Auxin controls seed dormancy through stimulation of abscisic acid signaling by inducing ARF-mediated *ABI3* activation in *Arabidopsis*

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The transition from dormancy to germination in seeds is a key physiological process during the lifecycle of plants. Abscisic acid (ABA) is the sole plant hormone known to maintain seed dormancy; it acts through a gene expression network involving the transcription factor ABSCISIC ACID INSENSITIVE 3 (ABI3). However, whether other phytohormone pathways function in the maintenance of seed dormancy in response to environmental and internal signals remains an important question. Here, we show that the plant growth hormone auxin, which acts as a versatile trigger in many developmental processes, also plays a critical role in seed dormancy in *Arabidopsis*. We show that disruptions in auxin signaling in *MIR160*-overexpressing plants, auxin receptor mutants, or auxin biosynthesis mutants dramatically release seed dormancy, whereas increases in auxin signaling or biosynthesis greatly enhance seed dormancy. Auxin action in seed dormancy requires the ABA signaling pathway (and vice versa), indicating that the roles of auxin and ABA in seed dormancy are interdependent. Furthermore, we show that auxin acts upstream of the major regulator of seed dormancy, ABI3, by recruiting the auxin response factors AUXIN RESPONSE FACTOR 10 and AUXIN RESPONSE FACTOR 16 to control the expression of *ABI3* during seed germination. Our study, thus, uncovers a previously unrecognized regulatory factor of seed dormancy and a coordinating network of auxin and ABA signaling in this important process.

hormones | interaction | preharvest sprouting | agriculture | evolutionary mechanism

Seed plants must be equipped with mechanisms to maintain the dormancy of freshly matured seeds until the proper season for propagation. The transition of the seed from dormancy to germination is a critical step in the lifecycle of plants. Dormancy is crucial to the survival of plant species, because it ensures that seed germination will occur only when environmental conditions are optimal for growth. Seed dormancy is also important for agriculture, because defective seed dormancy causes preharvest sprouting when humid conditions persist before harvest.

It has long been known that the relative levels of plant hormones control seed dormancy and germination. Gibberellins (GAs) break seed dormancy and promote germination (1, 2), and several other hormones, including brassinosteroids, ethylene, and cytokinin, have also been shown to promote seed germination (3, 4). However, abscisic acid (ABA) is the only hormone known to induce and maintain seed dormancy. ABA acts through the PYR/RCAR-PP2C-SnRK2 signaling cascade (5, 6). A major downstream component of ABA signaling, ABSCISIC ACID INSENSITIVE 3 (ABI3), has been long recognized as a major regulator of seed dormancy and ABA inhibition of seed germination (2).

The hormone auxin regulates many aspects of plant growth and development through the Transport inhibitor response1 (TIR1)/Additional F box protein (AFB)-Aux/indole-3-acetic acid (IAA) –AUXIN RESPONSE FACTOR (ARF) signaling system (7, 8). Recent studies have also suggested the potential involvement of auxin in seed dormancy maintenance. For example, exogenous application of auxin enhanced the inhibition of seed germination by ABA in *Arabidopsis* (9, 10) and also delayed seed germination of wheat (11, 12). However, whether auxin is directly required for seed dormancy is unclear, and the underlying mechanism of auxin function in seed dormancy remains unknown. During our previous studies on auxin-related growth and defense (13, 14), we found that *Arabidopsis* auxin mutants displayed either accelerated or inhibited germination of freshly harvested seeds, suggesting an active role of auxin in seed dormancy. In the current study, through extensive analysis of the auxin and ABA pathways and their roles in seed dormancy, we show that auxin is required for seed dormancy and ABA inhibition of seed germination. We have elucidated a molecular link through which auxin activates ABA signaling to inhibit seed germination.

Results

Seed Dormancy Depends On Auxin Levels. During our studies of auxin mutants, we found that freshly harvested seeds from unopened siliques (termed fresh wet seeds hereinafter) of transgenic plants overexpressing *MIR160* (*35S:MIR160*) (15) displayed greatly decreased dormancy compared with wild-type (WT) control based on cotyledon greening (Fig. S1 *A* and *B*) and radicle protrusion through the seed coat (Fig. S1 *C* and *D*). Because *MIR160* down-regulates the expression of three transcription factors, *ARF10*, *ARF16*, and *ARF17*, in auxin signaling (10, 15, 16), this observation suggested that the intrinsic auxin signal might play an important role in seed dormancy. This

Significance

Seed dormancy is a critical step in the lifecycle of plants, and it is crucial to the survival of plant species; this process is also important for agricultural practice to prevent preharvest sprouting when humid conditions persist before harvest. This study uncovers a previously unrecognized action of auxin in maintaining seed dormancy through AUXIN RESPONSE FACTOR 10/16-mediated expression of *ABSCISIC ACID INSENSITIVE 3*, a key regulator in the abscisic acid-mediated seed dormancy.

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The authors declare no conflict of interest.

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hypothesis was supported by our finding that the seed dormancy of the auxin-overproducing transgenic line *iaaM-OX* (17) was stronger than the seed dormancy of the WT by the cotyledon greening assay (Fig. 1*A*). Similarly, the radicle protrusion assay also revealed that fresh wet seeds of *iaaM-OX* plants did not even germinate at 30 d after imbibition, whereas 50% of similar seeds from WT plants germinated in 5 d (Fig. 1*C*). Consistent with transgenic experiments, exogenous application of IAA effectively enhanced, in a dose-dependent manner, the dormancy of fresh wet WT seeds (Fig. 1*D*). Interestingly, 4-d stratification at 4 °C nullified the inhibitory effect of exogenous IAA application (Fig. S1*E*), indicating that the auxin-mediated maintenance of seed dormancy could be prevented by stratification at 4 °C.

We next examined whether endogenous auxin levels also affect seed dormancy. The YUCCA (YUC) family of flavin monooxygenases are key enzymes in auxin biosynthesis (17, 18), and seed dormancy is established during seed maturation (1). Therefore, we searched the *Arabidopsis* microarray database (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), which revealed that *YUC1*, *YUC2*, and *YUC6* are expressed during seed development, with peak levels in the later stages of seed development (Fig. S2). Because the *yuc1yuc2yuc6* mutant exhibits low fertility (17), we used the *yuc1yuc6* double mutant to test seed dormancy. The fresh wet seeds of auxin-deficient *yuc1yuc6* double mutant displayed significantly decreased dormancy compared with similar seeds of the WT by both cotyledon greening (Fig. 1*B*) and radicle protrusion (Fig. 1*C*). To determine whether the effect of these mutants on seed development could somehow affect seed germination, we quantified germination rates of seeds after 4-d stratification at 4 °C but did not observe a difference in seed germination between the mutants and WT (Fig. S1*F*). Together, these results show that seed dormancy is strictly regulated by auxin.

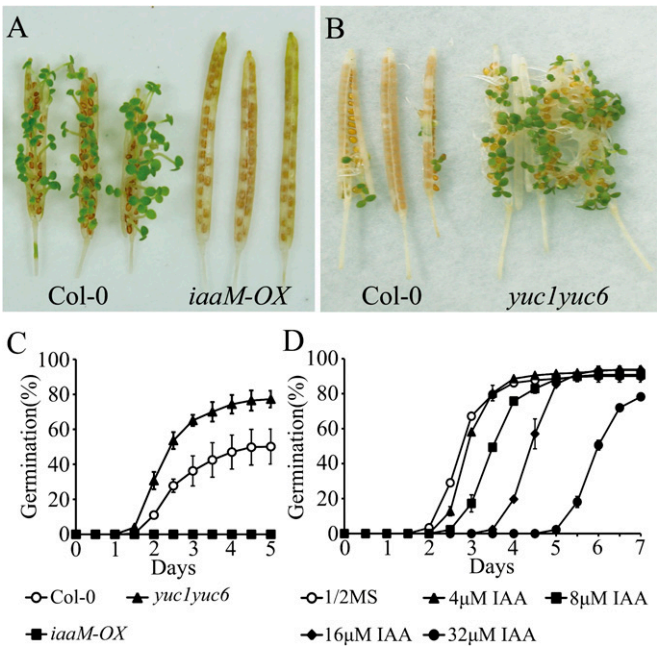

Fig. 1. Seed dormancy is controlled by auxin levels. (*A* and *B*) Visualization of seed germination by cotyledon greening in fresh mature siliques of WT Col-0, (*A*) *iaaM-OX*, and (*B*) *yuc1yuc6* plants after 7 d on water-saturated filter papers at 22 °C without stratification. (*C* and *D*) Radicle protrusion-based quantification of germination rates of non-stratified fresh wet seeds taken from unopened siliques on 1/2 MS containing no or various concentrations of IAA. (*C* and *D*) The average (± SD) values are shown.

TIR1/AFB-Mediated Auxin Pathway Is Required for Seed Dormancy. The auxin signaling pathway has been well-studied and involves a cascade mediated by the auxin receptors TIR1/AFB, the AUX/IAA transcriptional repressors, and the ARF transcription factors (8). We tested the role of TIR1/AFB in the control of seed dormancy. First, we assayed the seed dormancy of the auxin receptor mutants (19–21). Both the triple *tir1afb2afb3* and the quadruple mutant *tir1afb1afb2afb3* (22) failed to develop a hypocotyl and root meristem (Fig. S3*A*), probably because of a requirement for auxin in embryogenesis, similar to the auxin biosynthetic mutant *yuc1yuc4yuc10yuc11* (23). The majority of seeds from this mutant did not germinate normally (Fig. S3). Instead, we examined the dormancy of fresh wet seeds harvested from the *tir1afb2* and *tir1afb3* double mutants, which germinated normally like the WT seeds. The results showed that fresh wet seeds from these mutants germinated earlier than WT seeds assayed by both cotyledon greening (Fig. 2*A* and Fig. S4*A*) and radicle protrusion (Fig. 2*B*). IAA7/AXR2 and IAA17/AXR3 are two well-characterized transcriptional repressors of the TIR1/AFB-mediated auxin pathway, and the mutants *axr2-1* and *axr3-1* display a constitutive repression of auxin-responsive genes (24, 25). We observed that fresh wet seeds of the two mutants also germinated earlier than fresh wet seeds of the WT (Fig. 2 *C* and *D* and Fig. S4*B*).

We also examined the effects of downstream auxin signaling components on seed dormancy. Consistent with the phenotype of *35S:MIR160* transgenic plants, in which *ARF10*, *ARF16*, and *ARF17* were down-regulated, the *arf10arf16* double mutants (15) displayed a significant decrease in seed dormancy (Fig. 2 *E* and *F*). Consistently, two transgenic lines that express *MIR160*-resistant forms of *ARF10* and *ARF16* (*mARF10* and *mARF16*, respectively) (10, 15) displayed significantly increased seed dormancy (Fig. 2*F* and Fig. S4 *C* and *D*), confirming that *MIR160*, indeed, reduces seed dormancy through down-regulation of auxin signaling. Taken together, these results show that the TIR1/AFB-mediated auxin pathway, in which ARF10 and ARF16 act as key downstream components, is required for seed dormancy.

Auxin Signaling Pathway Is Essential for ABA Inhibition of Seed Germination. ABI4 and ABI5 are two important transcription factors, and their loss-of-function mutants *abi4* and *abi5-1* are insensitive to ABA-mediated inhibition of seed germination. However, the seed dormancy of the mutants does not change (26), suggesting that there may be distinct signaling pathways for ABA-mediated seed dormancy and ABA-inhibited seed germination. We, therefore, examined the role of auxin in ABA-inhibited germination. Indeed, we found that both exogenous and endogenous IAA inhibited seed germination, assayed by radicle protrusion, in an ABA-dependent manner (Fig. 3*A* and Fig. S5 *A* and *B*), indicating that auxin and ABA act synergistically to inhibit seed germination. Furthermore, we observed that the synergistic effect was IAA dose-dependent, with the maximal effect with 4 μM IAA in the presence of 1 μM ABA (Fig. 3*B*). An equal amount of IAA did not inhibit seed germination in the absence of ABA (Fig. S1*E*), indicating that the auxin-mediated inhibition of seed germination is dependent on ABA.

We further tested whether ABA inhibition of seed germination also depends on auxin function. Our results showed that seeds of *yuc1yuc6* germinated slightly earlier than seeds of the WT in the presence of 1 μM ABA (Fig. S5 *C* and *D*). Although the majority of seeds of *tir1afb1afb2afb3* were defective, a minority of the quadruple mutant seeds and almost all seeds of the single and double mutants were normal and germinated similarly to the WT seeds (Fig. S3). The normal seeds of the quadruple mutant were selected and found to be insensitive to ABA, with a close positive correlation between auxin signaling defect and ABA insensitivity (Fig. 3 *C* and *D* and Fig. S5*E*). Moreover, the *axr2-1* and *axr3-1* mutant seeds were also less sensitive to ABA than the WT seeds (Fig. S5 *F*–*I*).

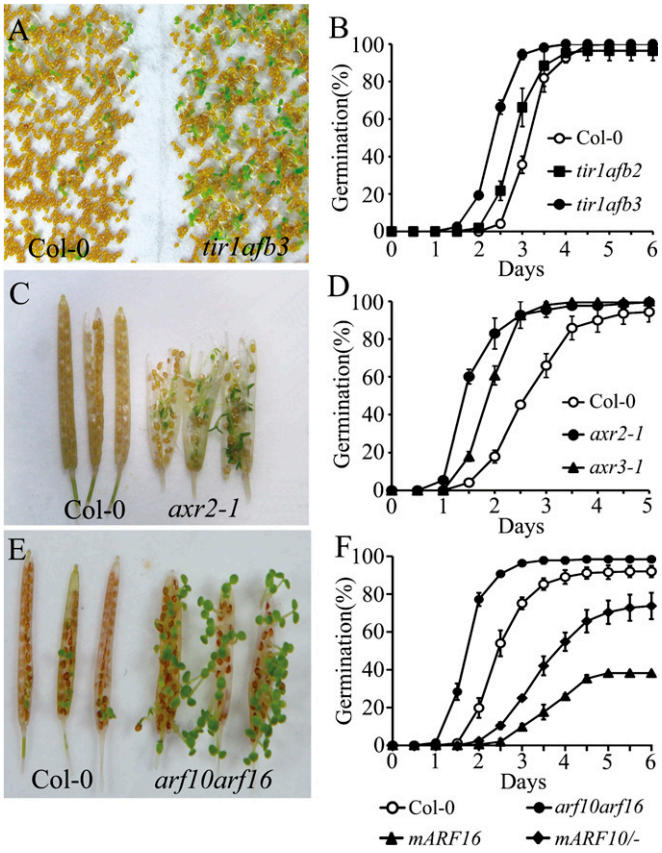

Fig. 2. Auxin detection and signaling are required for seed dormancy. (*A*, *C*, and *E*) Visualization of seed germination by cotyledon greening in fresh mature seeds or siliques from (*A*) the auxin receptor mutant *tir1afb3* and the auxin signaling mutants (*C*) *axr2* and (*E*) *arf10arf16* on water-saturated filter papers without stratification. Images were acquired (*A* and *C*) 4 and (*E*) 5 d after incubation at 22 °C. (*B*, *D*, and *F*) Radicle protrusion-based quantification of germination rates of nonstratified fresh wet seeds taken from unopened siliques of the auxin signaling mutants on 1/2 MS. The average rates (± SD) are shown.

ARFs activate/repress downstream genes to execute auxin responses. We observed that *arf10* and *arf16* were less sensitive and the *arf10arf16* double mutant was dramatically less sensitive to ABA during seed germination (Fig. 3*E*). This result was in agreement with the seed dormancy of *35S:MIR160* and *arf10arf16* (Fig. 2*E* and Fig. S1). Again, the effect of expressing the *MIR160*-resistant *mARF10* and *mARF16* was opposite to the effect of *arf10* and *arf16* mutations (Fig. 3*F*). Taken together, our results indicate that ABA function in seed germination is largely dependent on the TIR1/AFB-AUX/IAA-ARF–mediated auxin signaling pathway.

Auxin Stimulates *ABI3* Expression, and *ARF10* and *ARF16* Are Required for Maintenance of *ABI3* Expression. There are two possible mechanisms by which auxin functions in these ABA-mediated processes: either auxin stimulates ABA biosynthesis, or it activates the ABA response. First, we examined the effects of endogenous IAA on stomatal control, which is mediated by ABA levels, with water loss as readout. We found that the transpiration rate did not change in *iaaM-OX*, *tir1afb1afb2afb3*, and *arf10arf16* (Fig. S6 *A* and *B*), indicating that IAA unlikely enhances ABA levels in leaves. We could exclude ABA biosynthesis as a target of auxin action in seed dormancy and germination, because the effect of IAA on seed dormancy and germination persisted in *aba2-1*, an ABA synthesis-deficient mutant (27) (Fig. S6 *C* and *D*).

Therefore, the auxin function in the ABA-mediated response is likely specific to seed dormancy/germination inhibition by regulating a seed-specific ABA signaling component. ABI3, ABI4, and ABI5 are three key components of seed-specific ABA signaling. However, only ABI3 functions in both ABA-mediated seed dormancy and inhibition of seed germination (Fig. S7 *A*–*C*) but not stomatal regulation (26, 28, 29). We also proposed that auxin might regulate ABA action through transcriptional activation of *ABI3* by ARF10 and ARF16. To test our hypotheses, we first examined *ABI3* expression patterns in the WT and auxin mutants. As previously reported (30, 31), *ABI3* mRNA levels were high in seeds of the WT before germination and rapidly decreased after germination (Fig. 4). By contrast, its levels were maintained for a longer time (up to 108 h) in imbibited seeds from opened siliques (termed fresh dry seeds hereafter) of *iaaM-OX* and *mARF10* without stratification but decreased more rapidly in the *arf10arf16* double mutant than the WT (Fig. 4*A*). Similar results were also observed during seed germination in the presence of ABA (Fig. 4*B*). Moreover, exogenous IAA also enhanced *ABI3* mRNA levels during imbibition of fresh dry seeds without stratification but had little effect on the ABI3 protein stability (Fig. S7 *D* and *E*). Taken together, these results indicate that auxin and ARF10/16 are required for the maintenance of *ABI3* expression in the seed after imbibition.

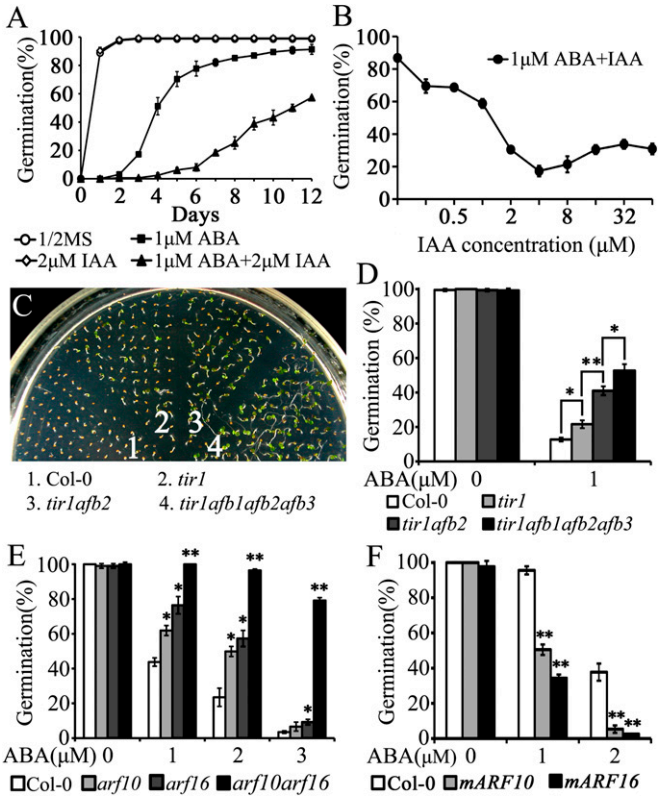

Fig. 3. Auxin is required for ABA-mediated inhibition of seed germination. (*A*) Germination of WT seeds on 1/2 MS with 2 μM IAA, 1 μM ABA, or both. (*B*) Germination rates of WT seeds with increasing concentrations of IAA in the presence of 1 μM ABA at 8 d. (*C*) Seed germination of Col-0 and the auxin receptor mutants on 1/2 MS with 1 μM ABA. The image was acquired after 5 d of incubation. (*D*) Seed germination rates were determined after 3 d on 1/2 MS medium with or without ABA. (*E* and *F*) Seed germination rates of Col-0 and the *ARF* mutants on 1/2 MS medium with or without ABA at (*E*) 6 and (*F*) 8 d. Significant differences were determined by Student *t* test (*$P < 0.05$; **$P < 0.01$). (*A*, *B*, and *D*–*F*) Quantification of seed germination was based on radicle protrusion through the seed coat.

Auxin-Enhanced Seed Dormancy and ABA Inhibition of Seed Germination Depend On ABI3. The transcription factor ABI3 is a key positive regulator of seed dormancy, and our results showed that auxin regulates the expression of *ABI3* in nonstratified imbibed seeds. We next examined the relationship between the synergistic effect of IAA and ABA on seed dormancy/germination and ABI3 activity. Our experiments showed that the synergistic inhibition effect of IAA and ABA on seed germination was lost in the *abi3-1* mutant (Fig. S8 *A* and *B*). Furthermore, the strong seed dormancy and ABA hypersensitivity of the *iaaM-OX* line were also compromised in the *iaaM-OX*/*abi3* double mutant (Fig. 5 *A* and *B* and Fig. S8*C*). A similar compromise in seed dormancy and ABA sensitivity was also observed in the *mARF16*/*abi3* double mutant (Fig. 5 *C* and *D* and Fig. S8*D*). Furthermore, auxin induced the strong accumulation of the ABI5 protein in germinating seeds (Fig. S8*E*), which acts downstream of ABI3 to inhibit seed germination (32). These results show that the enhancement by auxin of seed dormancy and ABA inhibition of seed germination depend on ABI3 function.

ARF10 and ARF16 Regulate *ABI3* Expression Indirectly. ARFs regulate the expression of a large set of auxin-responsive genes by binding to auxin response elements (AuxREs) in their promoters (33, 34). The AuxRE elements have the consensus sequence TGTCTC (35). We identified a potential AuxRE element in the promoter of *ABI3*. To assess the potential role of the AuxRE element in mediating *ABI3* expression *in planta*, we transformed *abi3-1* with either the WT *ABI3:ABI3* or the mutated *mABI3:ABI3*, in which the TGTCTC sequence is mutated to AAGCTC. The genetic complementation experiment showed that the mutated *mABI3:ABI3* construct, like the WT construct, could complement the phenotype of ABA insensitivity in *abi3-1* (Fig. S9*A*). This result suggests that the TGTCTC sequence is not required for the expression of *ABI3* during seed germination. This interpretation was in agreement with our yeast one-hybrid assays, which showed that ARF10 and ARF16 did not bind to the DNA region containing the TGTCTC element in the *ABI3* promoter or other regions covering a 2.7-kb *ABI3* promoter (Fig. S9*B*).

To confirm these in vitro results, we transiently activated *ABI3* expression in mesophyll protoplasts of 30-d-old *Arabidopsis* plants with expression of *35S:GUS* (β-glucuronidase) as a background control. We observed that expression of the *ABI3:LUC* (luciferase) fusion reporter was not significantly affected in the protoplast by cotransfection of *35S:ARF10* or *35S:ARF16* (Fig. S9*C*). The result is also consistent with the fact that *ABI3* is only highly activated in nongerminated and imbibing seeds and not activated by ABA in seedlings (31, 36, 37). We also performed chromatin immunoprecipitation (ChIP) assays using the nongerminated seeds of the myc-tagged ARF16 transgenic line after 12 h of imbibition. As shown in Fig. S9*D*, consistent with the yeast one-hybrid data, the anti-myc antibody did not precipitate the *ABI3* promoter fragments. These results suggest that ARF10 or ARF16 may not directly bind to the *ABI3* promoter and may recruit or activate an additional seed-specific transcription factor to stimulate *ABI3* expression (Fig. 5*E*). Additional seed dormancy mutant screening in the *mARF10* or *mARF16* background could identify the missing links in the *ARF10/16-ABI3* signaling cascade.

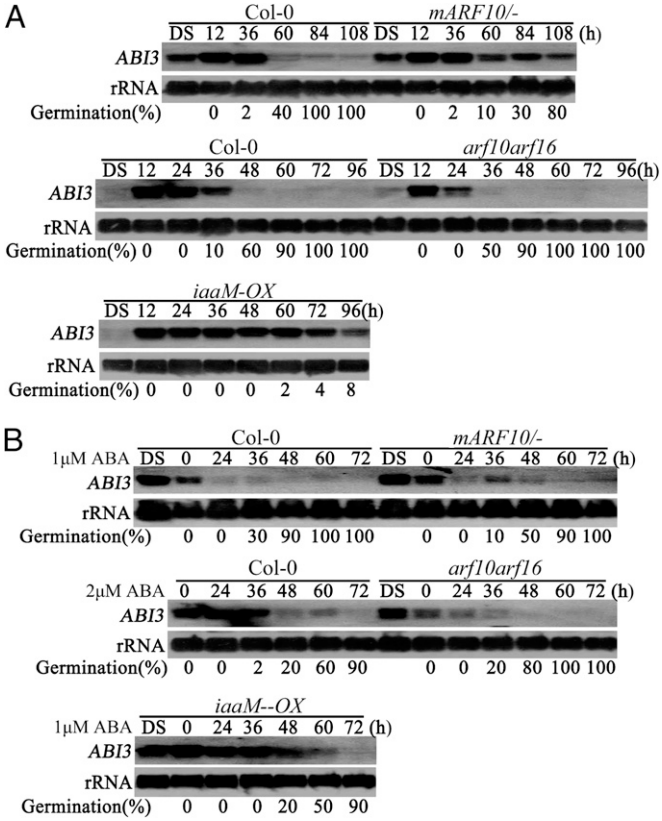

Fig. 4. ARF10 and ARF16 are required to maintain *ABI3* expression. (*A*) RNA blotting detection of *ABI3* transcript levels in Col-0, *mARF10/–*, *arf10arf16*, and *iaaM-OX* during imbibition of nonstratified fresh dry seeds in the absence of ABA. (*B*) RNA blotting detection of *ABI3* transcript levels in Col-0, *mARF10/–*, *arf10arf16*, and *iaaM-OX* during seed germination in the presence of ABA after 4-d stratification at 4 °C. The germination percentage at each time point assayed by radicle protrusion is indicated under the blots. DS, dry seed.

Discussion

Seed dormancy release and germination are complex biological processes that are affected by both developmental and environmental factors. In this study, we uncovered a previously unrecognized action of auxin in seed dormancy by which auxin enhances ABA-mediated seed dormancy through the recruitment of *ARF10/16* to maintain *ABI3* expression during seed imbibition. Therefore, our study establishes a molecular link between two important hormone pathways. Auxin promotes dormancy and inhibits germination by enhancing ABA action, thereby adding another protective level of control in the regulation of seed dormancy. This auxin-mediated seed dormancy is likely an evolutionary mechanism that prevents seed germination in unfavorable seasons, and it could also be crucial to the evolution and diversity conservation of seed plant species.

The observation that ARF10 and ARF16 act as positive regulators of the ABA signal pathway contributes to the emerging map of hormone signaling integration in plant development and environmental adaption (38–40), and also, it elucidates a crucial mechanistic role of auxin in this well-studied biological process. It has been proposed that different ARFs act as either activators or repressors of target genes to generate context-specific responses (41). Interestingly, another *ARF* gene, *ARF2*, was induced by ABA, and the *arf2* mutants displayed enhanced ABA sensitivity during seed germination and primary root growth, suggesting that ARF2 is a repressor of the ABA signaling pathway (42). Our study indicates that ARF10 and ARF16 function as the activators of *ABI3* transcription. Therefore, the cross-talk between the auxin and the ABA pathways may specify different biological processes through recruitment of different interacting components.

A recent study reported that low concentration of 1-naphthaleneacetic acid (NAA) or 2,4-dchlorophenoxyacetic acid (2,4-D) could promote seed germination assayed by cotyledon greening (43). However, we observed that low concentration of NAA or IAA also slightly inhibited seed germination assayed by radicle protrusion (Fig. S10). This contradiction was likely caused by the different ways to define seed germination (radicle protrusion or cotyledon greening). As a matter of fact, when using cotyledon greening to assay seed germination, we found

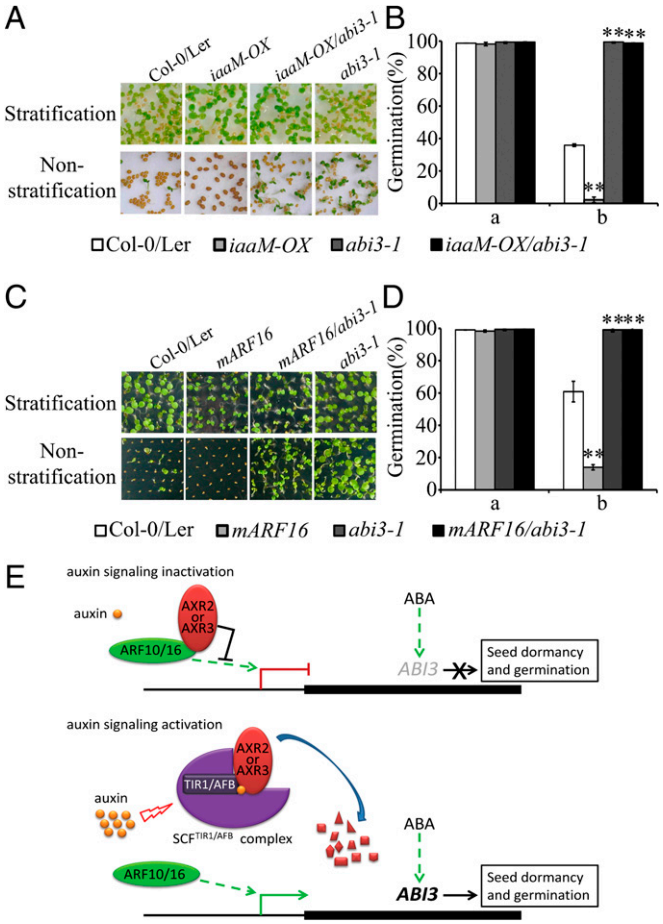

Fig. 5. Auxin-mediated control of seed dormancy depends on *ABI3*. (*A* and *B*) Fresh seeds of *iaaM-OX*, *iaaM-OX* /*abi3-1*, and *abi3-1* were germinated on 1/2 MS medium with or without stratification. The Col-0/L*er* hybrid was used as a control, because *iaaM-OX* is in the Col-0 background, whereas *abi3-1* is in the L*er* background. (*C* and *D*) Germination of fresh seeds of *mARF16* and *mARF16*/*abi3-1* as well as the *abi3-1* and Col-0/L*er* controls with or without stratification. Seed germination was (*A* and *C*) visualized by cotyledon greening and (*B* and *D*) quantified by radicle protrusion after 6 d of incubation on 1/2 MS medium at 22 °C. Values are presented as averages (± SDs) with Student *t* test (**$P < 0.01$). (*E*) A proposed model for the effect of the ABA–auxin interaction on the control of seed dormancy/germination. When auxin signaling is inactivated by low auxin level or signaling disruption, ARF10 and ARF16 are inactivated by the Aux/IAA repressors AXR2 and AXR3. *ABI3* expression cannot be maintained, and seed dormancy is released. With auxin signaling activation, auxin binds to the auxin receptor TIR1/AFB F-box proteins and promotes the degradation of IAA7/AXR2 and IAA17/AXR3. The degradation releases the activity of ARF10 and ARF16 and maintains the expression of *ABI3*, which protects seed dormancy and inhibits seed germination. The solid arrows and lines indicate direct regulation, and the dotted arrows indicate indirect regulation. The black box indicates the ABI3 gene region.

that low concentrations of NAA or IAA indeed could promote seed germination (Fig. S10*F*), likely caused by auxin stimulation of chloroplast development. ABI4 is also a seed-specific regulator of ABA signaling and was previously shown to have no effect on seed dormancy (26, 29). However, a recent study reported that ABI4 could also regulate primary seed dormancy in *Arabidopsis* (44). Under our experimental conditions, we found that *abi4* mutation had no detectable effect on seed dormancy (Fig. S7*C*). Many external factors are known to affect seed dormancy: low temperature, nitrogenous compounds, duration of the after-ripening process, and environmental conditions during seed maturation on the mother plant (45). These factors are likely responsible for the inconstancy on the involvement of ABI4 in regulating seed dormancy.

The mechanism by which seed dormancy evolved in seed plants remains unknown. Recent large-scale genome sequencing projects have facilitated the comparison of hormone signaling pathways in the plant kingdom and yielded important insights into the conservation and evolution of hormone signaling pathways (39). Interestingly, it has been suggested that the signaling machinery for GA, ethylene, and brassinosteroid probably did not evolve until after the evolutionary split of moss and vascular plants (46, 47). Moreover, the moss genome encodes proteins that function in auxin, ABA, and cytokinin signaling, whereas the genome of green algae does not (46, 47). These genome-wide comparisons suggest that these hormone pathways could have emerged during the early colonization of land by plants (39). We propose that the auxin and ABA signaling pathways might have coevolved to synergically control seed dormancy in ancient seed plants, allowing them to survive unfavorable environmental or seasonal factors during their early evolution.

Auxin is involved in almost all aspects of plant development as well as responses to a multitude of environmental situations (48). The GA pathway was recently shown to be subject to regulation by auxin (49). We show here that seed dormancy and ABA control of seed germination are also subject to strict modification by auxin. Seed primary dormancy is induced during seed development and also depends on environmental conditions (45). It will be interesting to further investigate whether and how developmental/environmental stimuli are integrated into auxin levels, distribution, and/or signaling to induce seed dormancy during seed development.

Materials and Methods

Plant Material and Growth Conditions. *Arabidopsis* ecotype Col-0 was used in all experiments, with the exception of the *abi3-1* and *abi5-1* mutants, which are in the Landsberg *erecta* (L*er*) and Wassilewskija backgrounds, respectively (29). The *tir1afb2*, *tir1afb3*, and *tir1afb1afb2afb3* mutants were generated by crossing *tir1* (CS3798), *afb1* (SALK_070172), *afb2* (SALK_137151), and *afb3* (SALK_068787), which were obtained from the ABRC. The *iaaM-OX*/*abi3-1* and *mARF16*/*abi3-1* double mutants were generated by crossing *iaaM-OX* and *mARF16* with *abi3-1*. All plants were grown under 16 h light and 8 h dark conditions at 22 °C.

Germination Experiments. For seed dormancy analysis, to make sure that all fresh wet seeds mature at the same time, we carefully selected plants with early siliques that matured at the same time. Seeds or siliques were directly sown without stratification on 1/2 MS medium with or without IAA or water-saturated filter paper for seed germination and then placed in the growth chamber. For the germination assay, only seeds that matured at the same time were used. Seeds were first sown on 1/2 MS medium with or without supplementation of ABA and/or IAA and incubated at 4 °C for 4 d, and they were then germinated under 16 h light and 8 h dark conditions at 22 °C. Seeds were counted as germinated when the radicle tip had fully penetrated the seed coat (radicle protrusion), and germinated seeds were scored at the indicated times; statistical analysis was performed with three biological replicates. All results were confirmed using harvested seeds from the next generation, and similar results were obtained.

***ABI3* Promoter Mutants, Genetic Complementation, and Protoplast Transient Expression.** A 6.4-kb genomic fragment containing the full-length ABI3 gene plus the 2,692-bp upstream and 828-bp downstream sequences was amplified by PCR and cloned into the vector pCAMBIA1300. The point mutations of the *ABI3* promoter were created by PCR and confirmed by sequencing. Transgenic *Arabidopsis* plants were generated by the floral dip method. Protoplast transient expression assays were performed as described (50).

Yeast One-Hybrid Experiment. Five DNA fragments covering a 2.7-kb *ABI3* promoter were inserted into the reporter vector pG221. The full-length coding regions of *ARF10* and *ARF16* were ligated into the vector pGADT7. The yeast strain EGY48 was used for transformation. Minimal medium (−Leu and −Ura) was used for selecting positive transformants. Positive colonies were then plated onto the selection medium and assayed for β-gal activity.

RNA Extraction, Northern Blot Analysis, Western Blot Analysis, and GUS Activity. Total RNA was extracted from seeds with extraction buffer [0.1 M Tris·HCl, pH 8.0, 0.05 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 2% (wt/vol) hexadecyltrimethylammonium bromide (CTAB), 2% (wt/vol) polyvinylpyrrolidone (PVP), 2 M NaCl, 3% β-mercaptoethanol (vol/vol)]. For Northern blots, 10 μg total RNA were separated on a 1% (wt/vol) denaturing agarose gel and transferred to Hybond-N^+ membranes (Amersham Pharmacia Biotech). A 345-bp fragment of *ABI3* was labeled with the DIG Labeling and Detection Starter Kit II (Roche) for RNA hybridizations. After stripping the probes, the filters were reprobed with a 2.5-kb cDNA fragment of *Arabidopsis* 18S *rRNA* to serve as a loading control; *35S:ABI3-6myc* (36) was germinated and grown on selective media for 5 d before transfer to liquid MS medium supplemented with the indicated concentrations of cycloheximide (CHX) and IAA (Sigma). Seedlings were harvested, and proteins were extracted at the indicated times for Western blot assay. For the GUS activity assay, seeds were dissected, and embryos were stained overnight to detect GUS activity.

Gene Expression Analysis. For real-time PCR assays, reactions were set up with SYBR Green Supermix (TAKARA). Gene expression was quantified at the logarithmic phase using the expression of the housekeeping *ACTIN2* as an internal control. Three biological replicates were performed for each experiment.

ChIP. The transgenic lines of myc-tagged ARF16 were used. ChIP assays were performed with seeds after imbibition for 12 h without stratification using the EpiQuik Plant ChIP Kit (Epigentek Group Inc.). An myc tag-specific monoclonal antibody was used for ChIP analysis. The 2.1-kb region upstream of *ABI3* was divided into five overlapping fragments for ChIP analysis: P1, −2,156 to −1,664; P2, −1,712 to −1,256; P3, −1,287 to −812; P4, −832 to −357, and P5, −386 to +66.

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