatal aperture. Data are mean ± SD, n = 20. Bar = 10 μm.

(B) Water loss from detached leaves. Leaves from 4-week-old plants of various genotypes were measured at different periods of time. Data are means ± SD, n = 5.

(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 re-watered normally for 3 d. Three independent assays were performed with similar results and representative images are shown.
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