Integration of light and abscisic acid signaling during seed germination and early seedling development

Hao Chen*, Jingyu Zhang†, Michael M. Neff‡, Suk-Whan Hong§, Huiyong Zhang¶, Xing-Wang Deng∥, and Liming Xiong*†

*Donald Danforth Plant Science Center, St Louis, MO 63132; †Department of Biology, Washington University, St. Louis, MO 63130; ‡Department of Crop and Soil Sciences, Washington State University, Pullman, WA 99164; ¶Department of Applied Plant Science, Chonnam National University, Gwangju 500-757, South Korea; and ∥Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06520

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Seed germination is regulated by endogenous hormonal cues and external environmental stimuli such as water, low temperature, and light. After germination, the young seedling must rapidly establish its root system and the photoautotrophic capability appropriate to its surrounding environment. Light and the phytohormone abscisic acid (ABA) both regulate seed germination and seedling development, although how light and ABA signals are integrated at the molecular level is not understood. Here, we found that the previously described light-signaling component HY5 also mediates ABA response in seed germination, early seedling growth, and root development in Arabidopsis. HY5 binds to the promoter of the transcription factor ABI5 gene with high affinity and is required for the expression of ABI5 and ABI5-targeted late embryogenesis-abundant genes in seeds. Chromatin immunoprecipitation also indicated that the binding of HY5 to the ABI5 promoter is significantly enhanced by ABA. Overexpression of ABI5 restores ABA sensitivity in hy5 and results in enhanced light responses and shorter hypocotyls in the wild type. Our studies identified an unexpected mode of light and ABA signal integration that may help young seedlings better adapt to environmental stresses.

light response | signal transduction

The plant hormone abscisic acid (ABA) plays essential roles in several aspects of plant growth and development, including seed maturation, seed dormancy, and adaptation to environmental stresses such as drought and high salinity (1–4). Genetic studies of ABA regulation of seed germination and gene expression have identified a number of Arabidopsis mutants with altered ABA sensitivities. One of the ABA insensitive mutants, abi5, was isolated for its ability to germinate in the presence of higher concentrations of exogenous ABA (5). ABI5 encodes a basic leucine zipper (bZIP) transcription factor whose accumulation inhibits seed germination and early seedling establishment (6–8). ABI5 regulates the expression of ABA induced, mostly seed-specific, AtEM genes that encode class I late embryogenesis-abundant (LEA) proteins important for seed maturation (9, 10). Interestingly, the ABI5 gene was also expressed in young seedlings, suggesting that ABI5 may have additional functions beyond seed development and germination (11). It is known that seed germination and early seedling development are also regulated by light (12). Yet little is known how light and ABA signals are integrated in the regulation of this common developmental transition from seeds to seedlings.

Whereas the role of ABA in seedling photomorphogenesis is unclear, ABA is well recognized for its critical roles in plant adaptation to drought stress. In addition to inducing stomatal closure and the activation of stress-responsive genes, ABA was recently found to be involved in lateral root development (13–15). Genetic studies indicated that the inhibition of lateral root elongation and potential promotion of primary root growth by drought stress and ABA are adaptive responses of roots to drought stress that are linked to whole plant drought tolerance (15). To reveal the molecular mechanisms underlying this adaptive response, we investigated genes potentially involved in this process by studying ABA response in Arabidopsis mutants with altered lateral root development. One of the mutants we examined is hy5, a mutant originally isolated for its long hypocotyl under light (16) but also with more elongated lateral roots (17). The HY5 gene encodes a bZIP transcription factor and is responsible for the activation of many light-regulated genes (18). We found that the hy5 mutant is tolerant to the inhibitory effect of ABA on the elongation of lateral roots, seedling growth, and seed germination. Furthermore, the steady-state transcript levels of the ABI5 and its target genes were greatly reduced in hy5 seeds. HY5 protein interacts with the ABI5 promoter, and the in vivo interaction was enhanced by ABA. Ectopic expression of ABI5 in the hy5 mutant background restored its ABA sensitivity and overexpression of ABI5 in the wild-type background-enhanced light response. These results indicate that HY5 integrates both ABA and light signal transduction pathways partially through direct activation of ABI5.

Results

HY5 Mediates ABA Response in Roots and Is Required for Osmotic Stress Tolerance in Seedlings. To determine whether HY5 is involved in ABA inhibition of lateral root development, we tested the response of a hy5 knockout mutant (SALK_096651; see Materials and Methods) to ABA. At either 0.5 or 1.0 μM ABA, the growth of the wild-type seedlings was inhibited in the primary root elongation and shoot growth were slower than that of the control plants, particularly at the 0.5-μM ABA level (Fig. 1 A and B). In contrast, the hy5 seedlings were much more tolerant to ABA in both primary root elongation and shoot growth (Fig. 1 A and B). After 3-week growth, the shoot fresh weights of the wild-type seedlings reduced by 30% and 60% with 0.5- and 1.0-μM ABA treatment, respectively; whereas no statistic difference in shoot fresh weights of hy5 seedlings was found among the treatments. As expected, ABA inhibits lateral root growth in wild-type plants (Fig. 1 A and C) and the average total length of lateral roots decreased 80% by even 0.5-μM ABA treatment (Fig. 1C). However, the growth of lateral roots of hy5 was much less inhibited by ABA (Fig. 1 A and C). Thus, hy5 mutant seedlings are much more sensitive to ABA both in shoot and root growth than the wild type.


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†To whom correspondence should be addressed. E-mail: lxiong@danforthcenter.org.

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This inhibition is significantly reduced in
whereas NaCl inhibits the growth of lateral roots in the wild type,
inhibition of ionic stress and osmotic stress. Interestingly,
indicated concentrations of NaCl. (%
seedlings were transferred to the shown plates. (%
steps were taken 5 days after the transfer. (B) Relative root length of wild-type and hy5 seedlings at 5 days after
being transferred to plates with the indicated concentrations of ABA. (C) Length of total lateral roots of two-week-old wild-type and hy5 seedlings as shown in A. (D) Morphology of wild-type and hy5 seedlings on plates without or with 50 or 100 mM mannitol. The pictures were taken 3 weeks after
seedlings were transferred to the shown plates. (E) Relative root length of wild-type and hy5 seedlings at 8 days after being transferred to plates with the indicated concentrations of NaCl. (F) Length of lateral roots of the wild-type and hy5 seedlings on media supplemented with the indicated concentrations of NaCl. Data in B, C, E, and F represent means and SE (n = 6).

Given that ABA mediates many aspects of abiotic stress response, we examined whether hy5 seedlings are compromised in stress tolerance. We first checked the sensitivity of hy5 to osmotic stress. When transferred to media supplemented with 50 or 100 mM mannitol, the shoot and root growth of hy5 was more severely retarded than that of the wild type (Fig. 1D). We then tested whether hy5 mutants are sensitive to ionic stress as well. We found that hy5 seedlings have slower growth rates of the primary roots and also yellowish leaves under salt stress (Fig. 1E and data not shown). Thus, hy5 mutants are more sensitive to the inhibition of ionic stress and osmotic stress. Interestingly, whereas NaCl inhibits the growth of lateral roots in the wild type, this inhibition is significantly reduced in hy5 seedlings (Fig. 1F).

This observation implicates that ABA may play a more important role in NaCl inhibition of lateral root growth than in the inhibition of primary root growth.

Germination of hy5 Mutant Seeds Is More Tolerant to ABA and Glucose. On media supplemented with 0.5 or 1.0 μM ABA, it was found that hy5 seeds germinated more rapidly and at a higher rate than the wild type, although wild-type seeds eventually germinated at these ABA levels (data not shown). In the presence of ABA, the germinated wild-type embryos were arrested, whereas those of hy5 were able to develop into normal green seedlings (Fig. 2B). This is not an indirect consequence of earlier seed germination of hy5 than the wild type, because the germinated wild-type embryos failed to develop into normal green seedlings even after prolonged incubation under the same conditions.

Seed germination is also sensitive to salt stress and sugars. These stimuli inhibit germination partly through enhanced biosynthesis of and responsiveness to ABA. Consistent with their resistance to ABA during germination, hy5 seeds have significantly higher germination rates than the wild type at either 150 mM NaCl or 333 mM glucose (Fig. 2 C and D).

**HY5 Is Required for ABA-Inducible Gene Expression in Seeds and During Seed Germination.** The impaired ABA responses of hy5 mutants in roots and seed germination suggest that HY5 may play unexpected roles in ABA signal transduction. The fact that HY5 is a DNA-binding transcription factor (18) prompted us to ask whether HY5 regulates ABA-inducible gene expression. Although hy5 seedlings are more sensitive to osmotic stress (Fig. 1D), no clear difference in gene expression was detected between young seedlings of hy5 and the wild type after ABA or osmotic stress treatments for eight known ABA up-regulated genes expressed. These genes include RD29A, RD22, DREB2A, DREB2B, CBFI, CBF2, CBF3, and ABF3 (data not shown). An exception is RD29B, whose transcript level was lower in hy5 than in the wild type under the ABA treatment (Fig. 3A). We then went on to investigate whether the expression of ABA-regulated seed-specific genes is altered in hy5. The transcription factors ABI3 and ABI5 are known to regulate ABA sensitivity during and immediately after seed germination. We thus checked the transcript levels of ABI3 and ABI5 in dry seeds. Although very little difference in ABI3 transcript level was detected, ABI5
mRNA level was greatly reduced in hy5 seeds (Fig. 3B). Subsequently, we monitored the transcript levels of three LEA genes, RAB18, AtEM1, and AtEM6, in hy5 seeds. AtEm1 and AtEm6 are likely the direct target genes of ABI5, and RAB18 transcript level is down-regulated in abi5 mutants (10, 19, 20). Consistent with the reduction in ABI5 transcript level, the steady-state levels of all of these ABI5-regulated genes are significantly down-regulated in hy5 seeds (Fig. 3B).

**HY5 Protein Binds to the ABI5 Promoter with High Affinity and Specificity.** Because ABI5 and its target genes are down-regulated in hy5 seeds (Fig. 3), and hy5 has reduced ABA sensitivity reminiscent of the abi5 mutant (Fig. 2), we speculated that HY5 may directly regulate ABI5 transcription. HY5 is known to bind to the G-box CACGTG in light-regulated gene promoters (18). In searching the ABI5 promoter, four G-box core sequences were found in the 1.7-kb fragment upstream of the translation start codon (Fig. 3C). To test whether any of these G-boxes is involved in HY5 binding, we did a gel retardation assay using *Escherichia coli* expressed recombinant HY5 protein together with the four ABI5 promoter fragments. The P1 and P4 fragments each harbor one G-box core, and the P2 contains two G-box cores, whereas P3 does not contain any consensus G-box motifs. Recombinant HY5 protein retarded the mobility of P1, P3, and P4, but not P2 (Fig. 3 D and E). Among the HY5 retarded fragments, P1 and HY5 gave the strongest signal intensity (Fig. 3D). Competition assays were performed with labeled P1 and unlabeled P4. With increasing unlabeled P1, the P1–HY5 complex quickly diminished (Fig. 3F). With unlabeled P4, however, even at a concentration 300-fold more than the labeled P1, the shifted band was still present (Fig. 3F). Therefore, HY5 does bind to ABI5 promoter with the highest affinity in the P1 region from −1,754 to −1,294.

To confirm these *in vitro* binding results, we performed ChIP assays using HA-tagged HY5 transgenic seedlings (21). Fig. 3G shows that consistent with the gel retardation data, anti-HA antibody specifically precipitated the ABI5 promoter P1 fragment, but not P2, P3, or P4. Interestingly, ABA treatment significantly enhanced the binding of HY5 protein to the P1 region of the ABI5 promoter. In contrast, ABA failed to enhance the binding of HY5 to the promoters of two other light-regulated HY5 target genes, CHS and RbcS (Fig. 3G). This result was further confirmed by quantitative real-time PCR. Compared with no enrichment of the CHS promoter, a 3.1-fold (*P* < 0.05) enrichment of the P1 fragment was detected in ABA-treated relative to ABA-untreated samples. Because ABA does not enhance HY5 expression nor increases HY5 protein stability (data not shown), the specific promotion of HY5 binding to the ABI5 promoter may underlie the mechanism for ABA up-regulation of ABI5 expression (21).

To examine the effect of HY5 on ABI5 transcription, we did a transient transcription assay using tobacco BY-2 protoplasts. However, the ABI5 promoter-driven β-glucuronidase (GUS) activity is very limited in this system, and HY5 protein did not exhibit a significant effect on ABI5 transcription (data not shown). Although almost similar levels of HY5 transcript can be detected in all of the tissues examined, including mature leaves (17), high expression of ABI5 is detected mainly during seed maturation and at the early stage of seedling development (6, 11). Therefore additional seed or early seedling-specific factors may be required to activate ABI5 transcription together with HY5. Indeed, despite no evidence for direct regulation, ABI5 promoter mRNA accumulation is altered in abi3, fua3, and lec1 mutants (6, 22).

**ABI5::GUS Activity in hy5 Is Reduced and Is Less Responsive to Stress Induction.** To address whether there is any tissue-specific regulation of ABI5 transcription by HY5, we made an ABI5::GUS construct consisting of a 1.7-kb fragment of the ABI5 promoter-driven GUS gene and transferred it into wild-type Col-0 and hy5. Sixteen independent transformants from each background were
examined for GUS activity, and similar GUS expression was found among different lines in the same background. Data from one representative line for each background are presented in Fig. 4. Similar to what was previously reported (11), ABI5 promoter activity was detected in cotyledons, hypocotyls, and roots of wild-type young seedlings (Fig. 4A). On the contrary, the ABI5::GUS activity was dramatically decreased in hy5 in all these tissues (Fig. 4B), which is also similar to what was seen with other previously characterized HY5 target genes in hy5 mutants (18).

As a critical regulator of photomorphogenesis, the HY5 protein level is tightly controlled by the COP1 E3 ubiquitin ligase in the dark, such that the HY5 protein accumulates only in the light (23). Therefore, if HY5 is essential for ABI5 transcription, dark-grown seedlings would have reduced ABI5::GUS activity. As expected, ABI5::GUS activity is much lower in wild-type etiolated seedlings (Fig. 4C and M). ABI5 transcripts could be detected in floral tissues (24), which is coincident with the high accumulation of HY5 protein in floral tissues (25). Consistently, ABI5::GUS activity is barely detectable in hy5 floral tissues, in contrast to the strong GUS staining in the wild type (Fig. 4D and E). Similarly, the GUS staining is weaker in hy5 siliques than in wild-type siliques (Fig. 4F).

The ABI5 promoter can be induced by ABA and salt stress (11). To investigate whether HY5 is also required for ABI5 induction by ABA and stress, we treated the seedlings with either 10 μM ABA or 100 mM NaCl for 1 day before staining or quantifying for GUS activity. Fig. 4G–L and N show that with both treatments wild-type seedlings display strong induction, whereas hy5 seedlings have only a limited augment of ABI5::GUS activity. These results indicate that the induction of ABI5 by both developmental cues and abiotic stresses requires HY5, which is consistent with the compromised stress tolerance of hy5 seedlings (Fig. 1).

Overexpression of ABI5 Confers ABA Sensitivity to hy5 and Inhibits Hypocotyl Elongation. If ABI5 is a direct target of HY5, one would expect that overexpression of ABI5 may be able to rescue some of the hy5 mutant phenotypes such as its reduced sensitivity to ABA. The ABI5 overexpression lines were generated in both Col-0 and hy5 background (Fig. SA). On medium without ABA, no obvious difference in growth between hy5 and hy5 transformed with 35S:ABI5 was noticed (Fig. 5B). With ABA supplement, however, seedlings of hy5 transformed with 35S:ABI5 exhibited sensitive responses to ABA in that their seedling development, seed germination, and primary root elongation are significantly inhibited relative to hy5 mutants (Fig. 5C–E). Thus, expression of ABI5 is able to confer ABA sensitivity to hy5 during seed germination and seedling growth. Nonetheless, the insensitivity to ABA in lateral root growth was not rescued in hy5 mutant overexpressing ABI5 (data not shown), suggesting that there are additional components controlling ABA response in lateral root development.

Because HY5 regulates the expression of ABI5, we asked whether ABI5 plays any role in light signal transduction. The abi5 mutant and 35S:ABI5 transgenic plants were hence investigated for their response to light. Under different light regimes such as blue light, red light, far-red light, or dark, no significant difference between hypocotyl lengths of abi5 and the wild type was observed (data not shown), suggesting that the absence of ABI5 alone does not compromise photomorphogenesis response. However, when ABI5 was overexpressed in the wild-type background, although there was no clear phenotypic alterations in the dark, these transgenic plants exhibited a significantly enhanced response to blue, red, and far-red light in repressing hypocotyl elongation (Fig. 5F–H), which is consistent with the accumulation of HY5 under a broad spectrum of light (26). Nonetheless, overexpression of ABI5 in hy5 was not sufficient to restore the light response of hy5, indicating that besides ABI5,
of the impaired ABA response, *hy5* seedlings were more sensitive in growth to salt and osmotic stress (Fig. 1 D and E). Nonetheless, *hy5* leaves did not exhibit any transpiration defects and the guard cell ABA responsiveness is unaltered (data not shown), which implies that either HY5 does not modulate ABA-induced stomata movement or that HY5 is not involved in the ABA response of mature plants.

Besides its role in photomorphogenesis, recent research also suggests that HY5 is involved in the activation of a subset of UV-B responsive genes. One of the down-regulated genes in *hy5* under UV-B stress is *DREB2A* (29), a well characterized ABA- and drought-inducible gene. Our results also demonstrate that *hy5* mutants are defective in the accumulation of *LEAs* and *ABI5* mRNAs in mature seeds and ABA induction of *RD29B* in young seedlings (Fig. 3A and B). Given that HY5 and its homolog HYH redundantly regulate target genes’ transcription (30, 31), it is not unexpected that only a limited number of ABA-responsive genes were altered in their expression in *hy5* young seedlings (Fig. S4 and data not shown). Indeed, transcript levels of several ABA-inducible genes including a dehydrin, a MYB-related gene, *RD20*, *COR47*, and *LTI29* are greatly reduced in *hy5-hyh* double mutant compared with single mutants where little reduction in the transcript levels of these genes was detected (30). The alteration of ABA responsive genes’ transcription in *hy5* or *hy5-hyh* mutant strongly suggests that HY5 may play critical roles in ABA signaling.

Intriguingly, genes with diminished accumulation in *hy5* seeds and young seedlings appear to be either direct or indirect targets of ABI5. In previous studies, ABI5 protein was shown to be able to activate the promoters of *AtEM1*, *AtEM6*, and *RD29B* in transient transcription assays (6, 32). These genes may be the direct targets of ABI5. On the other hand, although there was no direct evidence for the activation of *RAB18* transcription by ABI5, *RAB18* mRNA level is down-regulated in *abi5* mutant (19). Coincident with reduced transcript levels of these ABI5 target genes, the steady-state mRNA level of *ABI5* was significantly reduced in *hy5* seeds (Fig. 3B). Moreover, *ABI5::GUS* activities were much lower in *hy5* than in wild type under both normal or stress conditions (Fig. 4). Therefore, the decreased transcript levels of these ABA-responsive genes in *hy5* are most likely caused by the down-regulated ABI5 expression in the mutant. The fact that ABA insensitivity of *hy5* mutants could be restored by ectopically expressing ABI5 (Fig. 5 B–E) supports this notion. Importantly, HY5 can bind the ABI5 promoter in vitro and in vivo with high specificity (Fig. 3 D–G). This result is consistent with a recent ChIP–Chip study that showed that the ABI5 promoter was among the putative binding sites of HY5 (21). ABA enhancement of HY5 binding to the ABI5 promoter (Fig. 3G) further suggests that HY5 is very likely a transcriptional activator of *ABI5* expression in response to developmental and environmental cues.

Recently, it was reported that seeds in shallow soil could sense light through phytochrome B to down-regulate ABI3 and promote hypocotyl growth (33). Our results demonstrate that overexpression of *ABI5* inhibits hypocotyl elongation under blue, red, or far-red light (Fig. 5 F–H). Interestingly, the *rpm10* and *kehr* mutants, which stabilize the ABI5 protein, exhibit a decreased hypocotyl growth as well (34, 35). Thus, ABA may enhance seedling light response and inhibit hypocotyl elongation, which may confer increased resistance to drought and other stresses. In fact, drought and ABA inhibition of hypocotyl elongation has been documented, although the mechanism was previously unknown (36). Recent studies showed that light also positively regulates ABA signaling through the HY5-ABI5 regulon that may facilitate seedling establishment under abiotic stress.

**Discussion**

Based on the insensitivity of *hy5* mutants to light inhibition of hypocotyl elongation, HY5 was defined as a positive regulator downstream of diverse photoreceptors in the light signal transduction pathway (16, 27, 28). Interestingly, *hy5* mutants also have more lateral roots and these lateral roots grow more horizontally (17). Because drought stress and ABA inhibit lateral root elongation (13–15), we examined whether HY5 is involved in ABA-mediated inhibition of lateral root growth. Whereas ABA clearly represses lateral root growth in the wild type, it is much less effective in *hy5*. Thus, wild-type HY5 is required for this ABA response (Fig. 1 A and C). Furthermore, the *hy5* mutant seeds are less sensitive to the inhibitory effect of ABA, high salt, or glucose on seed germination (Fig. 2), indicating that HY5 partly mediates these ABA responses as well. Perhaps as a result other HY5 target genes are also required for the inhibition of hypocotyl elongation during photomorphogenesis.
Materials and Methods

Plant Materials, Plasmid Constructions, Plant Transformation, and Growth Conditions. Arabidopsis thaliana ecotype Columbia-0 was used in all experiments unless otherwise specified. T-DNA insertion line, SALK.096651, was obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Homozygous hys5 mutants, referred as hys5 in this study, were isolated by their long hypocotyls and confirmed by PCR-based genotyping. The full-length cDNA and the 1.7-kb promoter of the ABIS gene were amplified and the fragments were ligated into the pENTR-D-TOPO vector (Invitrogen). After sequence confirmation, the ABIS gene was inserted into the pMD162 vector to make the cauliflower mosaic virus 3SS promoter-driven overexpression construct, and the ABIS promoter was cloned into the pMD162 vector to generate the promoter GUS fusion construct. Agrobacterium tumefaciens GV3101 was transformed by electroporation with these constructs and was used to vacuum-infiltrate 4-week-old Arabidopsis seedlings. Seed germination assays and stress and ABA treatments of seedlings were conducted as described (39). Root growth measurement (15), histochemical staining, and GUS quantification (11) were performed as described.

RNA Blotting Analysis. Total RNA from dry seeds of Col-0, hys5, Ws, and abi5 was extracted and analyzed as described (39). Total RNA from young seedlings of Col-0 and hys5 was extracted by using TRI Reagent according to the manufacturer’s manual (Molecular Research). For stress treatments, whole seedlings were treated with 100 μM ABA for 0.5 or 2 h before extracting total RNA. The gene-specific probes were prepared by PCR amplification with the respective primers [supporting information (SI) Table 1].

Gel Retardation, Chip, and Real-Time PCR Assays. The ~1.7-kb region upstream of the translational start codon of the ABIS gene was divided into four overlapping fragments: P1, -1754 to -1294; P2, -1315 to -863; P3, -883 to -440; and P4, -460 to 0. These fragments were PCR-amplified with the respective primers (SI Table 1). Gel retardation was performed as described (18).

Chip assays were performed as described (21) with 2-day-old seedlings grown on Murashige and Skoog medium (MS) or MS supplemented with 0.5 μM ABA. The PCR primers for the P1–P4 fragments of the ABIS promoter were used to amplify the DNA fragments. Primers for real-time PCR of the ABIS P1 fragment were: 5′-TTTGGTGCGTGGGTCGATT and 5′-AACATGATTCCGAACTTCCAGT.

Light Responses. Assays of seedling light response (hypocotyl length) were conducted as described (40).

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