A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in *Arabidopsis*

Luis Lopez-Molina*†, Sébastien Mongrand*, and Nam-Hai Chua

Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399

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Seed dormancy is a trait of considerable adaptive significance because it maximizes seedling survival by preventing premature germination under unfavorable conditions. Understanding how seeds break dormancy and initiate growth is also of great agricultural and biotechnological interest. Abscisic acid (ABA) plays primary regulatory roles in the initiation and maintenance of seed dormancy. Here we report that the basic leucine zipper transcription factor ABI5 confers an enhanced response to exogenous ABA during germination, and seedling establishment, as well as subsequent vegetative growth. These responses correlate with total ABI5 levels. We show that ABI5 expression defines a narrow developmental window following germination, during which plants monitor the environmental osmotic status before initiating vegetative growth. ABI5 is necessary to maintain germinated embryos in a quiescent state thereby protecting plants from drought. As expected for a key player in ABA-triggered processes, ABI5 protein accumulation, phosphorylation, stability, and activity are highly regulated by ABA during germination and early seedling growth.

Abscisic acid (ABA) is a phytohormone regulating the initiation and maintenance of seed dormancy. It also plays an essential role in a plant's response to stress, particularly water deprivation, notably by regulating stomatal aperture (1). So far, ABA-insensitive screens have been widely used to identify molecular genetic components of the ABA signal transduction pathway (2, 3). In these screens, mutagenized *Arabidopsis* seeds were exposed to ABA concentrations that inhibit germination of wild-type (WT) seeds, and putative mutants that were able to germinate were isolated (2, 3). These screens have allowed the identification of several *ABI* (*AB*A-*i*nsensitive) genes (4–10) and recent studies have established that *ABI1* and *ABI3* are key players in vegetative and embryonic ABA responses, respectively (1, 11).

Nonetheless, few reports have clarified the physiological role of ABA and mechanisms of action triggered by ABA during germination and early seedling growth. We were led to address these issues by the recent cloning and analysis of *ABI5* by two independent groups (4, 5). The *abi5* mutation is recessive and *ABI5* encodes a putative transcription factor of the basic leucine zipper (bZIP) family (4, 5). The bZIP region of ABI5 shows extensive homology to previously characterized plant (bZIP) transcription factors capable of activating reporter genes containing *AB*A-*r*esponsive DNA *e*lements (ABREs) (12–14). ABI5 also binds to ABREs *in vitro* (unpublished results) and dry seeds of *abi5* show reduced transcript levels of ABA-responsive and ABRE-containing late embryonic genes such as *AtEm1* and *AtEm6* (4, 5). Together with the ABA insensitivity of *abi5* mutants, these results show that ABI5 is the first bZIP plant factor found to be required *in vivo* to signal ABA-elicited responses.

In the present work, we found that ABA regulates ABI5 accumulation and activity during a limited developmental window. On removal of ABA, ABI5 is rapidly degraded by a process that may involve the proteasome pathway and this coincides with the initiation of vegetative growth. Transgenic plants overexpressing ABI5 are hypersensitive to ABA, with respect to both germination and vegetative growth. We show that ABA delays germination, rather than blocking it altogether. This hormone also efficiently prevents vegetative growth by arresting development of mature germinated embryos. Moreover, ABI5 is a rate-limiting component of growth arrest, maintaining *Arabidopsis* seedlings quiescent for at least a month. We hypothesize that this ABA-induced and ABI5-dependent quiescence fulfills a developmental checkpoint during which *Arabidopsis* plants monitor the water status in the environment.

Materials and Methods

Plant Material and Growth Conditions. Seeds of *Arabidopsis thaliana*, ecotype Wassilewskija, Columbia or Landsberg erecta were surface sterilized as described (4) and then plated on Murashige and Skoog (MS) medium (15) containing 0.8% (wt/vol) Bacto-Agar (Difco). Plates were routinely kept for 3 days in the dark at 4°C to break dormancy (stratification) and transferred thereafter to a tissue culture room under constant white fluorescent light (27 μmol $m^{-2}{\cdot}s^{-1}$) at 22°C. Seeds of *abi1*, *abi3-1*, and *abi4* were obtained from the Nottingham *Arabidopsis* Stock Center (NASC). For ABA treatments, seedlings were transferred to plates supplemented with ABA (mixed isomers, Sigma A7393), which was dissolved in methanol. Control plates contained equal amounts of methanol.

Plasmid Constructions and Plant Transformation. DNA manipulations were performed according to standard methods (16). 35S constructs were generated by using a binary vector (17). Transgenic *Arabidopsis* lines [Wassilewskija ecotype (Ws)] were generated by using the *Agrobacterium tumefaciens* vacuum-infiltration method (18). Seeds (T1) from infiltrated plants were plated on MS medium containing 10 mg/L gluphosinate ammonium (Crescent Chemical, Hauppauge, NY) and 100 mg/L cefotaxime (Sigma).

RNA Extraction, Northern Blots, ABI5 Antibody Production, and Western Analysis. RNA extraction and Northern blot hybridizations were done as described (4). A DNA fragment encoding the ABI5 ORF was cloned into pET28a (Novagen) and the recombinant protein was induced and purified by using a commercial kit according to the manufacturer's instructions (His trap from Amersham Pharmacia). Polyclonal anti-ABI5 antiserum was obtained from rabbits immunized with His-tagged ABI5. Stan-

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Abbreviations: ABA, abscisic acid; ABI, ABA-insensitive gene; HA, hemagglutinin peptide tag; Ws, Wassilewskija ecotype; WT, wild type; MS, Murashige and Skoog.

*L.L.-M. and S.M. contributed equally to this work.

†To whom reprint requests should be addressed. E-mail: lopezl@rockvax.rockefeller.edu.

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dard methods using His-ABI5 coupled to a CNBr activated Sepharose 6-MB column (Amersham Pharmacia) were used to affinity purify specific ABI5 antibodies from sera.

Plant protein extracts were resolved under reducing conditions by using 10% SDS/polyacrylamide gels (19). Proteins were transferred onto poly(vinylidene difluoride) (PVDF) membranes (Immobilon-P from Millipore), which were incubated with primary affinity-purified-ABI5 antibody (diluted 1:3,000; 60 μg/ml) and secondary antibodies, peroxidase-conjugated anti-rabbit (Amersham Pharmacia; diluted 1:3,000), for 1 h at room temperature in TBS (25 mM Tris·HCl at pH 7.4; 137 mM NaCl, 5 mM KCl) supplemented with 4% nonfat dry milk. After incubation, membranes were washed twice (10 min each) with TBS containing 0.05% Tween 20. After the final wash, membrane-associated peroxidase activity was visualized by using the ECL kit (Amersham Pharmacia).

ABA Inhibition of Root Growth and Water Loss. WT Ws seedlings and transgenic Ws seedlings carrying *35S*::*HA-ABI5* (WT/*35S*::*HA-ABI5*, T3 generation) were germinated and grown for 5 days on MS medium without ABA. Seedlings were then transferred to plates containing 5 μM ABA, and subsequent root growth was scored after 5 days. Values were expressed as the average ± SD of 15 seedlings. For water loss measurement, young rosette leaves at the same developmental stage were excised from WT Ws, WT/*35S*::*HA-ABI5*, and *abi1* plants and weighed at the different times indicated (n = 3).

Cycloheximide Experiments. Eight-day-old WT/*35S*::*HA-ABI5* transgenic seedlings were incubated in liquid MS medium supplemented with 100 μM cycloheximide. After 15 h, 50 μM ABA was added to the medium and proteins were extracted at the indicated times. Equal amounts of protein were loaded on a 10% SDS/polyacrylamide (NOVEX, San Diego) gel and analyzed by Western blots using rabbit antibody to hemagglutinin peptide tag (HA; 1:3,000, Santa Cruz Biotechnology).

Cell-Free Degradation Assay. Eight-day-old WT/*35S*::*HA-ABI5* transgenic seedlings were ground in liquid nitrogen and proteins were extracted according to methods described in ref. 20. Cell debris was pelletted by centrifugation and equal amounts of extract were transferred to individual tubes with a mixture of anti-protease (complete Mini from Amersham Pharmacia) supplemented with 1 mM PMSF with or without 26S proteasome-specific inhibitors ALLN, MG115, MG132, and PS1 (10 μM each, Calbiochem). Samples were incubated at 30°C and an equal volume of SDS-loading buffer was added to stop the reactions. Equal amounts of sample were then analyzed by Western blots (rabbit polyclonal antibody to HA).

Immunoprecipitation and Phosphatase Experiments. Eight-day-old WT/*35S*::*HA-ABI5* transgenic seedlings (T3 generation) were treated in liquid MS medium with [^{32}P]orthophosphoric acid [100 μCi (1 Ci = 37 GBq), 285.5 Ci/mg, NEN] for 90 min. Seedlings were further treated with or without 50 μM ABA for 30 min. HA-ABI5 was immunoprecipitated by using rabbit antibody to HA coupled to beads according to methods described in ref. 21 for 5 h at 4°C using 5 μg of rabbit antibody to HA coupled on agarose beads (Santa Cruz Biotechnology). Beads were washed three times with 50 ml of immunoprecipitation buffer and once with 0.5 ml of λ phosphatase buffer (New England Biolabs). Beads were resuspended with 50 μl of λ phosphatase buffer and divided in two portions: one portion was treated with 200 units of λ phosphatase for 10 min at 30°C; the other portion was not treated. An equal volume of SDS-loading buffer was added to stop the reactions. Equal amounts of sample (10% of the total) were then analyzed by Western blot using mouse monoclonal antibody against HA as first antibody. The remaining sample was analyzed by SDS/PAGE, and PhosphorImager (Bio-Rad) was used to determine relative radioactive incorporation.

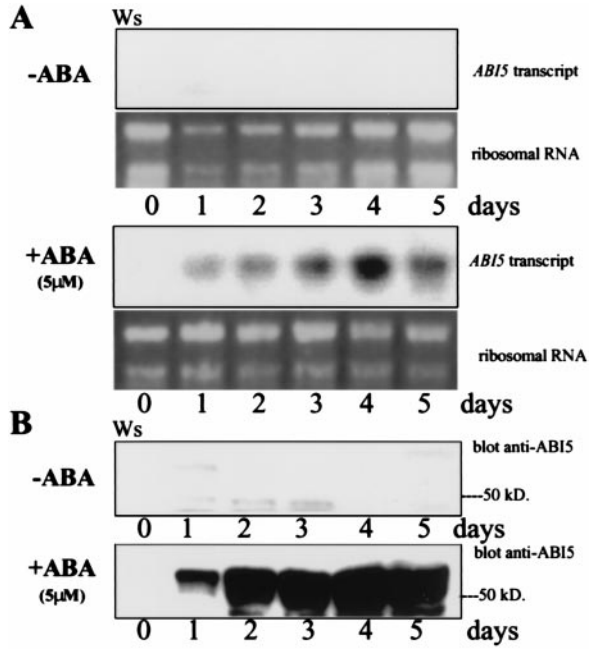

Fig. 1. ABI5 protein and transcript accumulation is highly regulated by ABA. WT Ws seeds were kept in darkness at 4°C for 3 days with or without ABA (5 μM) and then transferred to constant light at 22°C for 1, 2, 3, 4, and 5 days (0 indicates the time immediately following transfer). Total protein and RNA were isolated. (*A*) Northern blot analyses. Each lane contained 3 μg total RNA. (*B*) Western blot analyses using antibodies to ABI5. Each lane contained 10 μg protein.

Results

ABA Induces ABI5 Expression Early in Development. *abi5-4* mutants were selected on the basis of their ability to germinate and grow in otherwise inhibitory ABA concentrations (4). In the absence of ABA, seed germination and plant growth were indistinguishable between WT Ws and *abi5-4*. We hypothesized that ABI5 could act as a repressor of germination and growth, and that ABA might trigger its accumulation. To this end, we used Northern and Western blot analyses to investigate whether ABA would regulate *ABI5* transcript levels and protein expression during the first few days poststratification. ABA concentrations (3–10 μM) that discriminate between *abi5-4* mutants and WT under normal growth conditions were selected. WT seeds were plated on a germination medium in the presence or absence of 5 μM ABA and kept in the dark at 4°C for 3 days to break dormancy (stratification) before being transferred to 22°C under light (see *Materials and Methods*). As shown in Fig. 1*A*, ABI5 transcript was undetectable in the absence of ABA but was induced after one day in the presence of ABA. Fig. 1*B* shows that ABA also induced accumulation of ABI5. Indeed, by immunoblot analysis, the ABI5 protein with a molecular mass of about 50 *K*D was detected 2 days after stratification in the presence of ABA. This is in agreement with the predicted M_r of 47,000 for ABI5. Control experiments with *abi5-4* null mutants confirmed that this band is specific (data not shown). In the absence of ABA, this protein was absent (Fig. 1*B*) and undetectable even after prolonged film exposure (data not shown). In the presence of ABA, the protein level increased at least 10-fold during the first two days and remained constant thereafter. To rule out the possibility that the absence of the ABI5 protein might be due to

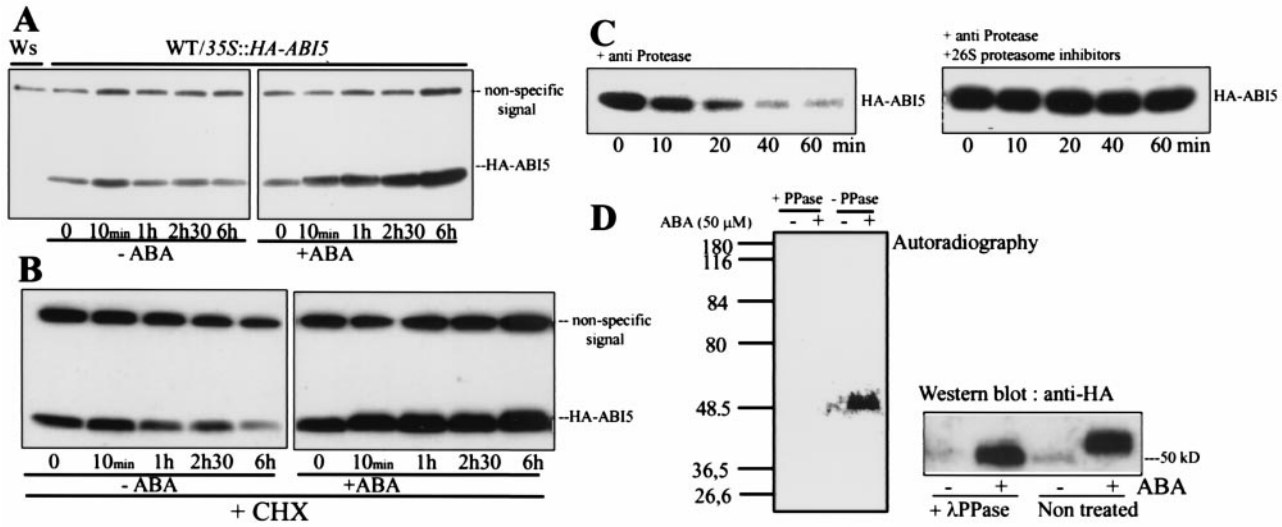

Fig. 2. ABA stabilizes ABI5 protein. (*A*) Eight-day-old Ws/*35S*::*HA-ABI5* seedlings (T3 generation) transferred to MS medium with or without 50 μM ABA. Proteins were extracted at the time points indicated and ABI5 protein levels were monitored by Western blot analysis using antibodies to HA. Each lane contained 5 μg protein. (*B*) Eight-day-old Ws/*35S*::*HA-ABI5* seedlings were incubated in liquid MS medium supplemented with 100 μM cycloheximide for 15 h. After the treatment, 50 μM ABA was added to the medium and proteins were extracted at the times indicated. Equal amounts (5 μg) of protein were loaded on a 10% SDS/polyacrylamide (NOVEX) gel and analyzed by Western blot using an anti-HA antibody. (*C*) Extracts prepared from 8-day-old Ws/*35S*::*HA-ABI5* seedlings were treated with a mixture of anti-protease (complete Mini from Amersham Pharmacia) supplemented with 1 mM PMSF with or without proteasome inhibitors ALLN, MG115, MG132, and PS1 (10 μM each, Calbiochem), as described in *Materials and Methods*. An equal volume of SDS-loading buffer was added to stop the reactions. Equal amounts of sample were then analyzed by Western blots using rabbit antibody to HA. (*D*) Eight-day-old Ws/*35S*::*HA-ABI5* transgenic seedlings were treated in liquid MS medium with [^{32}P]orthophosphoric acid (100 μCi) for 1 h 30 min. Seedlings were further treated with or without 50 μM ABA for 30 min. HA-ABI5 was immunoprecipitated by using rabbit antibody to HA coupled to beads. Beads were further treated with or without γ phosphatase. 90% of the sample was then analyzed by PhosphorImager (Bio-Rad); 10% of the sample was analyzed by Western blot using mouse monoclonal antibody to HA (Santa Cruz Biotechnology) as the first antibody.

a dilution caused by protein accumulation during the first five days without ABA, we compared total protein levels in *Arabidopsis* plants grown in the presence or absence of ABA for 6 days and found only a 2-fold difference in their protein content (data not shown). As this is insufficient to explain the observed differences in ABI5 accumulation level, we conclude that ABI5 accumulation is strongly induced by ABA.

ABA Stabilizes ABI5. Given the strong ABA-dependent ABI5 protein accumulation, we were interested to determine whether ABI5 protein stability is altered in the presence of ABA. For this purpose we generated WT/*35S*::*HA-ABI5* transgenic lines constitutively expressing *HA-ABI5* transcript. In these lines, HA-ABI5 accumulation is 5- to 10-fold higher in the presence of ABA as shown by immunoblot analysis using anti-HA antibodies (Fig. 2*A*). Similar results were obtained at all stages of development in *abi5-4*/*35S*::*ABI5* transformants when using antibodies to ABI5 (data not shown). This suggests that ABA can regulate ABI5 accumulation posttranscriptionally because the transgene RNA levels were unaffected by ABA (data not shown). Several mechanisms can be proposed to explain this observation, including an increase in protein synthesis and/or stability. To test the protein stability hypothesis, 8-day-old WT/*35S*::*HA-ABI5* transgenic seedlings were treated with the protein synthesis inhibitor cycloheximide for 15 h before addition of ABA (see *Materials and Methods*). Fig. 2*B* shows that ABA treatment prevented the decrease in ABI5 level observed in untreated plants, suggesting that ABA prevents ABI5 degradation.

To better understand the mechanism underlying this phenomenon, we measured ABI5 levels by using a cell-free assay (20). We observed a rapid degradation of ABI5 within 1 h even in the presence of a mixture of nonspecific protease inhibitors (Fig. 2*C*). However, this degradation can be prevented by inhibitors specific to the 26S proteasome (see *Materials and Methods*) suggesting that the latter is responsible for the degradation.

ABA Induces ABI5 Phosphorylation. In addition to the ABI5 accumulation effect observed on ABA exposure in WT/*35S*::*HA-ABI5* transgenic lines, we systematically observed a slight decrease in the mobility of the HA-ABI5 protein on SDS/PAGE Western blots when using rabbit polyclonal antibody to HA. This effect was rapid and was observed in seedlings treated with ABA at all developmental stages (data not shown). Indeed, after 10 min of ABA treatment, the mobility shift was already apparent and persisted for at least 48 h (data not shown). To test whether this mobility shift is due to ABA-triggered phosphorylation of ABI5, ABA treated 8-day-old seedlings were incubated with [^{32}P]orthophosphoric acid (see *Materials and Methods*) and antibodies to HA were used to immunoprecipitate HA-ABI5 protein. Subsequently, the immunoprecipitates were treated with recombinant lambda phosphatase (see *Materials and Methods*) and proteins were separated by SDS/PAGE for autoradiography and Western blot analysis. Fig. 2*D* shows that ABI5 is phosphorylated *in vivo* both in the presence and absence of ABA, although the specific activity of ABI5 does not increase on ABA exposure (Fig. 2*D* and data not shown). The phosphatase treatment was sufficient to remove the radiolabeling and to restore the mobility of the HA-ABI5 protein to that of HA-ABI5 in protein extracts of control plants not treated with ABA.

The ABA-Dependent ABI5 Accumulation Occurs Within a Limited Developmental Time Window Poststratification. To determine whether the observed ABA-dependent accumulation of ABI5 levels was restricted to early development, we stratified WT seeds in the absence of ABA and then transferred the seedlings at different time intervals to a medium supplemented with 5 μM ABA (Fig. 3*A*). Fig. 3*A* shows that ABI5 accumulation was

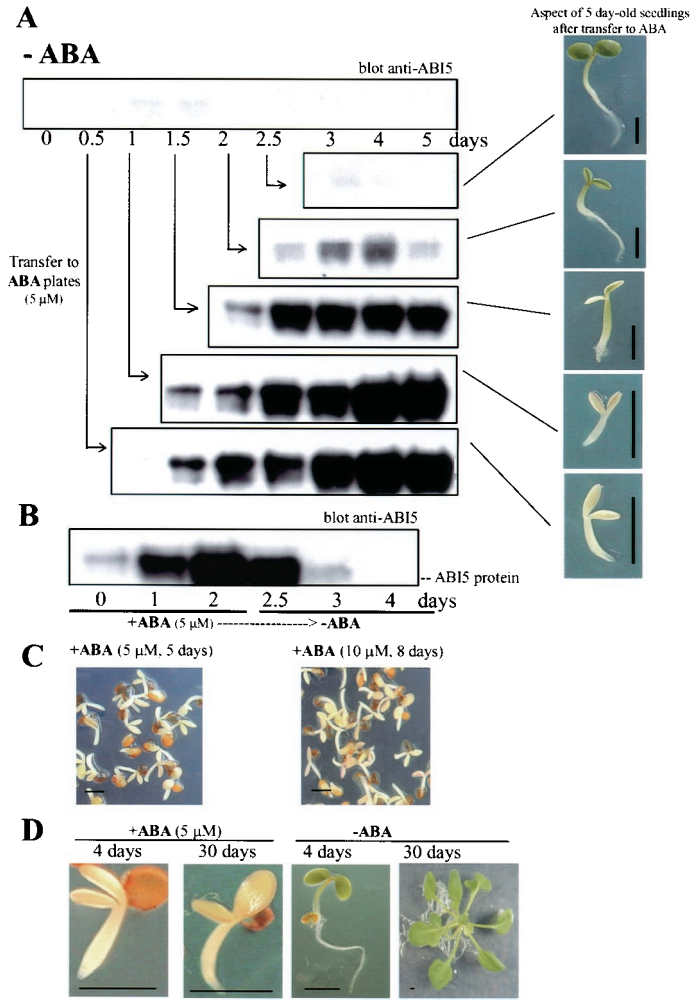

Fig. 3. ABA regulates ABI5 accumulation within a narrow developmental window. (*A*) WT Ws seeds were kept in darkness at 4°C for 3 days without ABA and then transferred to 5 μM ABA plates at the indicated times poststratification. ABI5 levels were monitored by Western blot analysis. Photographs depict representative seedlings 5 days after the different treatments. (Scale bar, 0.5 mm.) (*B*) ABI5 protein level rapidly decreased on ABA removal. WT Ws seeds treated with ABA (5 μM) as described in Fig. 1*B* were transferred to plates without ABA, 2 days poststratification. ABI5 levels were monitored by Western blot analyses as described in Fig. 1. (*C*) WT Ws seeds eventually germinated in the presence of ABA (5 and 10 μM). (Scale bar, 0.5 mm.) (*D*) WT Ws seedlings 4 and 30 days poststratification in the presence or absence of ABA (5 μM). (Scale bar, 0.5 mm.)

efficiently induced during a limited developmental window between 48 and 60 h poststratification. After 60 h, little or no induction was observed. Fig. 3*B* shows that ABA removal was accompanied by a rapid decrease in ABI5 levels.

ABA Efficiently Triggers Growth Arrest Within a Limited Developmental Time Window Poststratification. Given the ABA insensitivity of *abi5-4* mutant and the timing of ABI5 induction on ABA exposure, we studied the effect of ABA on growth during early development. Application of 5 μM ABA within 60 h poststratification did not prevent germination, but maintained the germinated embryos in an arrested state for several days, whereas ABA applied outside the 60-h time frame failed to arrest growth and prevent greening (Fig. 3*A*). Virtually all WT seeds were fully germinated on 5 μM ABA only 5 days poststratification (Fig. 3*C*). Similar results were obtained with different Ws seed batches and for the ecotypes Landsberg and Columbia (data not shown). At 10 μM ABA, complete germination was observed 8 days poststratification (Fig. 3*C*). Thereafter, ABA efficiently blocked further growth for at least a month (Fig. 3*D*). ABI5 levels remained constant as long as ABA was present (data not shown). During this period, almost all embryos (95% $\pm$ 3, n = 200) remained white and quiescent, and did not grow (data not shown). On ABA removal, all the embryos started to green and resumed normal growth (data not shown).

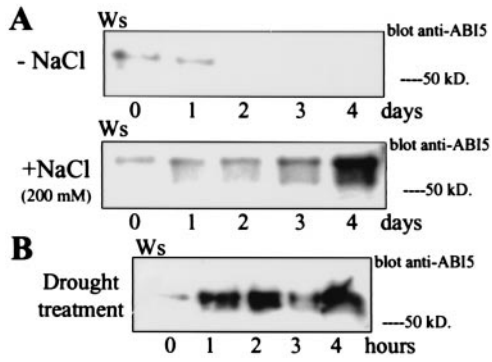

Fig. 4. ABI5 protein accumulation is induced by salt and drought stress. (*A*) WT Ws seeds were kept in darkness at 4°C for 3 days with or without NaCl (200 mM) and then transferred to constant light at 22°C for 1, 2, 3, 4, and 5 days (0 indicates the time immediately following transfer). Western blot analyses using antibodies to ABI5 were performed as described in Fig. 1. (*B*) One-day-old seeds were transferred to absorbing paper for 1, 2, 3, and 4 h. Western blot analyses using antibodies to ABI5 were further performed as described in Fig. 1.

Taken together, these results suggest that ABA is more efficient as an early growth inhibitor than as an inhibitor of germination. They also provide evidence that ABA, in addition to delaying germination, can also reversibly block growth during a narrow developmental time interval following germination and before the onset of vegetative growth.

ABI5 Expression Is Induced by Drought and High Salt Stress. Because ABA mediates stress responses in *Arabidopsis*, we examined ABI5 expression on water stress. ABI5 accumulation was measured in seeds treated with high salt concentrations or subjected to water deprivation. Both treatments resulted in a rapid and robust increase of ABI5 accumulation (Fig. 4).

Germinated and ABA-Arrested Mature Embryos Are Resistant to Extended Drought. The induction of ABI5 accumulation by drought suggested that this protein plays a protective role under this condition. To test this hypothesis, we broke embryo dormancy of WT and *abi5-4* seeds in the absence of ABA for 24 h. Seeds were then transferred for an additional 10 days to media with or without ABA. After 10 days, ABA-treated WT mature embryos had germinated but were arrested in growth as described above, whereas *abi5-4* or nontreated WT Ws plants were green and continued to grow (data not shown). Plants were then water-stressed for different time intervals on absorbent papers before being allowed to recover in normal medium. Table 1 shows that the majority of the quiescent WT mature embryos were able to survive after 36 h of water stress. This is in sharp contrast with *abi5-4* embryos, none of which survived. *abi5-4* plants were able to survive better with than without ABA (Table 1), although not to the same extent as WT. This observation probably reflects the fact that *abi5-4* (like all other *abi* mutants, data not shown) is not completely insensitive to ABA, suggesting that ABI5 is not the only protein mediating ABA-dependent processes early in development. Indeed, *abi5-4* plants grow more slowly on 5 μM ABA than on medium lacking it, whereas the

Table 1. Arrested and germinated embryos are more resistant to drought

	Percentage of survival after different period of drought treatment (h)				
	0	8	12	24	36
Ws					
−ABA	100	53	2	0	0
+ABA (5 μM)	100	100	98	90	84
abi5-4					
−ABA	100	50	0	0	0
+ABA (5 μM)	100	97	55	20	0

growth of WT plants is completely arrested. This developmental difference may be responsible for the higher resistance to water depletion of ABA-treated *abi5-4* plants (Table 1).

ABI5 Overexpressing Plants Are Hypersensitive to ABA. To determine whether the presence of ABI5 is sufficient to arrest growth in the absence of ABA, we expressed the *ABI5* gene from a constitutive promoter. Transgenic *Arabidopsis* lines expressing *ABI5* under the control of the cauliflower mosaic virus 35S promoter in WT Ws or *abi5-4* backgrounds were generated and several independent lines expressing various levels of ABI5 obtained. Despite the accumulation of ABI5, the lines germinated and developed normally in the absence of ABA (data not shown). In the transgenic line shown in Fig. 5*A*, ABI5 levels in absence of ABA were at least 5-fold higher than that in WT plants grown in the presence of ABA (data not shown). These results show that the presence of ABI5 is necessary, but not sufficient for growth arrest and that ABA is required for a further activation step.

We next investigated the effect of ABA in ABI5 overexpressing lines. Addition of 5 μM ABA severely delayed germination by at least 7 days and blocked mature embryo growth in several lines (data not shown). To measure more precisely the ABA response, we plated WT/*35S*::*HA-ABI5* transgenic seeds on growth media containing different ABA concentrations. Treatment of WT/*35S*::*HA-ABI5* plants with 0.5 μM ABA was sufficient to phenocopy the response of WT plants exposed to 5 μM ABA (Fig. 5*A*). At 0.5 μM ABA, WT plants grew normally (Fig. 5*A*). Inhibition of root growth by ABA was unaffected in *abi5-4* mutants (data not shown) but was exacerbated in ABI5-overexpressing lines (Fig. 5*B*). ABI5-overexpressing plants also retained water more efficiently than WT plants (Fig. 5*C*). These results show that transgenic plants overexpressing ABI5 are hypersensitive to ABA.

The Extent of Growth Arrest Is Correlated with ABI5 Protein Levels. We used *abi5-4* lines expressing the *35S*::*ABI5* transgene (*abi5-4*/*35S*::*ABI5*) to investigate the relationship between protein accumulation and the extent of growth arrest in response to ABA. Fig. 5*D* shows that *abi5-4*/*35S*::*ABI5* transgenic lines expressing intermediate ABI5 levels also displayed an intermediate phenotype in the presence of ABA. These results suggest that ABI5 is a rate-limiting factor conveying the ABA-mediated developmental growth arrest.

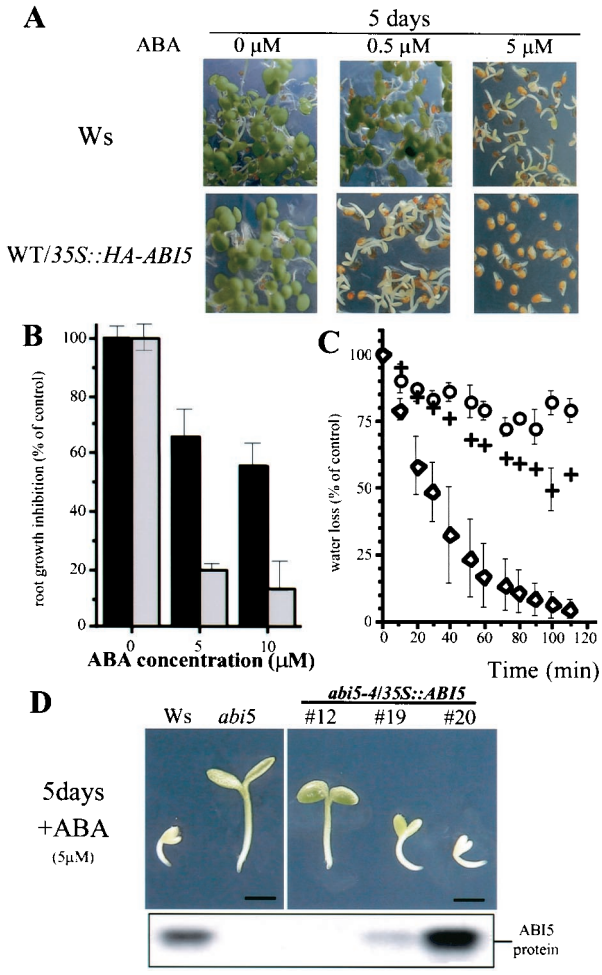

Fig. 5. Transgenic plants overexpressing ABI5 are hypersensitive to ABA. (*A*) WT Ws and Ws/*35S*::*HA-ABI5* transgenic plants were grown for 5 days with 0, 0.5, and 5 μM ABA. Transgenic seeds were arrested in development at 0.5 μM ABA and only started to geminate after 5 days. WT Ws seeds were insensitive to 0.5 μM ABA and were fully germinated in 5 μM ABA after 5 days. (*B*) Inhibition of root growth by exogenous ABA is exacerbated in Ws/*35S*::*HA-ABI5* transgenic lines (dotted bars) as compared with WT Ws plants (black bars). Five-day-old seedlings grown in absence of ABA were transferred to 0, 5, and 10 μM ABA plates for 5 days. The root length of ABA-treated seedlings was expressed as a percentage of nontreated controls ($n = 15$). Bars indicate standard deviations. (*C*) Ws/*35S*::*HA-ABI5* (open circles) transgenic plants retained water more efficiently. Young rosette leaves from WT Ws control (crosses) and transgenic plants at the same developmental stage (one month) were excised and weighed at different times indicated ($n = 3$). *abi1* measurements (lozenges) are included as a control for water loss. (*D*) Seeds from WT Ws, *abi5-4* and 3 independent *abi5-4* transgenic lines carrying a *35S*::*HA-ABI5* transgene were treated with 5 μM ABA as described in Fig. 1. The picture depicts representative seedlings after 5 days. Western blots using anti-ABI5 antibodies were performed on the same plants. Each lane contained 5 μg protein.

Discussion

Although the physiological roles of ABA in the establishment and maintenance of seed dormancy are well characterized, little is known about the mechanisms by which ABA signals these processes. In the classical ABA-insensitive screen (2, 3), researchers have exploited the inhibitory effect of applied ABA on germination and early growth following stratification. However, it is unclear what the physiological relevance of this effect is. It is reasonable to hypothesize that applying ABA may activate the same signal transduction pathways that promote and maintain dormancy during embryogenesis. However, it is equally reasonable to assume that the effect of ABA is in fact multifaceted when applied to mature embryos. This is suggested by the fact that ABI1 and ABI2 are primarily involved with ABA signaling in vegetative tissues, although *abi1* and *abi2* were recovered in the classical germination screen (2, 9, 22, 23).

Our work reveals additional levels of complexity underlying the transition of a dormant seed to a young seedling committed to auxotrophy. Indeed, we have delineated a narrow developmental window following stratification during which ABA reg-

ulates endogenous ABI5 protein accumulation. This accumulation seems to be due primarily to an increase in *ABI5* transcript abundance on ABA application, but ABA-mediated protein stabilization is also likely to contribute. We used ABA application to reveal that ABI5 serves as a molecular marker for a window period when seeds in their normal environment employ ABA responsiveness as an important adaptive mechanism. We suggest that shortly after stratification, winter annual plants such as *Arabidopsis*, which have already germinated, are still able to prevent adult growth if external environmental conditions suddenly become potentially fatal. Therefore, one can define at least two early growth checkpoints in *Arabidopsis*. Passage through the first checkpoint results in germination, as defined by the emergence of the radicle from the seed coat. After germination, a second checkpoint comes into play where water availability in the environment is monitored. Results presented here provide evidence that ABA and ABI5 are key players in the latter process. We found that the narrow developmental window allowing growth arrest to occur still operates in darkness (data not shown). This suggests that the window of responsiveness is not a simple consequence of a down regulation of ABA-mediated gene expression by light and strengthens the idea that the growth arrest is a bona fide adaptive response of *Arabidopsis*.

We present several lines of evidence supporting this hypothesis and the key role played by ABI5 in the growth arrest. First, in the concentration range (3–10 μM) used to isolate *ABI* mutants (2, 4, 5), we found that ABA is in fact more efficient as an early growth inhibitor than as an inhibitor of germination. Indeed, we have shown that in the presence of ABA, all seeds eventually fully germinate after 10 days, but that the germinated embryos remain quiescent for much longer. As soon as ABA is removed, growth and greening are initiated. Second, germinated and ABA-arrested embryos are more tolerant to water stress than control plants not treated with ABA. In nature, winter annual species such as *Arabidopsis* may be exposed to sufficiently cold and humid summer days to break dormancy and trigger germination, thereby bypassing the first checkpoint. The ABA-mediated growth arrest described here would then increase the chance of the germinated seedlings to survive in the event of a subsequent prolonged drought. Accordingly, *abi5-4* seeds were also able to germinate and green in 200 mM NaCl, suggesting that high salt concentration cannot arrest growth in these mutants (data not shown). Third, *ABI5* expression is regulated by drought and salt exposure. In WT plants, ABI5 abundance closely reflects environmental conditions during the 60 h following stratification. Within this time period, ABI5 levels increase on imposition of water stress and decrease on stress removal. Fourth, in addition to being necessary for the ABA-mediated growth arrest, ABI5 overexpression results in an enhanced response to ABA, suggesting that ABI5 limits transduction of a stress-responsive signal *in planta*.

Our work raises several questions for future investigation. It is unclear how ABI5 arrests growth on ABA treatment. Clearly, the cell cycle is arrested, but we do not know whether this is a direct or indirect effect of ABI5 on the cell-cycle machinery. ABI5 might control the expression of the recently reported (24) cyclin-dependent kinase inhibitor. Another area of future research is the mechanism of ABI5 activation by ABA. In the present work, we have shown that ABI5 accumulation *per se* is not sufficient to arrest growth because transgenic plants overexpressing ABI5 grow normally in the absence of ABA. ABI5 could be activated by recruitment of a partner protein, by nucleocytoplasmic shuttling, or by phosphorylation. In the latter case, the ABI5 mobility shift suggests that ABI5 is phosphorylated *de novo* on ABA exposure. However, the number of phosphate groups per ABI5 molecule appears to be unchanged. Therefore, our data do not rule out whether the mobility shift is a result of a novel and unique phosphorylation event on newly translated ABI5 or of a rearrangement of phosphate groups in a given ABI5 molecule. In the latter case, ABI5 would then be dephosphorylated and phosphorylated in novel sites *in vivo*.

Resolution of these issues and the identification of additional molecular components of the ABA response pathway will potentially open the way for the direct manipulation of seed germination and seedling hardiness. The ability to engineer the earliest and most critical events of the plant life cycle would not only have a substantial effect on agriculture (e.g., crop production or weed control), but might also be of considerable ecological significance.

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