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# Abscisic Acid Regulates Early Seed Development in *Arabidopsis* by ABI5-Mediated Transcription of *SHORT HYPOCOTYL UNDER BLUE1*[C][W][OPEN]

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**Seed development includes an early stage of endosperm proliferation and a late stage of embryo growth at the expense of the endosperm in *Arabidopsis thaliana*. Abscisic acid (ABA) has known functions during late seed development, but its roles in early seed development remain elusive. In this study, we report that ABA-deficient mutants produced seeds with increased size, mass, and embryo cell number but delayed endosperm cellularization. *ABSCISIC ACID DEFICIENT2* (*ABA2*) encodes a unique short-chain dehydrogenase/reductase that functions in ABA biosynthesis, and its expression pattern overlaps that of *SHORT HYPOCOTYL UNDER BLUE1* (*SHB1*) during seed development. *SHB1* RNA accumulation was significantly upregulated in the *aba2-1* mutant and was downregulated by the application of exogenous ABA. Furthermore, RNA accumulation of the basic/region leucine zipper transcription factor ABSCISIC ACID-INSENSITIVE5 (ABI5), involved in ABA signaling, was decreased in *aba2-1*. Consistent with this, seed size was also increased in *abi5*. We further show that ABI5 directly binds to two discrete regions in the *SHB1* promoter. Our results suggest that ABA negatively regulates *SHB1* expression, at least in part, through the action of its downstream signaling component ABI5. Our findings provide insights into the molecular mechanisms by which ABA regulates early seed development.**

## INTRODUCTION

Double fertilization in higher plants initiates the formation of seeds, which have a diploid embryo and a triploid endosperm that develop in concert. This developmental process consists of several stages in *Arabidopsis thaliana* (Baud et al., 2002). Following fertilization, the basic embryonic pattern becomes established, while the endosperm proliferates, undergoing a syncytial phase and a cellularization phase (Boisnard-Lorig et al., 2001). The timing of endosperm cellularization correlates with nuclear proliferation and therefore may influence seed size (Sundaresan, 2005; Kang et al., 2008). Indeed, precocious cellularization of the endosperm led to decreased nuclear proliferation and seed size reduction, whereas delayed cellularization promoted nuclear proliferation and increased seed size (Garcia et al., 2003, 2005; Luo et al., 2005). Following endosperm proliferation, in the early maturation stage, the embryo grows rapidly by absorbing nutrients from the endosperm (Baud et al., 2002). During the last stage, or late maturation stage, the embryo appears metabolically quiescent and becomes tolerant to desiccation (Baud et al., 2002), resulting in a mature seed containing the embryo, wrapped with an endosperm and an outside coat (Scott et al., 1998; Haughn and Chaudhury, 2005).

A number of genes that regulate integument development, embryonic pattern formation, and endosperm development have been described (Le et al., 2010; Belmonte et al., 2013). Mutants of *AUXIN RESPONSE FACTOR2* have large seeds through the promotion of cell division in the integument and other organs (Schruff et al., 2006). The transcription factors LEAFY COTYLEDON2 and FUSCA3 play critical roles in embryogenesis (Keith et al., 1994; Meinke et al., 1994). AGAMOUS-like 62 regulates endosperm cellularization, and its functional loss results in small seeds due to premature endosperm cellularization (Kang et al., 2008). The signaling regulator SURROUNDING REGION-RELATED8, together with WUSCHEL-LIKE HOMEOBOX8, coordinately modulates seed growth and size (Fiume and Fletcher, 2012). Functional loss of *HAIKU2* (*IKU2*) and *MINISEED3* (*MINI3*), encoding a receptor-like kinase and a WRKY transcription factor, respectively, also produces small seeds with precocious endosperm cellularization and reduced embryo growth (Luo et al., 2005). Recently, *IKU2* and *MINI3* were found to be in a genetic pathway regulated by *SHORT HYPOCOTYL UNDER BLUE1* (*SHB1*), whose gain-of-function mutants produce larger seeds due to enhanced endosperm proliferation and delayed endosperm cellularization (Zhou et al., 2009).

The phytohormone abscisic acid (ABA) mediates several aspects of seed development, such as biosynthesis of storage compounds in the embryo, seed dormancy, and the inhibition of precocious germination (McCarty, 1995; Finkelstein et al., 2002; Kanno et al., 2010). During seed development, ABA accumulation exhibits two peaks. During early seed maturation, ABA mainly

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originates from the maternal tissues and reaches its first maximal expression peak (Finkelstein et al., 2002; Finkelstein, 2004). Subsequently, ABA biosynthesis initiates in the embryo, and its levels accumulate to reach its second peak during the late maturation stage.

Earlier studies identified a number of ABA biosynthetic genes that are expressed during seed development. For instance, *ABSCISIC ACID DEFICIENT1* (*ABA1*) encodes a zeaxanthin epoxidase that functions in ABA biosynthesis and is expressed ubiquitously in *Arabidopsis* seed (Audran et al., 2001). The enzymes 9-*cis*-epoxycarotenoid dioxygenases catalyze the cleavage of 9-*cis*-epoxycarotenoids to xanthoxin, the key regulatory step in ABA biosynthesis. *Arabidopsis* NCED6 is one of the nine 9-*cis*-epoxycarotenoid dioxygenases required for ABA biosynthesis during seed development (Lefebvre et al., 2006). *Arabidopsis ABA2* encodes a unique short-chain dehydrogenase/reductase that specifically catalyzes the conversion of xanthoxin to abscisic aldehyde during ABA biosynthesis (Cheng et al., 2002). The *aba2* mutant exhibits typical ABA-deficient phenotypes, such as decreased seed dormancy, early flowering, and growth retardation under stress conditions (Léon-Kloosterziel, et al., 1996; Zhou et al., 1998).

ABA signaling consists of three layers including ABA metabolism and transport, signal perception and transduction, as well as signal response and modulation (Hauser et al., 2011). ABA perception and signal transduction depend on a core signaling pathway comprising three classes of proteins, including the PYRABACTIN RESISTANCE (PYR)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) (Ma et al., 2009; Park et al., 2009), PROTEIN PHOSPHATASE 2C (PP2C) (Schweighofer et al., 2004), and SNF1-RELATED PROTEIN KINASE2 (SnRK2) kinases (Mustilli et al., 2002; Yoshida et al., 2006; Umezawa et al., 2009). In this core pathway, PYR/RCAR acts as an ABA receptor, while PP2Cs and SnRK2s act as negative or positive regulators, respectively. The formation of PYR/RCAR-PP2C complexes inhibits the activity of the PP2Cs, thereby activating SnRK2s, which target the AREB/ABF-type basic/region leucine zipper (bZIP) transcription factors (Hubbard et al., 2010). These transcription factors bind to the ABA response elements (ABREs; ACGT) to mediate the expression of their downstream genes. Mutations in the bZIP transcription factor ABI5 caused pleiotropic defects characteristic of ABA insensitivity (Finkelstein and Lynch, 2000).

Although many of the biochemical and physiological aspects of ABA have now been characterized, its involvement in early seed development remains unclear. In our study, we found that ABA negatively mediates seed size by influencing the timing of endosperm cellularization. Furthermore, we demonstrated that the ABA signaling component ABI5 directly binds to the promoter region of *SHB1* during early seed development. Our results thus showed that ABA regulates early seed development by ABI5-regulated *SHB1* expression.

## RESULTS

### ABA-Deficient Mutants Exhibit Increased Seed Size and Mass

To further elucidate the relationship between ABA and seed development, we closely examined the phenotypes of the ABA-deficient mutant *aba2-1*. Seeds of *aba2-1* were larger in size at both early green maturation and late dry maturation stages compared with the wild type (Figures 1A to 1E). Next, we analyzed the average mass of the *aba2-1* mutant seeds compared with the wild type by weighing batches of 100 mature dry seeds. The seed mass of *aba2-1* mutant was significantly greater than the wild type (Figure 1F). In addition to its seed phenotypes, the *aba2-1* mutant also exhibited phenotypes characteristic of ABA deficiency, including early flowering and developmental retardation (Léon-Kloosterziel et al., 1996; Cheng et al., 2002). Similar phenotypes were also observed in its allelic mutant *aba2-3* (Supplemental Figures 1A and 1B). To confirm the involvement of ABA in seed development, we characterized two more ABA-deficient mutants, *aba1* and *nced6*, and found out that, similar to *aba2*,

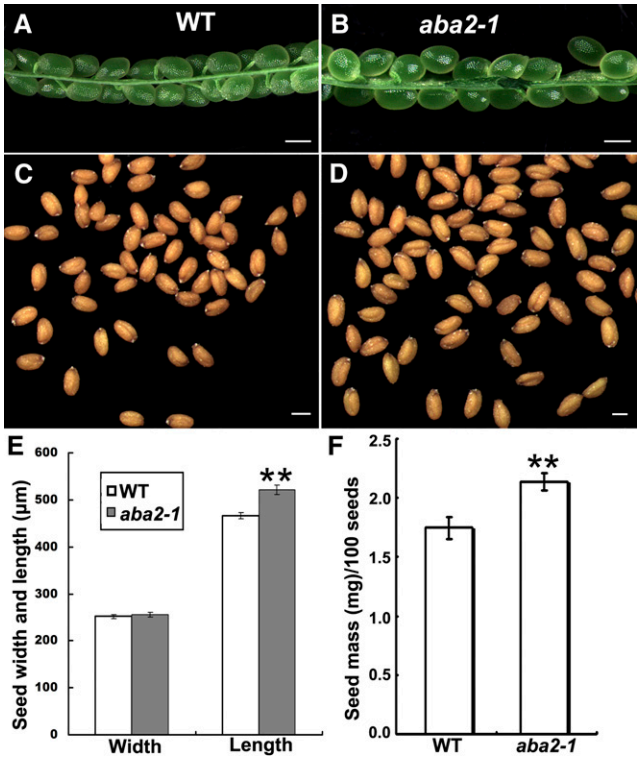


**Figure 1.** Mutation of *ABA2* Results in an Increased Seed Size and Mass.

**(A)** Mature green seeds of the wild type.
**(B)** Mature green seeds of *aba2-1*.
**(C)** Mature dry seeds of the wild type.
**(D)** Mature dry seeds of *aba2-1*.
**(E)** Average seed width and length for the wild type and *aba2-1*.
**(F)** Average seed mass per 100 seeds for the wild type and *aba2-1*. The error bars in **(E)** and **(F)** represent standard errors, which were calculated from three sets of biological replicates. In each replicate, at least 100 seeds were examined. Asterisks denote Student's *t* test significant difference compared with wild-type plants, with two asterisks denoting $P < 0.01$. Bars = 500 μm.
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both mutants produced larger seeds than the wild type (Supplemental Figures 1C to 1F).

As *aba2-1* exhibited the most pronounced phenotype in terms of seed size among the ABA-deficient mutants examined, we chose this mutant for further analyses. We examined the ABA content in siliques of *aba2-1* through radioimmunoassay analysis. As shown in Figure 2A, the ABA content in *aba2-1* siliques was significantly lower than that of the wild type at 3 and 6 d after pollination (DAP). Next, we confirmed the ABA content using liquid chromatography–tandem mass spectrometry. The results also indicated that the ABA content in *aba2-1* siliques was lower than that of the wild type (Supplemental Figure 2). The *aba2-1* mutant also exhibited retardation of silique elongation (Figure 2B). We hypothesized that if a deficiency in ABA accumulation causes the variation of the seed size in *aba2-1*, then exogenous ABA would rescue this phenotype. To test this hypothesis,

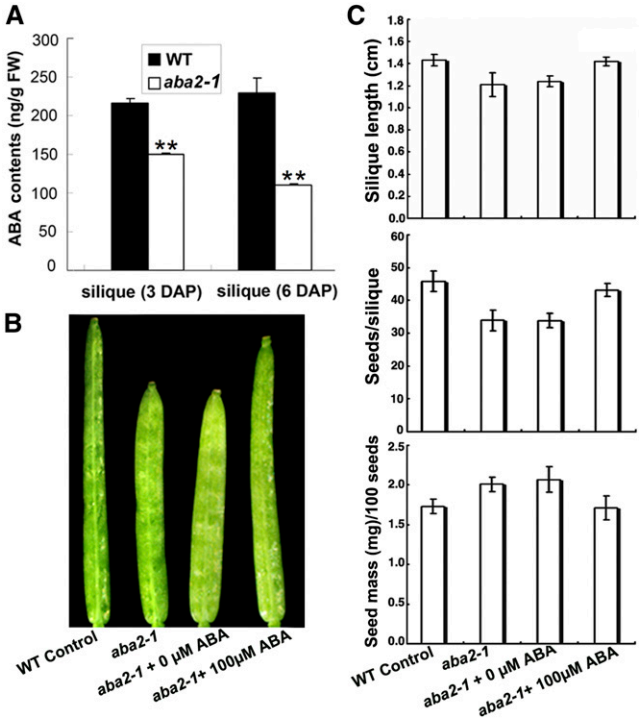


**Figure 2.** Mutation of *ABA2* Reduces the ABA Contents of Seeds.

**(A)** ABA contents in the siliques of the wild type and *aba2-1* as determined by immunoassay. About 50 mg of fresh sample from each genotype was used in each experiment. The data are the mean ± SD of three biological replicates. Asterisks denote Student's *t* test significant difference compared with wild-type plants, with two asterisks denoting $P < 0.01$. FW, fresh weight.

**(B)** Application of 100 μM ABA rescues the silique-length phenotypes of *aba2-1*.

**(C)** Silique length, seed number per silique, and seed mass per 100 seeds of *aba2-1* after treatment with 100 μM ABA. The error bars represent SE.

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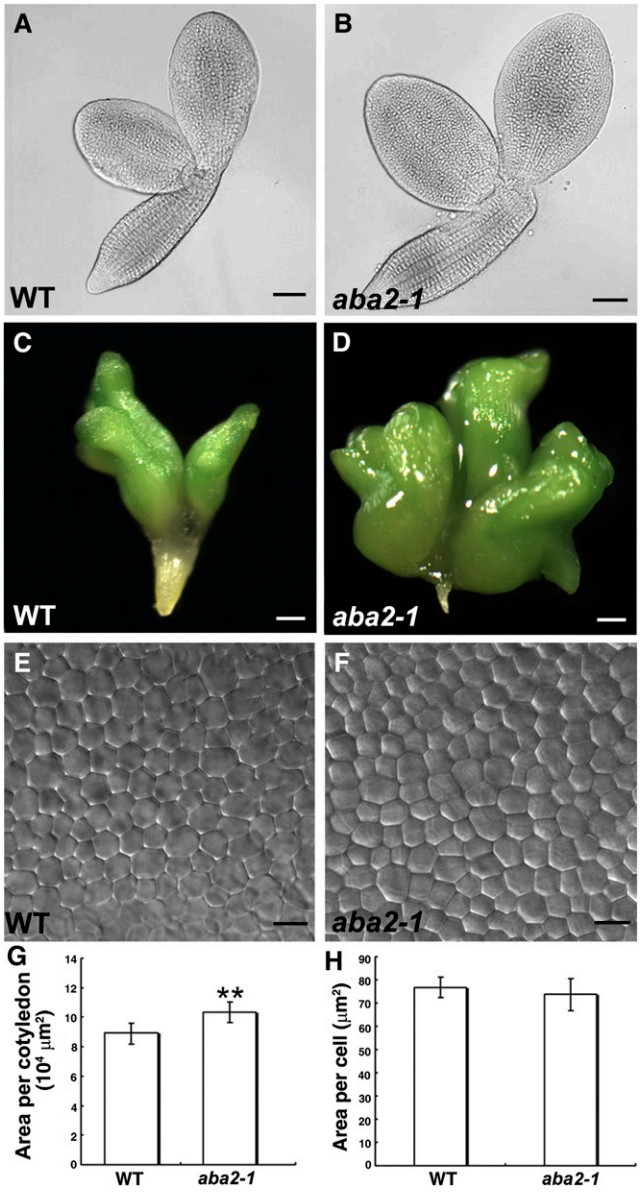


**Figure 3.** Increased Cell Number Causes the Enlarged Embryo of *aba2-1* Seeds.

**(A)** and **(B)** Mature embryos from the wild type **(A)** and *aba2-1* **(B)** imaged using differential interference contrast optics.

**(C)** and **(D)** Somatic embryos regenerated from the callus of the wild type **(C)** and *aba2-1* **(D)** using immature zygotic embryos as explants.

**(E)** and **(F)** Epidermal cells in the central regions of wild-type **(E)** and *aba2-1* **(F)** cotyledons observed under a scanning electron microscope.

**(G)** Comparison of cotyledon areas between the wild type and *aba2-1*.

**(H)** Comparison of epidermal cell areas in the cotyledons between the wild type and *aba2-1*. The data presented in **(G)** and **(H)** are the means ± SE from at least 50 independently collected seeds from the wild type or *aba2-1*. Asterisks denote Student's *t* test significant difference compared with wild-type plants, with two asterisks denoting $P < 0.01$.

Bars = 50 μm in **(A)** to **(D)** and 10 μm in **(E)** and **(F)**.

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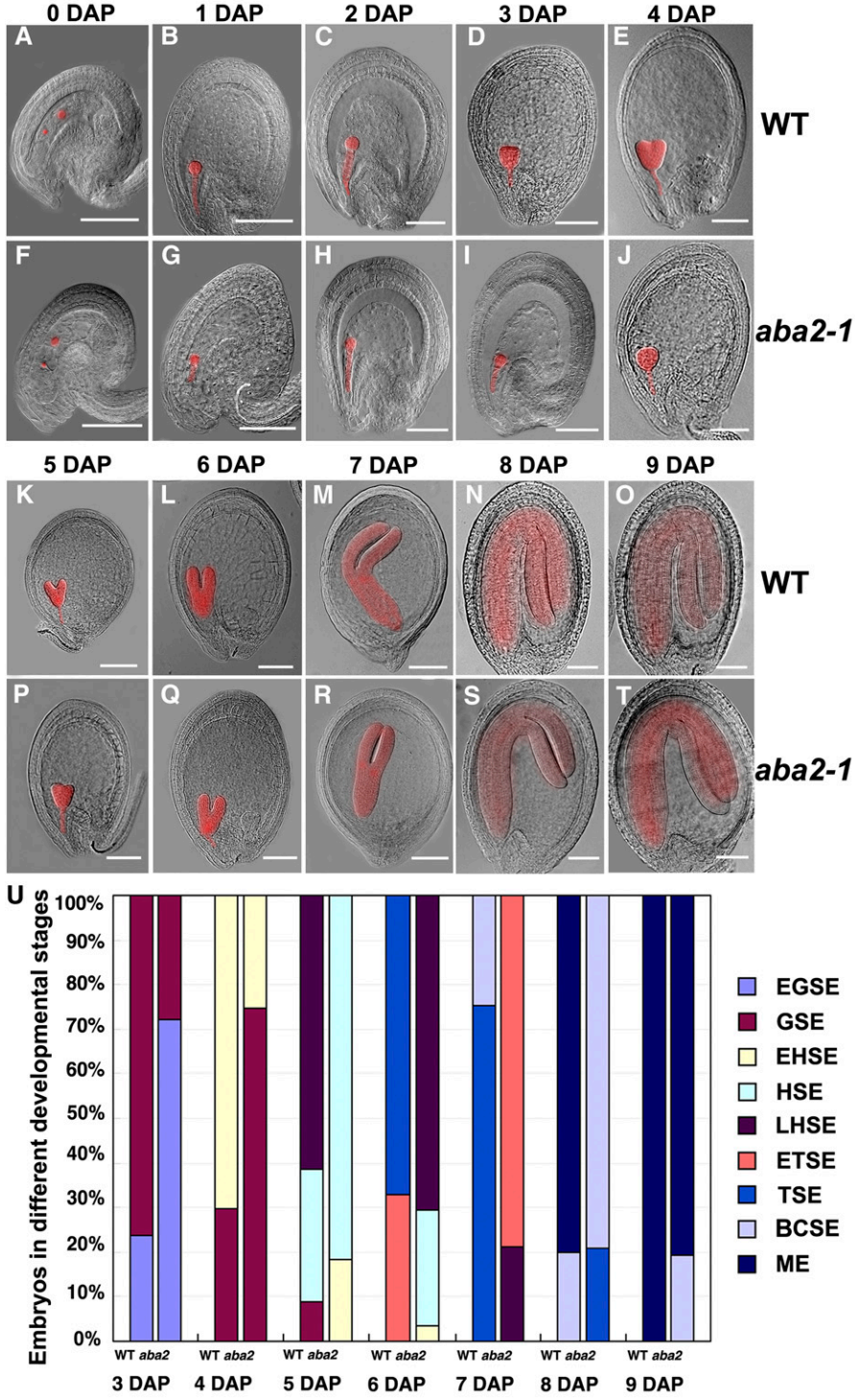


**Figure 4.** Seed Embryo Development Is Delayed in *aba2-1* Mutants.

**(A)** to **(T)** Whole-mount seeds were observed with differential interference contrast optics at different stages of embryogenesis in the wild type (**[A]** to **[E]** and **[K]** to **[O]**) and *aba2-1* (**[F]** to **[J]** and **[P]** to **[T]**). Bars = 50 μm.

**(U)** Percentage of embryos at different stages was recorded for the wild type and *aba2-1*, and at least 50 seeds were examined for each genotype. EGSE, early globular stage embryo; GSE, globular stage embryo; EHSE, early heart stage embryo; HSE, heart stage embryo; LHSE, late torpedo stage embryo; ETSE, early torpedo stage embryo; TSE, torpedo stage embryo; BCSE, bent cotyledon stage embryo; GE, green stage embryo. Developing embryos were pseudo-colored in red.

100 μM exogenous ABA was applied daily to the developing siliques of *aba2-1* after pollination. By 9 DAP, the silique lengths of the ABA-treated *aba2-1* were comparable to that of the wild type (Figure 2B). Statistical data revealed that both the silique length and the seed number per silique in *aba2-1* increased to wild-type levels after ABA treatment (Figures 2C). Interestingly, the seed mass of *aba2-1* was also reduced to wild-type levels by ABA treatment (Figure 2C). These results indicated that seed size increase in *aba2-1* was indeed due to ABA deficiency.

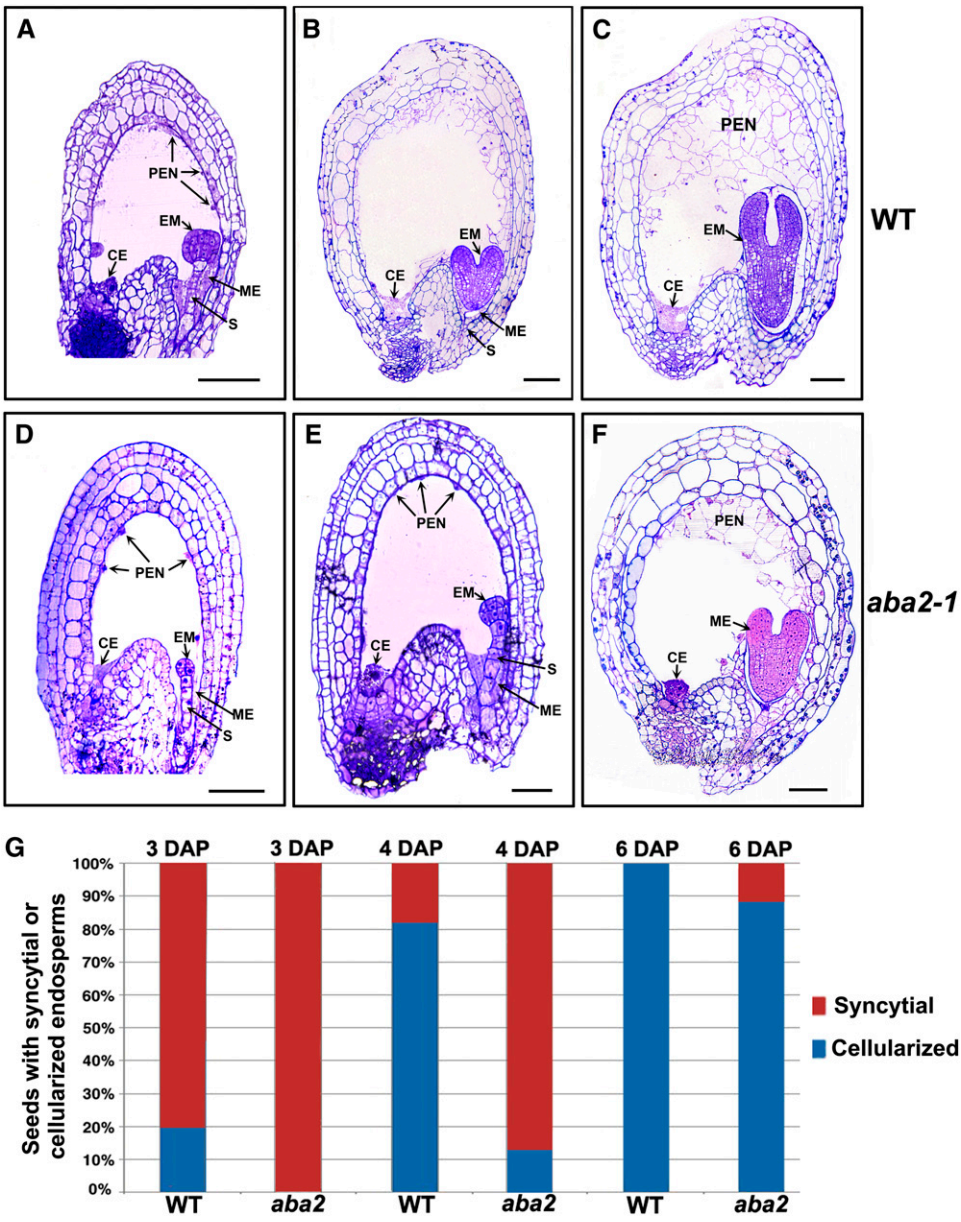


**Figure 5.** Endosperm Cellularization Is Delayed in *aba2-1*.

**(A)** Wild-type seed at 3 DAP showing the uncellularized endosperm and embryo at the mid-globular stage.
**(B)** Wild-type seed at 4 DAP showing a partially cellularized endosperm.
**(C)** Wild-type seed at 6 DAP showing a mid-torpedo stage embryo and almost 70% of cellularized endosperm.
**(D)** *aba2-1* seed showing uncellularized endosperm and embryo at the preglobular stage.
**(E)** *aba2-1* seed at 4 DAP showing an increased seed cavity and delayed endosperm cellularization.
**(F)** *aba2-1* seed at 6 DAP showing a late-heart stage embryo and only 40% of cellularized endosperm.
**(G)** Percentage of seeds with syncytial or cellularized endosperms was recorded. At least 50 endosperms were examined at each time point.
EM, embryo; PEN, peripheral endosperm; CE, chalazal endosperm; En, endosperm; ME, micropylar endosperm; S, suspensor. Bars = 50 μm.

### Seed Size Increase in *aba2-1* Is Due to Increased Embryonic Cell Number

The embryo occupies the majority of the mature *Arabidopsis* seed volume. To identify the cellular basis of the enlarged seeds in *aba2-1*, we compared embryo sizes between *aba2-1* and wild-type seeds. The cotyledons, embryonic shoots, hypocotyls, and radicle in *aba2-1* were all larger than those of wild-type seeds (Figures 3A, 3B, and 3G). We also compared the size of the somatic embryo regenerated from *aba2-1* or from the wild type. By culturing the explants on medium containing 2,4-D for 10 d, we regenerated several green somatic embryos from the explants (Su et al., 2009). Interestingly, somatic embryos regenerated from *aba2-1* were larger than those from the wild type (Figures 3C and 3D), indicating that *ABA2* mutations also affected embryo development in vitro. We further measured the size of epidermal cells from cotyledons of *aba2-1* or the wild type (Figures 3E, 3F, and 3H). We did not find a significant difference in the size of the cotyledon cells between *aba2-1* and the wild type (Figures 3E, 3F, and 3H), suggesting that the enlarged seed size of *aba2-1* resulted from enhanced cell division rather than cell elongation in the embryo.

### Mutation of *ABA2* Results in Delayed Embryogenesis and Endosperm Cellularization

We further examined the development of the endosperm and embryo in *aba2-1*. The development of *aba2-1* embryos was delayed compared with the wild type, although the mutant embryos displayed normal morphology (Figures 4A to 4T). At 1 and 2 DAP, when wild-type embryos were at 8-cell and 16-cell stages (Figures 4B and 4C), the *aba2* embryos were at 1-cell and 4-cell stages (Figures 4G and 4H), respectively. At 3 DAP, ~80% of wild-type embryos had reached the globular stage (Figures 4D and 4U), whereas ~70% of *aba2-1* embryos were still at the early globular stage (Figures 4I and 4U). At 4 DAP, ~80% of

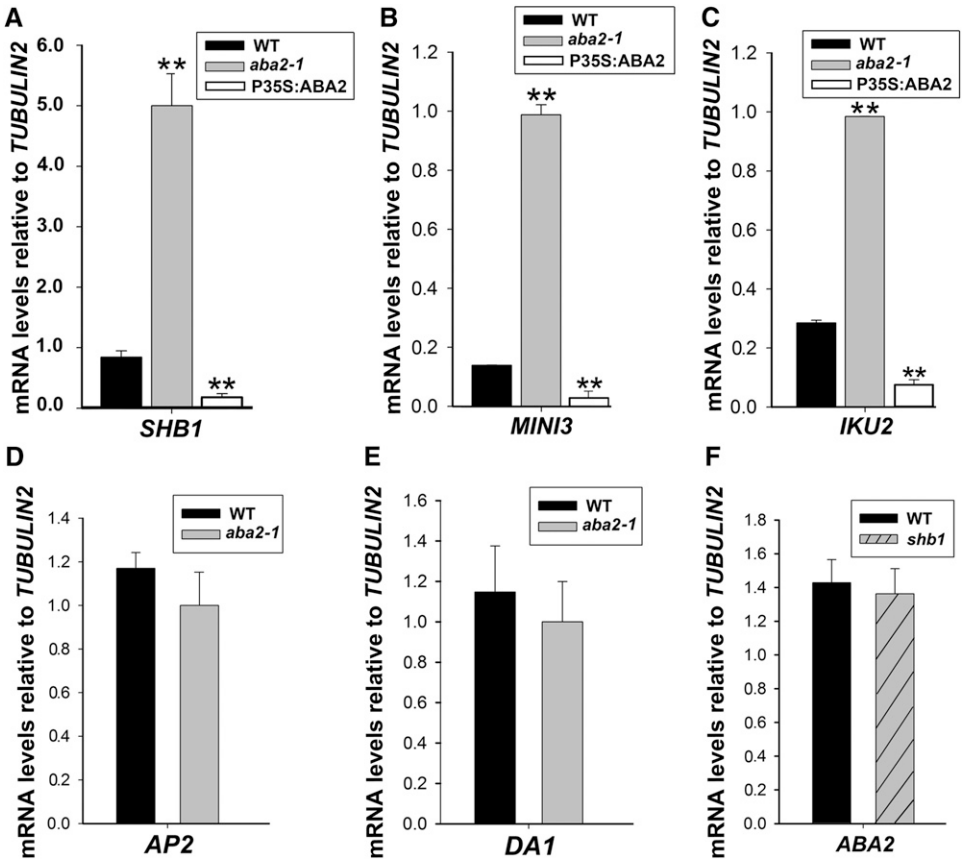


**Figure 6.** Increased RNA Accumulation of *SHB1*, *MINI3*, and *IKU2* in *aba2-1* Mutants.

**(A)** RNA accumulation of *SHB1* in wild-type, *aba2-1*, and P35S:ABA2 transgenic plants by qRT-PCR.
**(B)** and **(C)** RNA accumulation of *MINI3* **(B)** or *IKU2* **(C)** was upregulated in *aba2-1* but downregulated in the P35S:ABA2 transgenic plants by qRT-PCR.
**(D)** and **(E)** RNA accumulation of *AP2* and *DA1* was not significantly altered in *aba2-1*.
**(F)** RNA accumulation of *ABA2* was not changed in *shb1*. Seeds separated from siliques at 6 DAP were used for qRT-PCR analyses. RNA accumulation of each gene was normalized to that of *TUBULIN2*. RNA accumulation data shown are the mean values of three biological replicates, each of which biological replicate was examined in triplicates. The error bars represent SE. Asterisks denote Student's *t* test significant difference compared with wild-type plants, with two asterisks denoting $P < 0.01$.

*aba2-1* embryos had reached the globular stage (Figures 4J and 4U), whereas wild-type embryos had already developed into the early-heart stage (Figures 4E and 4U). From 5 to 8 DAP, *aba2-1* embryos still showed delayed development (Figures 4K to 4N and 4P to 4S). In addition, wild-type embryos reached the green embryo stage at 9 DAP (Figures 4O and 4U), but *aba2-1* embryos did not show any green color at the same time point (Figures 4T and 4U). Furthermore, the wild-type embryo reached the mature green embryo stage at 10 DAP, while the *aba2-1* embryos reached the same stage at 12 DAP (Supplemental Figures 3A, 3E, and 3F). As seed developed, wild-type seeds reached dry seed stage at 14 DAP (Supplemental Figures 3B and 3C), but the *aba2-1* seed did not reach this stage (Supplemental Figures 3G). At 16 DAP, 2 d after the wild-type seed dried (see Supplemental Figure 3D), *aba2-1* seeds reached the dry seed stage (Supplemental Figures 3H), and the dry seeds of *aba2-1* were larger than those of the wild type (cf. Supplemental Figure 3H to Supplemental Figures 3C and 3D).

A previous study revealed an association between embryo development and endosperm cellularization in seeds (Zhou et al., 2009). We thus examined the development of the endosperm in *aba2-1* seeds. When the wild-type embryos were at the mid-globular stage at 3 DAP, the endosperm began to cellularize in ~20% of wild-type seeds (Figures 5A and 5G), whereas the endosperms of *aba2-1* were still syncytial (Figures 5D and 5G). By 4 DAP, the wild-type embryos were at the heart stage, and nearly all of the endosperms were cellularized (Figures 5B and 5G). However, most of the *aba2-1* endosperms were still in the syncytial phase (Figures 5E and 5G). At 6 DAP, the total number of nuclei was increased in *aba2-1* endosperm compared with the wild type (Figures 5C, 5F, and 5G). It was shown previously that the size of the endosperm may be regulated through the timing of its cellularization (Zhou et al., 2009). Consistent with this, our results indicated that delayed endosperm cellularization in *aba2-1* caused an increase in nuclear proliferation.

### ABA Negatively Regulates *SHB1* RNA Accumulation

A number of genes involved in seed size control were previously described, such as *SHB1*, *MINI3*, *IKU2*, *DA1*, and *APETALA2* (*AP2*) (Jofuku et al., 2005; Luo et al., 2005; Ohto et al., 2005; Li et al., 2008). To determine their genetic interactions with *ABA2*, we first examined their RNA accumulation using quantitative RT-PCR (qRT-PCR). As shown in Figure 6, RNA accumulation of *SHB1*, *MINI3*, and *IKU2* was significantly increased in *aba2-1* but reduced in the P35S:ABA2 transgenic lines compared with the wild type (Figures 6A to 6C). The RNA accumulation of other seed-regulating genes, such as *AP2* and *DA1*, did not change significantly (Figures 6D and 6E). On the other hand, RNA accumulation of *ABA2* was not affected in *shb1* (Figure 6F). These results indicated that *ABA2* regulates seed development likely through *SHB1*.

Because *aba2* is an ABA-deficient mutant, we considered the possibility that *SHB1* might be negatively regulated by ABA biosynthesis. To test this possibility, we performed qRT-PCR analysis to examine the *SHB1* RNA accumulation in developing seeds upon exogenous ABA treatment. In wild-type control siliques, the first peak of *SHB1* RNA accumulation was detected at 1 DAP followed by a slight decrease at 3 DAP (Figure 7A). Subsequently, the *SHB1* transcript level increased at 6 DAP and reached its second peak at 9 DAP with a total of a 5-fold increase from 0 to 9 DAP in control siliques (Figure 7A). However, the *SHB1* RNA accumulation peaks disappeared upon treatment with 100 μM ABA (Figure 7A). Then, we examined the *SHB1* RNA accumulation in seedlings after 10 μM ABA treatment, speculating that they may also be sensitive to exogenous ABA. Interestingly, ABA treatment also decreased the accumulation of *SHB1* RNA (Figure 7B). Hence, the suppressed *SHB1* RNA accumulation by ABA is consistent with the enhanced *SHB1* RNA accumulation in the *aba2-1* mutant, suggesting that ABA negatively regulates *SHB1* expression.

To determine the genetic relationship between *ABA2* and *SHB1*, we generated the *aba2-1 shb1* double mutant. Introducing *shb1* completely suppressed the seed size increase of *aba2-1* such that seed width, length, and mass of the double mutant resembled those of *shb1* (Figures 8A to 8C). These results suggested that *SHB1* is genetically epistatic to *ABA2* in seed size control.

### The *ABA2* Expression Pattern Overlaps with That of *SHB1* during Seed Development

Due to the genetic epistasis between *ABA2* and *SHB1* in seed size control, we speculated that their expression patterns during seed development might overlap. Our qRT-PCR analysis showed

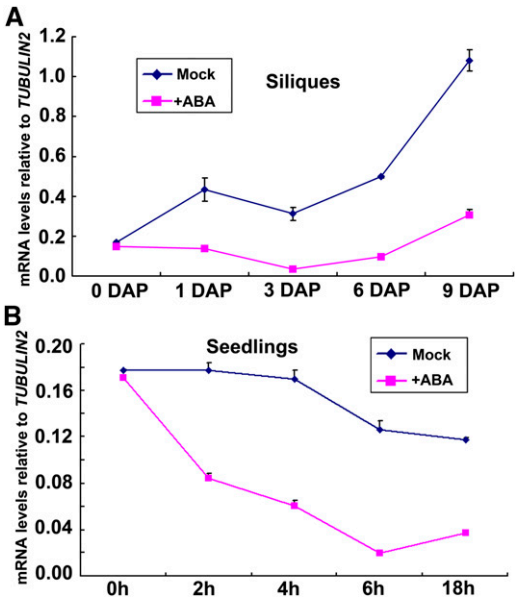


**Figure 7.** *SHB1* RNA Accumulation Is Inhibited by Exogenous ABA Application.

**(A)** RNA accumulation of *SHB1* was decreased upon ABA treatment by real-time qRT-PCR. Siliques were sprayed with 100 μM ABA at 0, 1, 3, 6, and 9 DAP.

**(B)** Ten-day-old wild-type seedlings grown on MS medium were treated with or without 10 μM ABA for 2, 4, 6, and 18 h. RNA accumulation of *SHB1* was normalized to that of *TUBULIN2*. RNA accumulation data shown are the mean values of three biological replicates, each of which biological replicate was examined in triplicates. The error bars represent SE.

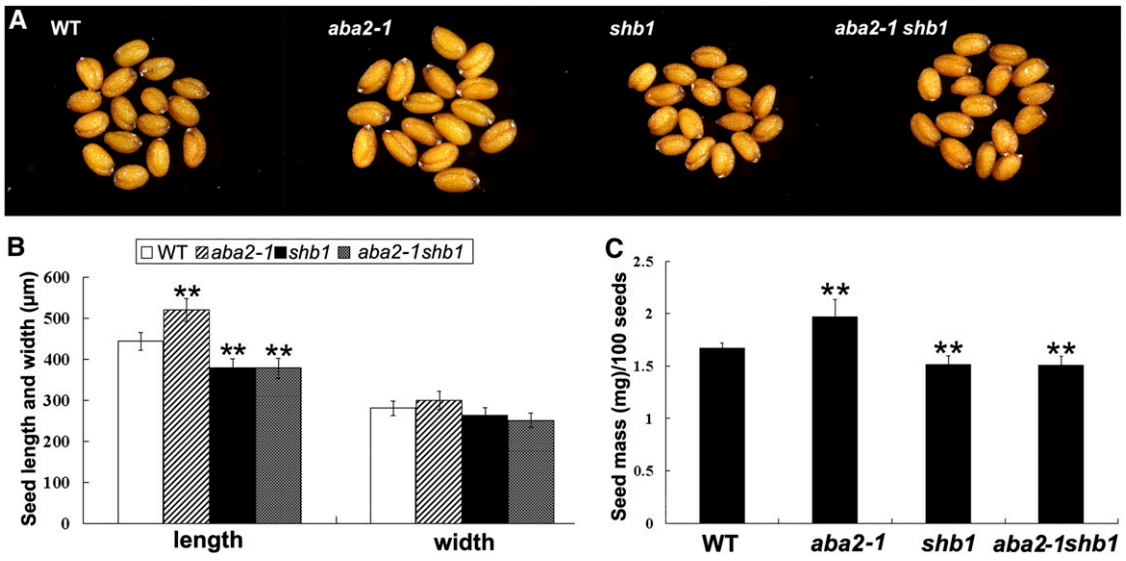


**Figure 8.** *shb1* Suppresses Seed Enlargement of *aba2-1*.

**(A)** Images of mature seeds of the wild type, *aba2-1*, *shb1*, and *aba2-1 shb1* double mutants.
**(B)** Seed length and width of the wild type, *aba2-1*, *shb1*, and *aba2-1 shb1* double mutants.
**(C)** Seed mass per 100 seeds of the wild type, *aba2-1*, *shb1*, and *aba2-1 shb1* double mutant.
The data in **(B)** and **(C)** are the means ± SE calculated from at least 50 seeds independently collected from the wild type and mutants. Asterisks denote Student's *t* test significant difference compared with wild-type plants, with two asterisks denoting $P < 0.01$.
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that *ABA2* is highly expressed in developing seeds (Supplemental Figure 4). We further examined the localization of *ABA2* transcripts in developing seeds using in situ hybridization and β-glucuronidase (GUS) staining with the PABA2:GUS reporter lines. *ABA2* transcripts were detected in unfertilized ovules (Figure 9A) and embryos immediately after pollination (Figures 9B to 9G and 9H). At 2 and 4 DAP, *ABA2* expression was clearly detected in the preglobular and globular embryos, as well as in the endosperm (Figures 9B and 9C). At a later stage, *ABA2* was found to be mainly expressed in the heart and torpedo embryos and in the cellularized endosperm (Figures 9D to 9F). In the green seeds, *ABA2* expression was evident in the whole embryo (Figure 9G). The GUS staining clearly showed that *ABA2* transcripts accumulated in both the endosperm and the embryo during early seed development (Figures 9I to 9M). It was reported that *SHB1* was expressed in developing and cellularized endosperm and in embryos from globular to torpedo stages (Zhou et al., 2009). The overlapping expression patterns of *ABA2* and *SHB1* provide further evidence that they function in the same pathway during seed development.

### ABI5 Mediates the Negative Regulation of *SHB1* RNA Accumulation by ABA

To explore the underlying mechanisms by which ABA regulates the expression of *SHB1* during seed development, we analyzed the promoter sequences of *SHB1* and found seven ACGT-containing ABREs within its 2.6-kb promoter region (Figure 11A). ABRE is a *cis*-acting element recognized by several bZIP transcription factors that function in ABA signal transduction, such as ABI5, AREB1, AREB2, and ABF3 (Finkelstein and Lynch, 2000). These bZIP transcription factors mediate downstream gene expression in *Arabidopsis* upon binding to the ABRE (Furihata et al., 2006; Yoshida et al., 2010). These findings suggest that the expression of *SHB1* may be regulated by bZIP transcription factors involved in ABA signaling. To determine this possibility, we investigated the transcript levels of *ABI5*, *AREB1*, *AREB2*, and *ABF3* in developing seeds of *aba2* and the wild type using qRT-PCR. We found that the transcript levels of all four genes were downregulated in *aba2* but that *ABI5* was the most significantly suppressed gene (Supplemental Figure 5). Correspondingly, we found that functional loss of *ABI5* resulted in seed size increase (Figures 10A to 10C). In addition, *SHB1* RNA accumulation was significantly enhanced or reduced in *ABI5* loss- or gain-of-function siliques, respectively (Figure 10D), implying that ABI5 might be the ABA-responsive negative regulator for *SHB1*.

To investigate whether ABI5 directly binds to the promoter of *SHB1*, we performed a chromatin immunoprecipitation (ChIP) assay followed by qRT-PCR analysis. We monitored the enrichment of different fragments of the *SHB1* promoter using ChIP analyses (Figure 11A) and found that S3 and S5 fragments, which span the −531 to −830 and −1707 to −2020 regions, respectively, were strongly enriched in experiments with either anti-ABI5 or anti-MYC antibodies. The S1 fragment, which spans the region from 0 to −233 in the *SHB1* promoter, was moderately enriched in experiments using both anti-ABI5 and anti-MYC antibodies. By contrast, we did not detect any

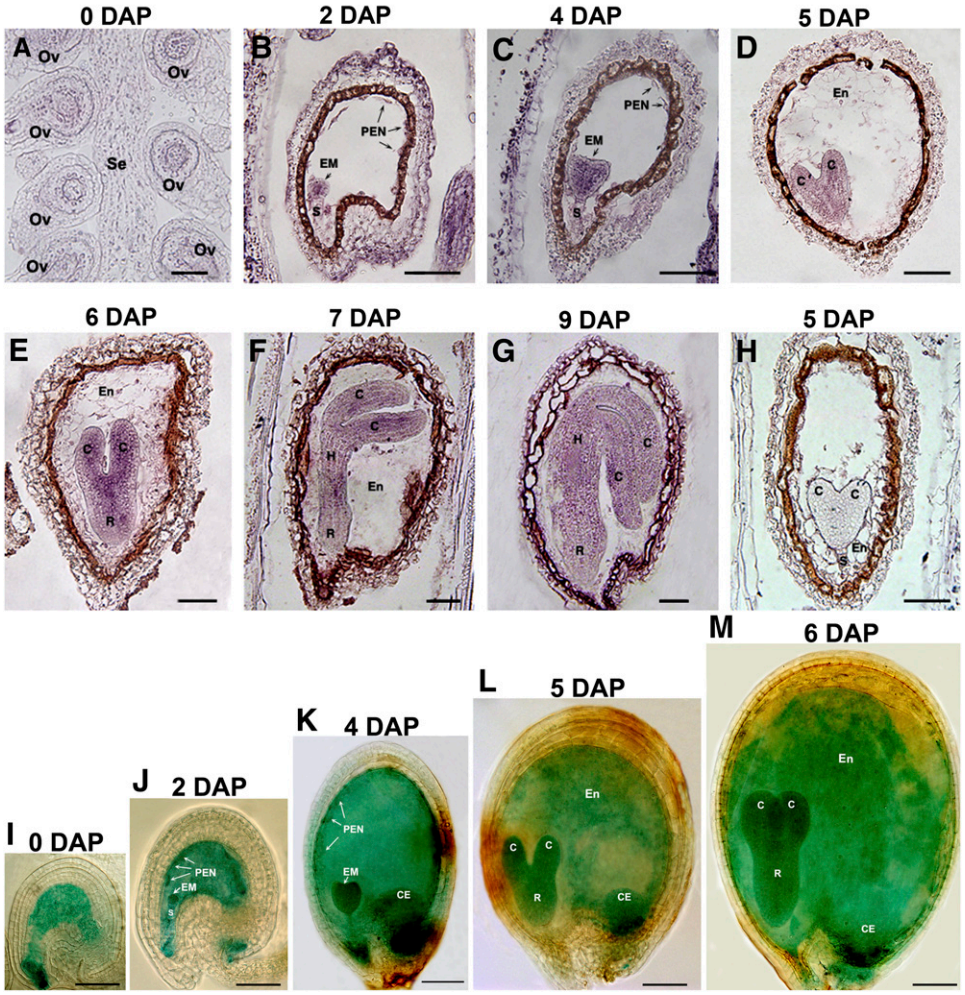


**Figure 9.** Localization of *ABA2* mRNA during Seed Development.

**(A)** to **(G)** As determined by in situ hybridization analysis, ABA2 was expressed both in the unfertilized ovule **(A)**, in the endosperm and embryo at the early globular stage **(B)**, early heart stage **(C)**, heart stage **(D)**, torpedo stage **(E)**, late torpedo stage **(F)**, and mature stage **(G)**.
**(H)** In situ hybridization with an *ABA2* sense probe control produced no visible signals.
**(I)** to **(M)** GUS signals were observed in the unfertilized ovule and the developing seeds of PABA2:GUS transgenic plants. Within the seeds, GUS signals were detected in the endosperms and the embryos.
Ov, ovule; Se, septum; EM, embryo; PEN, peripheral endosperm; En, endosperm; C, cotyledon; R, root; H, hypocotyl; S, suspensor; CE, chalazal endosperm. Bars = 50 μm.

significant enrichment of S2, S4, S6, and S7 fragments in the *SHB1* promoter with anti-MYC antibodies (Figures 11B and 11C). We also performed ChIP analysis of the promoter sequences of *YUCCA1* and *YUCCA4*, both of which encode key enzymes in auxin biosynthesis and are expressed during seed development (Cheng et al., 2007). As negative controls, neither of these sequences was significantly enriched (Supplemental Figure 6). This result indicated that ABI5 directly binds to the promoter of *SHB1* in vivo. These results were further confirmed by yeast one-hybrid analysis and electrophoretic mobility shift assays (EMSAs). Growth of the yeast on selection medium was detected using one-hybrid analysis, suggesting binding of ABI5 to the *SHB1* promoter (Figure 11D). In EMSA experiments using biotin-labeled 29-bp oligos (−669 to −697) covering two ABRE sites in the S3 fragment, a clear ABI5-dependent mobility shift was identified (Figure 11E). Mutated oligos failed to compete with the wild-type cognate oligos, suggesting a specific recognition of the elements in the *SHB1* promoter by ABI5 (Figure 11E).

## DISCUSSION

### ABA Is Required for Early Seed Development

A number of genes involved in ABA biosynthesis and signaling have been found to regulate seed maturation (Koornneef and Karssen, 1994; McCarty, 1995; Bewley, 1997; Finkelstein et al.,

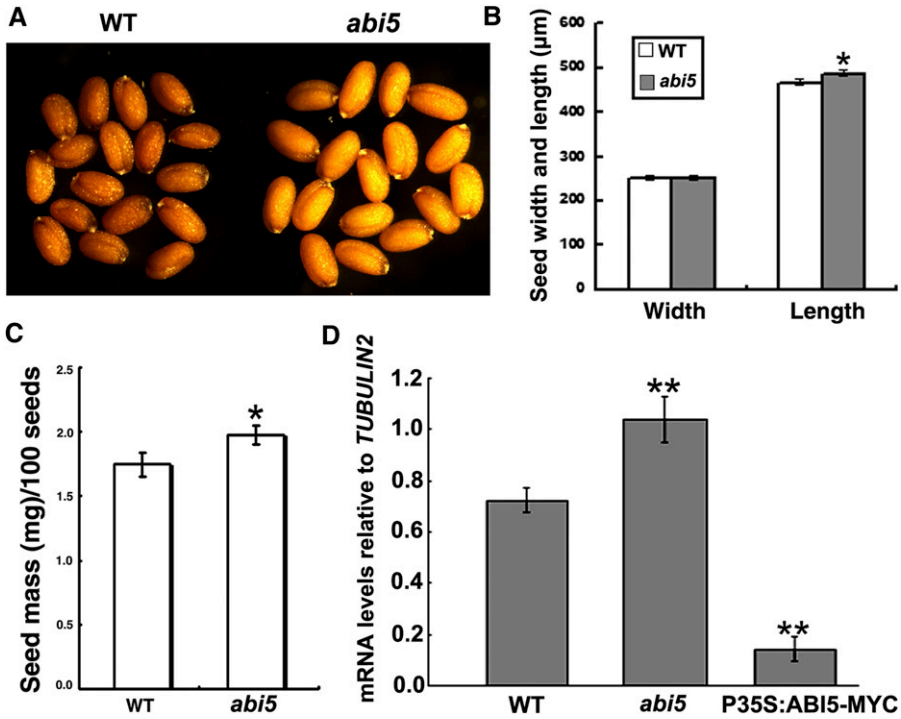


**Figure 10.** Mutation of *ABI5* Increases *SHB1* RNA Accumulation and Seed Mass.

**(A)** Mature dry seeds of the wild type (left) and *abi5* (right).
**(B)** Seed width and length in the wild type and *abi5*.
**(C)** Average seed mass per 100 seeds randomly selected from wild type and *abi5*. Standard errors in **(B)** and **(C)** were calculated from three sets of biological replicates. In each replicate, at least 100 seeds were examined.
**(D)** *SHB1* RNA accumulation in wild-type, *abi5*, and P35S:ABI5-MYC transgenic seeds was examined by qRT-PCR analysis and normalized to that of *TUBULIN2*. RNA accumulation data shown are the mean values of three biological replicates, each of which was examined in triplicate. In **(B)** to **(D)**, the error bars represent SE. Asterisks denote Student's *t* test significant difference compared with wild-type plants, with two asterisks and one asterisk denoting $P < 0.01$ and $P < 0.05$, respectively.
[See online article for color version of this figure.]

2002), indicating that ABA plays many important roles in seed development. Previous studies have suggested that ABA is mainly synthesized during seed maturation or after 9 DAP (Nambara and Marion-Poll, 2003). However, whether and how ABA biosynthesis and signaling are involved in early seed development prior to 9 DAP remain unknown.

*ABA2* encodes a key enzyme for ABA biosynthesis (González-Guzmán et al., 2002). In a previous study, the GUS signals in a PABA2:GUS transgenic plant were mainly detected in the seed funicule and in junction tissues between the pedicles and young siliques, but only faintly detected in developing seeds (Cheng et al., 2002). Recently, analyses of mRNA profiles revealed that *ABA2* was expressed in seeds from fertilization through maturation, with relatively high levels in subregions belonging to the embryo and endosperm (Belmonte et al., 2013). Here, we found by our current in situ hybridization and GUS staining experiments that *ABA2* is expressed in both the embryo and endosperm at 3 to 9 DAP in *Arabidopsis* seeds (Figure 9; Supplemental Figure 4), implying a function of *ABA2* in early seed development. Consistent with this notion, mutations in *ABA2* caused an increase in both seed length and seed mass, which could be suppressed by the application of exogenous ABA (Figure 2). Consistent with this, mutations in other genes involved in ABA biosynthesis, including *ABA1* and *NCED6*, produce larger seeds. These results indicated that both ABA synthesis and ABA accumulation are required for early seed development.

### ABA Mediates Endosperm Cellularization and Embryonic Cell Division in Early Seed Development

The first stage of seed development is characterized by proliferation and rapid growth of the endosperm, leading to a significant increase in the seed size (Boisnard-Lorig et al., 2001). Previous studies suggested that the timing of endosperm cellularization is important for final seed size (Garcia et al., 2003, 2005; Luo et al., 2005) such that delayed endosperm cellularization correlated well with increased nuclear proliferation and seed size increase (Kang et al., 2008). In this study, mutations of *ABA2* delayed endosperm cellularization and resulted in seed enlargement (Figures 1 and 5). These results implied that ABA negatively regulates endosperm proliferation by promoting the early cellularization of the endosperm in early seed development.

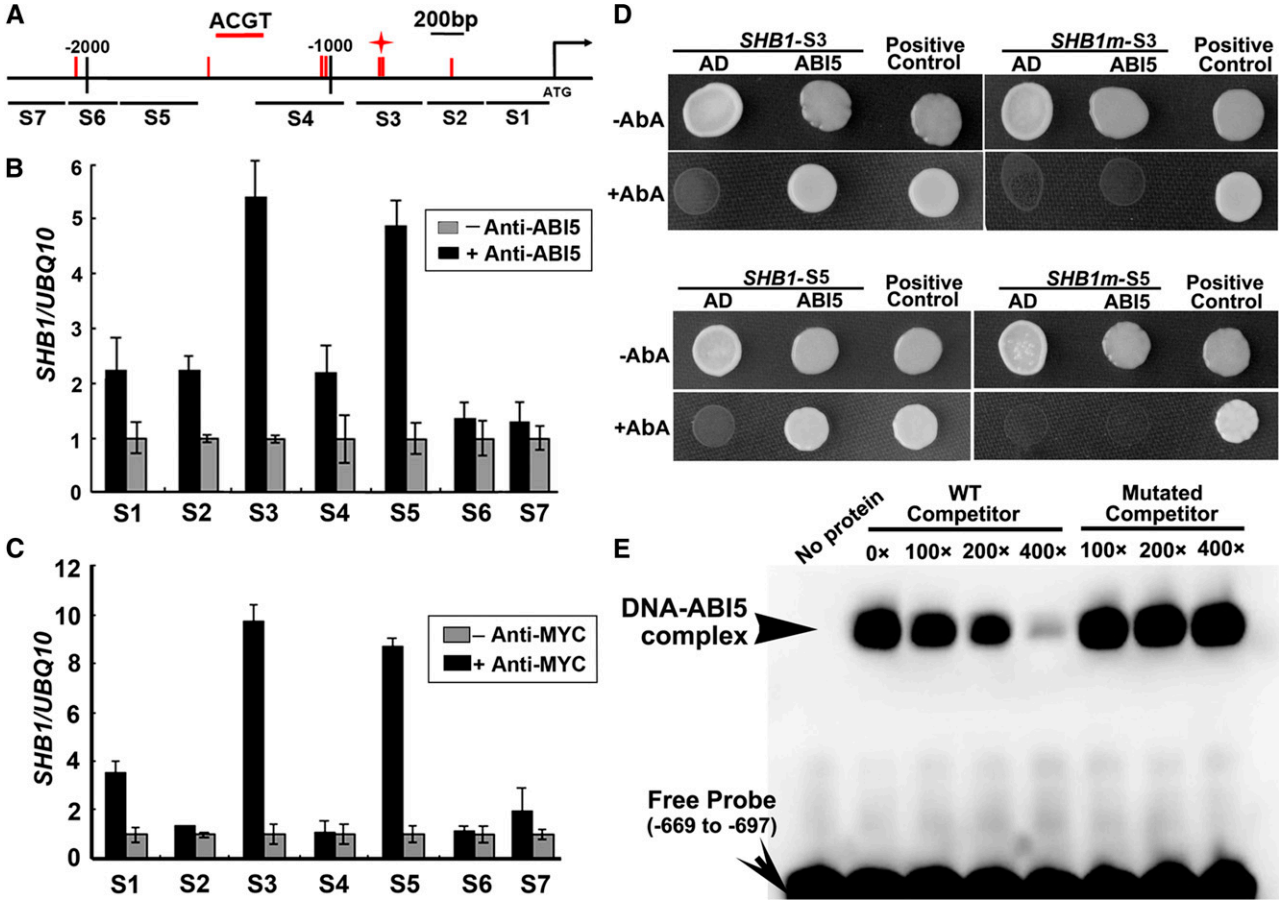


**Figure 11.** ABI5 Directly Binds to the *SHB1* Promoter.

**(A)** A 2.6-kb promoter region of *SHB1* upstream of its ATG codon contains several ABRE (ACGT) elements (red bars). A double ABRE element in fragment S3 is indicated by a red star. S1 to S7 indicate various PCR fragments used for ChIP–quantitative PCR analysis.

**(B)** ChIP–quantitative PCR was performed using an anti-ABI5 antibody in wild-type plants and specific primers to amplify various promoter regions of *SHB1*.

**(C)** ChIP–quantitative PCR was performed in the P35S:ABI5-MYC transgenic plants using an anti-MYC antibody. Preimmune serum was used as mock control. Means were calculated from three biological samples and each of which biological replicate was examined in triplicates. The error bars in **(B)** and **(C)** represent SE.

**(D)** Assessment of the interaction between ABI5 and the *SHB1* promoter by a yeast one-hybrid analysis. Yeast strains containing the S3 (above) or S5 fragments of the *SHB1* promoter and *SHB1m* promoter constructs were grown on media under selective (SD/-Leu, +300 ng/mL Aureobasidin A [AbA]) or nonselective (SD/-Leu, -AbA) conditions. Yeast cells transformed with full-length *ABI5* cDNAs were fused to pGADT7 AD and were grown on the plates with Aureobasidin A.

**(E)** Direct interaction between ABI5 and the *SHB1* promoter determined by EMSA. The retarded DNA-protein complex was reduced by competition using either the wild type or the mutated probes at a 100×, 200×, and 400× molar excess. The arrows indicate the position of the shifted bands and free probes, respectively.

ABA inhibits cell division in several different types of plant cells (Stewart and Smith, 1972; Newton, 1977). Specifically, ABA can arrest cell division by activating cyclin-dependent kinase inhibitor (ICK1) in 2-week-old seedlings (Wang et al., 1998). In this study, *aba2* mutant plants gave rise to seeds with enlarged embryos at the second stage of seed development through increased cell number but not cell size (Figure 3). Since *ABA2* is well expressed in the embryos (Figure 9), it may control cell division in the embryo by regulating cell cycle–related genes. On the other hand, the growth of the seed embryo occurs primarily during the second stage of seed development by absorbing nutrients from the endosperm (Boisnard-Lorig et al., 2001). Thus, the promoted endosperm proliferation caused by a mutation in *ABA2* might provide excessive nutrients for cell growth and thus augment the proliferation of the embryo at the second stage of seed development.

### ABA Negatively Regulates the Accumulation of *SHB1* RNA

Previous studies have provided genetic and molecular evidence that *SHB1* regulates seed size through the control of endosperm proliferation (Zhou et al., 2009). Recently, it was shown that

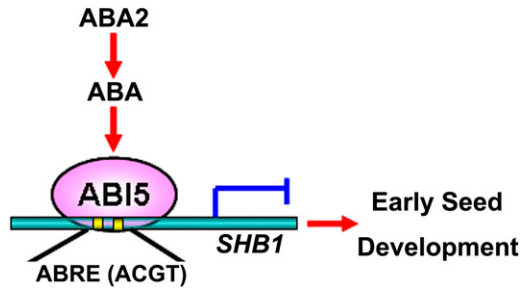


**Figure 12.** A Working Model for the ABA-Mediated Early Seed Development in *Arabidopsis*.

ABA2 is positively involved in ABA accumulation. ABA negatively regulates *SHB1* transcription through the ABA signaling component ABI5 directly binding to the ABRE *cis*-elements in the *SHB1* promoter region. The blue bar represents the promoter region of *SHB1*, and the yellow bars represent ABRE elements.

SHB1, through DNA binding, regulates the expression of *MINI3* and hence *IKU2*, both of which participate in the regulation of endosperm proliferation and cellularization (Zhou et al., 2009; Kang et al., 2013). The phenotypes of *aba2* were similar to those of *shb1-D* (Zhou et al., 2009). Our genetic and molecular analyses further indicated that *ABA2* is required for the proper expression of *SHB1*, *MINI3*, and *IKU2* (Figure 6) and the effects of *ABA2* on endosperm cellularization are largely dependent on *SHB1* function.

### Direct Regulation of *SHB1* Expression by ABI5

*Arabidopsis ABA2* encodes a dehydrogenase/reductase that catalyzes a step in the ABA biosynthesis pathway (Cheng et al., 2002). Hence, *ABA2* might regulate ABA accumulation while ABA signal transduction ultimately controls seed development. ABA signal transduction requires a class of bZIP transcription factors that regulate the expression of ABA-dependent downstream genes through directly binding to the ABA response elements in the promoter regions of these genes (Hubbard et al., 2010). Two copies of the ABRE are usually required to render a promoter ABA sensitive (Hubbard et al., 2010). In the promoter region of *SHB1*, four out of the seven ABRE elements are orientated as pairs. We used ChIP approaches to confirm that the bZIP transcription factor ABI5 binds to the S3 fragment that contains two copies of the ABRE (Figure 11). Moreover, we found that *ABI5* RNA accumulation was significantly lower in the *aba2* mutants (Supplemental Figure 5). Taken together, these data showed that ABI5 is involved in ABA-mediated early seed development.

Mutations in either *ABA2* or *ABI5* cause an increase in seed size (Figures 1 and 10A). However, the phenotype of *aba2* (25 to 30% increase in seed size) was more severe than that of *abi5* (10 to 15% increase in seed size), suggesting a degree of functional redundancy between these two factors during early seed development. In the *Arabidopsis* genome, 13 genes encode ABI5-related bZIP transcription factors, and seven of these show differential expression during seed development (Bensmihen et al., 2002). Moreover, hetero- or homodimers of AREB/ABFs can function in ABA signaling (Fujita et al., 2005). Indeed, we found in this regard that the expression levels of *AREB1*, *AREB2*, and *ABF3* are also downregulated in addition to *ABI5* in the *aba2-1* mutant (Supplemental Figure 5). Hence, it is likely that other factors, particularly the ABI5-related bZIPs, are involved in the regulation of *SHB1* expression in addition to ABI5.

### Control of Seed Size: A Working Model

Our genetic and molecular analyses suggest that the *ABA2*, *SHB1*, *MINI3*, and *IKU2* function in the same pathway to mediate seed development. Thus, we propose a working model for ABA-mediated early seed development (Figure 12). *ABA2* tunes the endogenous level of ABA and regulates the transcription of *ABI5*. ABI5 in turn directly represses the expression of *SHB1*. SHB1 interacting with MINI3 and other factors further controls the expression of both *MINI3* and *IKU2* (Zhou et al., 2009; Kang et al., 2013). It is likely that ABA mediates the expression of *SHB1* for the proper development of endosperm and embryo during the early seed development. Endosperm proliferation must be terminated at the appropriate time for a phase transition to embryo development. Hence, we identified an important regulatory cascade that connects ABA-regulated biosynthesis and signaling with the SHB1-MINI3-IKU2 pathway to control early seed development.

## METHODS

### Plant Materials, Growth Conditions, and Somatic Embryo Induction

We used *Arabidopsis thaliana* ecotype Columbia-0 as the wild type. Mutants used in this study, including *aba2-1* (CS156; Léon-Kloosterziel et al., 1996; González-Guzmán et al., 2002), *aba2-3* (CS3834; Laby et al., 2000), *shb1* (SALK_128406), *aba1* (SALK_022816), and *nced6* (SALK_062061), are all in the Columbia-0 background and were obtained from the ABRC (Ohio State University, Columbus). Seeds of the wild type and *aba2-1* were surface-sterilized in 10% sodium hypochlorite solution and 0.01% Triton X-100 for 5 min and then washed three times in sterile distilled water. They were plated on 0.8% (w/v) solid agar Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and cold-treated at 4°C for 3 d to overcome dormancy. Subsequently, they were grown under sterile conditions with a 16-h-light/8-h-dark cycle (light intensity 40 μM photons $m^{-2}$ $s^{-1}$) at 20 to 22°C for ~60 d until the last siliques on the inflorescences were dry. The P35S:ABI5-MYC and *abi5-1* plants were kindly provided by Qi Xie (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China; Zhang et al., 2007). *ABI5* expression in siliques of the P35S:ABI5-MYC transgenic plants was much higher than that of the wild type by qRT-PCR analysis (Supplemental Figure 7B).

Somatic embryo regeneration was performed using the method described by Su et al. (2009). Bent-cotyledon embryos were collected as explants and were cultured on agar-solidified B5 medium containing 4.5 μM 2,4-D (Pillon et al., 1996). Somatic embryos were induced from the surfaces of the explants after 10 d of culture in light.

### P35S:ABA2 Construction

To produce P35S:ABA2 transgenic plants, an 858-bp fragment with the *ABA2* cDNA was integrated into the vector pMDC43. The primers ABA2-F and ABA2-R used are shown in the Supplemental Table 1. Transgenic plants carrying P35S:ABA2 were generated by the floral dipping method

(Clough and Bent, 1998). RNA accumulation of *ABA2* in siliques of the P35S:ABA2 transgenic plants was much higher than that in the wild type (Supplemental Figure 7A).

### PABA:GUS Construction and GUS Staining

The 1161-kb region of genomic DNA upstream to the *ABA2* coding region was amplified by PCR using the primers PABA2-F and PABA2-R and was cloned into the pBI121 vector. The sequences of primers PABA2-F and PABA2-R are listed in Supplemental Table 1. For GUS staining, tissues were harvested and fixed in 90% acetone on ice for 15 to 20 min and were transferred to staining solution containing 50 mM $NaPO_4$, pH 7.2, 2 mM X-gluc (Sigma-Aldrich), 0.5 mM $K_3Fe(CN)_6$, and 0.5 mM $K_4Fe(CN)_6$, incubated at 37°C overnight. Then, the siliques were cleared and embedded in Hoyer's solution (3:0.8:0.4 of chloral hydrate:water:glycerol). Finally, the seeds in the siliques were photographed using an Olympus BH-2 microscope equipped with an Olympus DP12 digital camera.

### Scanning Electron Microscopy and Image Processing

Green embryos were dissected from wild-type and *aba2* seeds and fixed in freshly prepared FAA (10% formalin, 5% acetic acid, and 45% ethanol) at 4°C for 2 h. Samples were subsequently washed three times with PBS. The embryos were dehydrated in 30, 50, 75, 95, and 100% ethanol for ~30 min each at 4°C. The samples were then dried, gold-palladium coated, and graphed as described by Li et al. (2002). ImageJ software (W.S. Rasband, ImageJ, U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997 to 2008) was used to quantify the cotyledon area as well as average size of epidermal cells in the central region of cotyledons.

### Differential Interference Contrast Analysis

Seeds of the wild type and *aba2-1* were collected at various developmental stages and fixed in the FAA overnight at 4°C. The samples were then incubated in Hoyer's solution for 40 min. The development of endosperm and embryo in cleared seeds was observed by differential interference contrast optics of a Zeiss 510 Meta confocal laser scanning microscope as described previously (Cheng et al., 2013).

### Sectioning and Cytological Analysis

Seed samples for cytological analysis were fixed in FAA overnight at 4°C as described above. Dehydration was performed using ethanol with an increasing concentration ranging from 30 to 100%. The last step repeated three times. Sections were stained using 0.1% (w/v) toluidine blue O in double distilled water. Then, the sections were photographed with an Olympus BH-2 microscope equipped with an Olympus digital camera.

### ABA Treatment and Measurement

To determine the sensitivity of *SHB1* RNA accumulation to ABA treatment during early seedling development, 8-d-old wild-type seedlings were transferred onto solid MS medium with or without 10 μM ABA for 0, 2, 4, 6, and 18 h, respectively. To test the sensitivity of *SHB1* RNA accumulation to ABA treatment in seed development, 4-week-old wild-type plants were sprayed with or without 100 μM ABA mixed with 0.02% (w/v) Triton X-100 and then tented with plastic wrap. The plants were sprayed once per day until the seeds had developed to 9 DAP. The seeds were harvested at 1, 3, 6, and 9 d after hand-pollination. To examine whether the exogenous ABA rescued the phenotypes of the *aba2* mutant, 3-week-old wild-type and *aba2* mutant plants were sprayed with or without 100 μM ABA mixed with 0.02% (w/v) Triton X-100 and tented with plastic wrap once per day until 9 DAP. The seeds were then collected.

For endogenous ABA levels at various seed developmental stages, a radioimmunoassay method was used as previously described by Wang et al. (2011).

### RNA Isolation and qRT-PCR Analysis

Total RNA was extracted from the tissues using a commercially available kit (Total RNA Isolation System; Promega). The primers used in the qRT-PCR reactions are presented in Supplemental Table 1. The qRT-PCR amplifications were performed with each cDNA dilution using SYBR Green Master mix with Chromo4 as described in the manufacturer's protocol (Bio-Rad Laboratories). RNA accumulation of genes in each sample was normalized to that of *TUBULIN2*, and the measurements were performed using three biological replicates. The comparative CT method, means, and standard deviations were used to calculate and analyze the results.

### In Situ Hybridization Analysis

Flowers of the wild type were hand-pollinated and tagged with the date of pollination on each flower. The siliques at different days after pollination were collected and fixed in FAA overnight at 4°C. Following dehydration, the fixed tissues were embedded in Paraplast (Sigma-Aldrich) and sectioned at 8 μm. Antisense and sense RNA probes were synthesized in vitro with digoxigenin- UTP using SP6 and T7 RNA polymerases (digoxigenin RNA labeling kit; Boehringer Mannheim) and then used for hybridization according to the detailed procedures, as previously described (Zhao et al., 2006). The antisense probes ABA-T7-S, ABA-T7, ABA-SP6-S, and ABA-SP6 are listed in Supplemental Table 1.

### Double Mutant Analysis

The *aba2-1* mutant was crossed with the *shb1* mutant. The ethyl methanesulfonate–mutagenized *aba2-1* mutant results in a Ser-to-Asn substitution at position 264 in the C-terminal region of the enzyme. The *aba2-1 shb1* double mutants were obtained from F2 progenies, and the presence of *aba2-1* and *shb1-1* was monitored by PCR, as previously described (Bui and Liu, 2009). The PCR primers used are shown in the Supplemental Table 1. Seed weights and numbers per silique were determined from the inflorescences of four to five plants in sum.

### Yeast One-Hybrid Analysis

Yeast one-hybrid analysis was performed using a commercial kit (Clontech Laboratories) as described in the manufacturer's protocol. Briefly, pAbAi vectors harboring fragments of *SHB1* (−531 to −830 and −1707 to −2020) were integrated into the yeast genome (Y1HGold strain). A yeast reporter vector, p53-AbAi construct, was used as a positive control (Clontech Laboratories). Using total cDNA derived from *Arabidopsis* whole-plant RNA as the template, the coding sequence of *ABI5* was amplified and introduced into the pGADT7 AD vector (Clontech Laboratories). Yeast transformation and evaluations of interactions were performed as previously described (Cheng et al., 2013). After culture on selection plates for 3 d, activation of the yeast growth was observed (SD/-Leu) containing 300 ng/mL Aureobasidin A. The oligo sequences derived from *SHB1* promoter and primers to amplify *ABI5* cDNA are described in Supplemental Table 2.

### Fusion Protein Preparation and EMSA

For His-tagged ABI5 protein production, the pET28a-ABI5 construct was kindly provided by Yan Guo (China Agricultural University, Beijing, China; Zheng et al., 2012) and expressed in the *Escherichia coli* BL21 (DE3) cell line. Expressed proteins were extracted and purified using Ni Sepharose 6

Fast Flow (GE Healthcare Life Sciences) according to the manufacturer's instructions. Probe labeling and EMSA experiments were performed according to previous descriptions (Cheng et al., 2013). Briefly, the 29-bp single-stranded wild-type and mutated DNA oligonucleotides (oligos) of the *SHB1* promoter (−669 to −697) were synthesized. To generate double-stranded oligos, an equal amount of complementary single-stranded oligos was mixed, heated to 95°C for 5 min, and slowly cooled down to 25°C. For a binding reaction, the LightShift Chemiluminescent EMSA kit (Pierce) was used. For competition experiments, different amounts of nonlabeled wild-type and mutated double-stranded oligos were used.

Sequence information for the wild type and mutated synthetic oligonucleotide probes is provided in Supplemental Table 2.

### ChIP Assays

The immunoprecipitation of bound chromatin was performed using a ChIP kit (Upstate) according to the manufacturer's protocol. The seeds of the wild-type and the transgenic lines containing P35S:ABI5-MYC were harvested 4 to 5 DAP (from early stage to late heart stage), and the seeds were then fixed with 1% (v/v) formaldehyde in GB buffer (0.4 M Suc, 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, and 1 mM phenylmethanesulfonyl fluoride) under a vacuum for 10 min at room temperature. The cross-linking was stopped with 0.125 M Gly. Chromatin was then isolated from the tissues, resuspended in SDS lysis buffer with protease inhibitors, and sonicated to achieve an average DNA size of between 0.2 and 1 kb. Next, the chromatin extract was obtained by centrifugation. ChIP with antibodies against both ABI5 (kindly provided by Jianru Zuo; Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) and MYC (Sigma-Aldrich) were used to purify DNA fragments, which were washed with 70% ethanol and finally dissolved in 10 μL water with 20 mg/mL RNase. In the end, DNA fragments were analyzed in triplicate, with the qRT-PCR method described previously (Cheng et al., 2013). The fold enrichment of a specific chromatin fragment was normalized to that of the *UBQ10* and calculated according to the following equation: $2^{(Ct\ SHB1\ MOCK\text{-}Ct\ SHB1\ ChIP)}/2^{(Ct\ UBQ10\ MOCK\text{-}Ct\ UBQ10\ ChIP)}$. Mouse IgG was used as a mock control. The primers used to amplify *ABI5* cDNA (ABI5-F and ABI5-R) and *SHB1* promoter DNA are listed in Supplemental Table 2.

### Accession Numbers

Sequence data generated from the experiments described in this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ABA2* (AT1G52340), *SHB1* (AT4G25350), *ABI5* (AT2G36270), *MINI3* (AT1G55600), *IKU2* (AT3G19700), *AREB1* (AT1G45249), *AREB2* (AT3G19290), *ABF3* (AT4G34000), *DA1* (AT1G19270), *AP2* (AT4G36920), *YUCCA1* (AT4G32540), and *YUCCA4* (AT5G11320).

### Supplemental Data

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

**Supplemental Figure 1.** Seed Size and Mass Are Increased in ABA-Deficient Mutants.

**Supplemental Figure 2.** Quantification of ABA in the *aba2-1* Mutant.

**Supplemental Figure 3.** Mutation in *ABA2* Caused Delay in Seed Development and Enlargement in Seed Size.

**Supplemental Figure 4.** *ABA2* Expression Pattern Is Examined in Various Organs or Tissues Examined by qRT-PCR.

**Supplemental Figure 5.** RNA Accumulation of bZIP Transcription Factor Genes Is Decreased in the *aba2* Mutant.

**Supplemental Figure 6.** ABI5 Binds to the Promoter of *SHB1* but Not *YUCCA1* and *YUCCA4*.

**Supplemental Figure 7.** RNA Accumulation of *ABA2* and *ABI5* Was Determined by qRT-PCR.

**Supplemental Table 1.** Primers Used in This Study.

**Supplemental Table 2.** Oligo Sequences and Primers Used in the Yeast One-Hybrid, EMSA, and ChIP Assays.

## ACKNOWLEDGMENTS

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## AUTHOR CONTRIBUTIONS

X.S.Z. and X.Y.Z. designed the research. Z.J.C., X.Y.Z., X.X.S., F.W., and C.Z. performed different aspects of the research. X.S.Z., Z.J.C., and X.Y.Z. analyzed data. Y.G.L. performed ABA measurements. Z.J.C., X.S.Z., and Y.Z. wrote the article.

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