The Arabidopsis MYB96 Transcription Factor Is a Positive Regulator of *ABSCISIC ACID-INSENSITIVE4* in the Control of Seed Germination[1]

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Seed germination is a key developmental transition that initiates the plant life cycle. The timing of germination is determined by the coordinated action of two phytohormones, gibberellin and abscisic acid (ABA). In particular, ABA plays a key role in integrating environmental information and inhibiting the germination process. The utilization of embryonic lipid reserves contributes to seed germination by acting as an energy source, and ABA suppresses lipid degradation to modulate the germination process. Here, we report that the ABA-responsive R2R3-type MYB transcription factor MYB96, which is highly expressed in embryo, regulates seed germination by controlling the expression of *ABSCISIC ACID-INSENSITIVE4* (*ABI4*) in Arabidopsis (*Arabidopsis thaliana*). In the presence of ABA, germination was accelerated in *MYB96*-deficient *myb96-1* seeds, whereas the process was significantly delayed in *MYB96*-overexpressing activation-tagging *myb96-ox* seeds. Consistently, *myb96-1* seeds degraded a larger extent of lipid reserves even in the presence of ABA, while reduced lipid mobilization was observed in *myb96-ox* seeds. MYB96 directly regulates *ABI4*, which acts as a repressor of lipid breakdown, to define its spatial and temporal expression. Genetic analysis further demonstrated that *ABI4* is epistatic to *MYB96* in the control of seed germination. Taken together, the MYB96-ABI4 module regulates lipid mobilization specifically in the embryo to ensure proper seed germination under suboptimal conditions.

Seeds are products of plant sexual reproduction and facilitate the spread of offspring. Since plants are sessile organisms with a limited ability to seek favorable growth conditions, seed dispersal is an evolutionarily adaptive trait to disseminate their offspring to optimal locations (Bewley, 1997; Nonogaki, 2014). Thus, seeds enable plants to endure environmental disadvantages and confine growth until they encounter favorable environmental conditions (Bewley, 1997).

Seed embryogenesis is initiated by double fertilization (Gehring et al., 2004; Berger et al., 2008). While the zygote divides asymmetrically to form the diploid embryo, the triploid endosperm constantly proliferates inside the maternal ovule (Natesh and Rau, 1984; West and Harada, 1993). At the end of the embryonic growth phase, cell cycle activities in the embryo are arrested (Raz et al., 2001), and storage molecules, such as proteins, carbohydrates, and lipids, accumulate particularly in the cotyledons (Goldberg et al., 1989; Raz et al., 2001). This phase is referred to as seed maturation. Mature seeds become dehydrated and enter a quiescent state that is tolerant to desiccation (Crouch, 1987; Harada et al., 1988; Kermode, 1990; McCarty and Carson, 1991), establishing primary dormancy.

Seed dormancy can be broken when seeds experience stratification and/or after-ripening (Vleeshouwers et al., 1995; Bewley, 1997; Nonogaki, 2014). Germination is a key developmental transition to determine when to initiate the plant life cycle. Since it is an irreversible transition, seeds have to precisely monitor external environmental factors, such as light, temperature, water and nutrient availability, and high salinity (Huang et al., 2003; Qu et al., 2008; Joosen et al., 2013; Lim et al., 2013).

These environmental signals are integrated into internal developmental programs in seeds, which are operated primarily by antagonistic actions of two phytohormones: abscisic acid (ABA) and GA (Holdsworth et al., 1999; Finch-Savage and Leubner-Metzger, 2006; Seo et al., 2006). GA promotes germination, whereas ABA suppresses completion of the developmental transition (Garciarrubio et al., 1997; Debeaujon and Koornneef, 2000; Finkelstein et al., 2002; Peng and Harberd, 2002; Ogawa et al., 2003; Lau and Deng, 2010).

Transcriptional regulation is a fundamental regulation scheme for the seed germination process (Thomas, 1993; Nakabayashi et al., 2005). The GA function in promoting seed germination is accomplished partly through the action of central GA signaling repressors, DELLA-domain proteins, including GIBBERELLIN INSENSITIVE (GAI), REPRESSOR OF GIBBERELLIN1-3 (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3, all of which act as transcriptional regulators (Hussain and Peng, 2003; Tyler et al., 2004; Cao et al., 2006; Park et al., 2013). They interact with transcription factors to allow DELLAs to regulate gene expression by targeting specific gene promoters (de Lucas et al., 2008; Feng et al., 2008; Arnaud et al., 2010; Gallego-Bartolomé et al., 2010; Hong et al., 2012). Indeed, DELLAs bind to promoters of *GIBBERELLIN INSENSITIVE DWARF1*, *WRKY27*, and *SCARECROW-LIKE3* to control seed germination (Zentella et al., 2007; Gallego-Bartolomé et al., 2011; Zhang et al., 2011; Stamm et al., 2012). The DELLA proteins are rapidly degraded in response to GA and are thus considered as repressors of seed germination in the absence of GA (Dill et al., 2001; Tyler et al., 2004).

ABA regulation of seed germination also requires ABA-responsive transcription factors. ABSCISIC ACID INSENSITIVE3 (ABI3), ABI4, and ABI5, which encode transcription factors with the B3 domain, APETALA2 domain, and basic Leu zipper domain, respectively, are expressed highly in seeds and are involved in regulating sensitivity to ABA (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000). Indeed, genetic mutants at these loci exhibit reduced ABA sensitivity as well as accelerated seed germination in the presence of exogenous ABA (Finkelstein and Somerville, 1990). They act in concert with each other and form combinatorial networks to determine the proper time to germinate (Söderman et al., 2000; Nakamura et al., 2001).

In addition to their overlapping roles in ABA-dependent seed germination, ABI3, ABI4, and ABI5 have their own unique functions. ABI4 is particularly interesting in that it plays a unique role in embryonic lipid catabolism during the germination process (Penfield et al., 2006). Seeds utilize lipid reserves to fuel postgerminative seedling growth (Penfield et al., 2005; Quettier and Eastmond, 2009), but the lipid catabolic process is inhibited in the presence of ABA in order to delay seed germination, thereby minimizing environmental damage to early seedlings. Notably, ABA specifically represses embryonic lipid breakdown (Penfield et al., 2006). The differential responses of embryo and endosperm to ABA are accomplished by the confined expression of *ABI4* in the embryo (Penfield et al., 2006). However, the mechanisms by which *ABI4* is expressed specifically in the embryo and by which specific regulator(s) participate in defining its spatial and temporal expression remain unknown.

We previously showed that the MYB96 transcription factor mediates a variety of plant responses to ABA, including drought resistance, stomatal conductance, lateral root development, anthocyanin accumulation, and cuticular wax biosynthesis (Seo et al., 2009, 2011; Seo and Park, 2010, 2011). The expression of *MYB96* is considerably induced by drought stress and ABA (Seo et al., 2009), and it activates the expression of many ABA-responsive genes through direct binding to gene promoters to optimize plant growth and fitness under environmentally unfavorable conditions (Seo et al., 2009, 2011; Seo and Park, 2010).

Here, we report that MYB96 also plays a key role in the ABA control of seed germination in Arabidopsis (*Arabidopsis thaliana*). The *MYB96* gene was significantly expressed in the embryo and conferred embryonic ABA sensitivity in seed germination as well as triacylglycerol (TAG) breakdown. In the presence of ABA, *MYB96*-overexpressing *myb96-ox* seeds exhibited delayed germination with reduced lipid breakdown, whereas *myb96-1* seeds showed accelerated seed germination and lipid breakdown. The MYB96 regulation of seed germination was largely dependent on *ABI4*, a key repressor of embryonic lipid mobilization during the germination process. Together, our findings provide evidence that MYB96 is an unequivocal transcriptional regulator of *ABI4* and defines the spatial and temporal specificity of its expression to fine-tune seed germination under unfavorable environmental conditions.

RESULTS

MYB96 Is Expressed in Embryos

MYB96 mediates ABA signal transduction in a variety of physiological processes, such as drought response, lateral root development, and cuticular wax accumulation (Seo et al., 2009, 2011). Since seed germination is also a key developmental transition governed by ABA (Bewley, 1997; Nakashima and Yamaguchi-Shinozaki, 2013), it was highly plausible that MYB96 may also regulate seed germination in an ABA-dependent manner.

To explore the role of *MYB96* in seed germination, we first determined its expression patterns in seeds. Quantitative reverse transcription (qRT)-PCR analysis revealed that *MYB96* transcripts accumulated at high levels in dry seeds (Fig. 1A), and its transcript accumulation was drastically reduced after cold stratification and imbibition. The transcript levels of *MYB96* were reduced by 98% after stratification compared with those in dry seeds (Fig. 1A) and recovered at seedling stages (Supplemental Fig. S1), implying an inhibitory role of *MYB96* in seed germination.

Spatial expression patterns of *MYB96* were further examined using *pMYB96:GUS* transgenic seeds, in
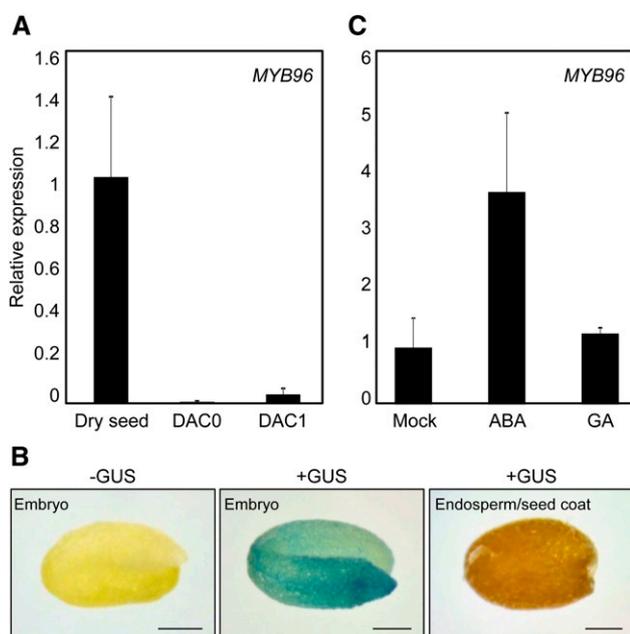
Figure 1. Expression of *MYB96* in seeds. A, *MYB96* expression in dry seeds and during seed germination. Wild-type seeds were kept in darkness at 4°C for 72 h (stratification) and transferred to long-day conditions at 22°C for germination. Transcript accumulation was analyzed by qRT-PCR. The *EUKARYOTIC TRANSLATION INITIATION FACTOR4A1* (*eIF4a*) gene (At3g13920) was used as an internal control. Biological triplicates were averaged. Error bars indicate SE. DAC, Days after cold stratification. B, Histochemical staining analysis. The *pMYB96*: *GUS* transgenic seeds, in which a promoter sequence covering an approximately 2-kb region upstream of the *MYB96* transcription start site was transcriptionally fused to a GUS-coding sequence, were subject to GUS staining. Bars = 0.7 μm. C, Induction of *MYB96* by ABA in seeds. Stratified wild-type seeds were transferred to MS medium supplemented with 5 μM ABA or GA and incubated for 1 d under long-day conditions. Transcript accumulation was analyzed by qRT-PCR. Biological triplicates were averaged. Error bars indicate SE.

which a promoter sequence covering an approximately 2-kb region upstream of the *MYB96* transcription start site was transcriptionally fused to a GUS-coding sequence. In agreement with the qRT-PCR results, GUS activities were strongly detected in dry seeds (Fig. 1B) and reduced after stratification and imbibition (Supplemental Fig. S2). Moreover, dissection of seeds showed that high levels of GUS expression were observed only in the embryo (Fig. 1B). GUS activity was undetectable in the endosperm and seed coat (Fig. 1B).

MYB96 Is Induced by ABA in Seeds But Not by GA

MYB96 is transcriptionally induced by ABA in seedlings (Seo et al., 2009). Considering that ABA content decreases in seeds following stratification and imbibition (Grappin et al., 2000; Shu et al., 2013), the decreased expression of *MYB96* during seed germination seemed likely to correlate with endogenous ABA levels in seeds.

Therefore, we asked whether ABA induction of *MYB96* is also relevant in seeds.

To examine the effect of ABA on *MYB96* expression in germinating seeds, stratified wild-type seeds were plated on Murashige and Skoog (MS) medium supplemented with or without ABA. qRT-PCR analysis showed that transcript levels of *MYB96* were significantly increased 3.5-fold by exogenous ABA treatment in wild-type seeds (Fig. 1C; Seo et al., 2009). Histochemical analysis also revealed that the ABA induction of *MYB96* was observed mainly in the embryo (Supplemental Fig. S3).

Seed germination is tightly regulated by antagonistic interactions between ABA and GA (Yano et al., 2009; Yaish et al., 2010). Thus, we next asked whether the promotive action of GA in seed germination is also associated with *MYB96* expression. Contrary to ABA, *MYB96* was unaffected by exogenous GA treatment (Fig. 1C). Furthermore, *MYB96* expression was also unchanged in quadruple-DELLA (*gai-t6 rga-t2 rgl1-1 rgl2-1*) and GA-deficient *ga1-3* mutants (Supplemental Fig. S4). These observations suggest that MYB96 plays a primary role in ABA signal transduction in seeds.

MYB96 Regulates ABA-Dependent Seed Germination

Our observation that seed-expressed *MYB96* was regulated by ABA suggested that it might have a physiological role in ABA-dependent seed germination. Therefore, we measured the germination rate of seeds of the wild type, the *MYB96*-overexpressing activation-tagging line *myb96-ox* (Seo et al., 2009), and the transfer-DNA insertional *MYB96*-deficient mutant *myb96-1* (Seo et al., 2009) at various concentrations of ABA.

The germination rate of *myb96-ox* and *myb96-1* seeds was similar to that of wild-type seeds in the absence of ABA (Fig. 2A). However, in the presence of ABA, the germination rate was substantially reduced in *myb96-ox* seeds but elevated in *myb96-1* seeds compared with wild-type seeds (Fig. 2A). The differential ABA responses of *myb96-ox* and *myb96-1* seeds were more evident at higher concentrations of ABA (Fig. 2A).

For ABA dose-response assays, seeds were germinated on MS medium plates supplemented with different concentrations of ABA, and germination percentages were scored 2 d after cold stratification. These assays clearly revealed that the *myb96-ox* seeds exhibited increased sensitivity to exogenous ABA and the *myb96-1* seeds were hyposensitive to ABA relative to wild-type seeds (Fig. 2B).

Cotyledon opening is also sensitive to exogenous ABA treatment in the association with seed germination. The cotyledon opening of *myb96-ox* and *myb96-1* seeds was indistinguishable from that of wild-type seeds in the absence of ABA (Supplemental Fig. S5). However, in agreement with the seed germination rates, the cotyledon opening of *myb96-ox* was hypersensitive to ABA, whereas the *myb96-1* mutants were hyposensitive (Supplemental Figs. S5 and S6). These observations indicate that MYB96 determines ABA sensitivity and regulates ABA-dependent seed germination.
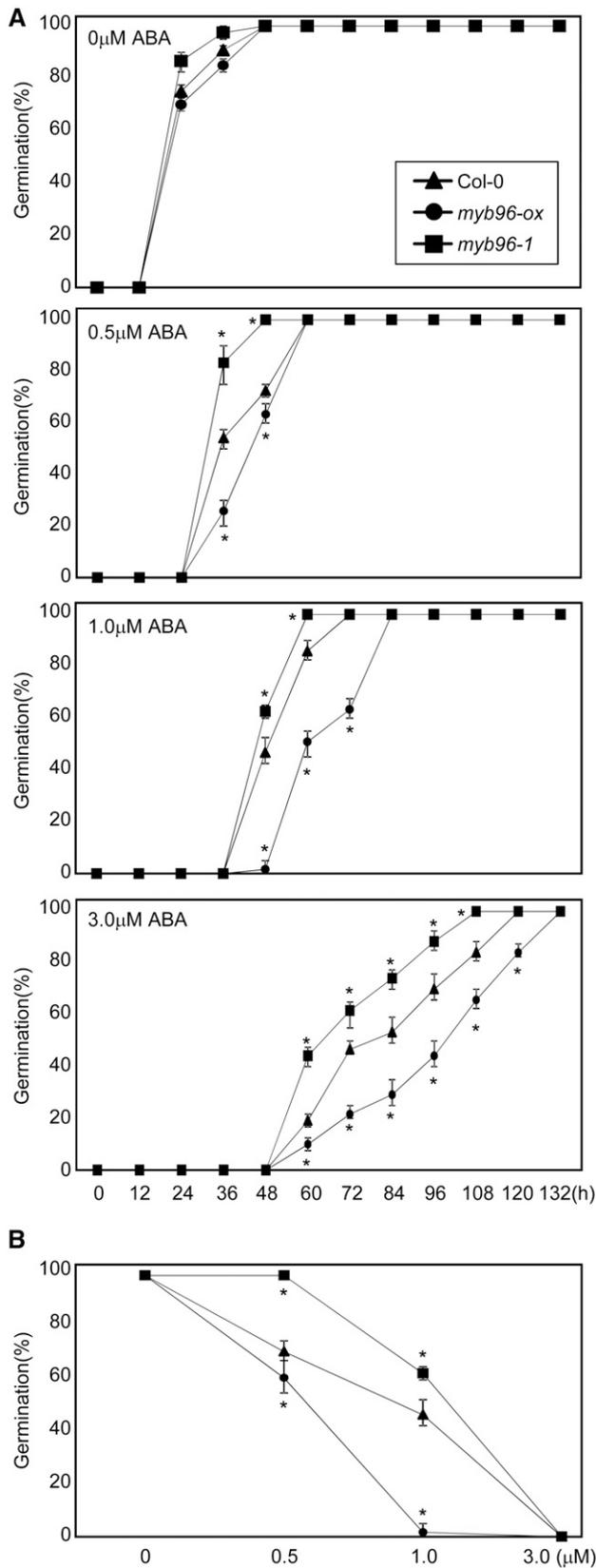
Figure 2. ABA sensitivity of *myb96-ox* and *myb96-1* in seed germination. A, Germination rate of *myb96-ox* and *myb96-1*. Seed germination

ABI4 Is Up-Regulated in myb96-ox

To establish the molecular networks underlying the MYB96 regulation of seed germination, we monitored the expression patterns of genes associated with ABA-dependent seed germination, such as *ABI1*, *ABI2*, *ABI3*, *ABI4*, *ABI5*, *ACYL-COENZYME A-BINDING PROTEIN1* (*ACBP1*), *ARABIDOPSIS HISTIDINE KINASE1* (*AHK1*), *BEL1-LIKE HOMEODOMAIN1* (*BLH1*), *FLOWERING LOCUS Y* (*FY*), *RING-H2 FINGER A2A* (*RHA2a*), *SNF1-RELATED PROTEIN KINASE2.2* (*SnRK2.2*), and *SnRK2.3* (Finkelstein and Somerville, 1990; Söderman et al., 2000; Carles et al., 2002; Fujii et al., 2007; Tran et al., 2007; Moes et al., 2008; Li et al., 2011; Jiang et al., 2012; Du et al., 2013; Kim et al., 2013), in the wild type, *myb96-ox*, and *myb96-1*, in the absence of ABA. In seeds, many genes were differentially expressed in *myb96-ox* and *myb96-1*, but MYB96 regulation of *ABI4* was most likely relevant (Fig. 3A). The expression of *ABI4* was reduced in *myb96-1* seeds, whereas *myb96-ox* increased *ABI4* expression. To further support this observation, we also analyzed the expression of these genes in wild-type, *myb96-ox*, and *myb96-1* seedlings. Most of the genes examined were transcriptionally unchanged in the mutants, but the expression of *ABI4* was elevated approximately 12-fold in the *myb96-ox* mutant (Fig. 3B). The *ABI3* and *ABI5* genes were also slightly up-regulated in *myb96-ox* (Fig. 3B). However, their expression was not down-regulated in *myb96-1*, possibly due to extremely low expression levels of the genes in wild-type seedlings under normal growth conditions.

Transcript levels of the same set of genes were analyzed in *myb96-1* seeds in the presence of ABA. Most of the genes, except for *AHK1*, *FY*, and *RHA2a*, were induced by ABA. Notably, the ABA induction of *ABI4* and *BLH1* genes was significantly reduced in the *myb96-1* seeds compared with wild-type seeds (Fig. 4), while the ABA induction of the others was not influenced. The expression of two genes was also analyzed in germinating *myb96-ox* seeds in the presence of ABA. Accordingly, their ABA induction was slightly hypersensitive in *myb96-ox* (Supplemental Fig. S7). While the expression of *BLH1* seems to be dependent upon MYB96, based on the close correlation between *ABI4* expression and MYB96 activity (Figs. 3 and 4), we hypothesized that MYB96 is intimately required for the proper expression of *ABI4* in the control of

percentages of the indicated genotypes grown on different concentrations of ABA were quantified after the end of stratification. Radicle emergence was used as a morphological marker for germination. At least 40 seeds per genotype were measured in each replicate. Biological triplicates were averaged. Error bars indicate sE. Statistically significant differences between wild-type Columbia-0 (Col-0) and mutants are indicated by asterisks (Student's *t* test, *P < 0.05). B, ABA dose-response assay. Wild-type, *myb96-ox*, and *myb96-1* seeds were germinated on an increasing concentration of ABA. Germination percentages were scored 2 d after cold stratification. Biological triplicates were averaged. Error bars indicate sE. Statistically significant differences between the wild type and mutants are indicated by asterisks (Student's *t* test, *P < 0.05).
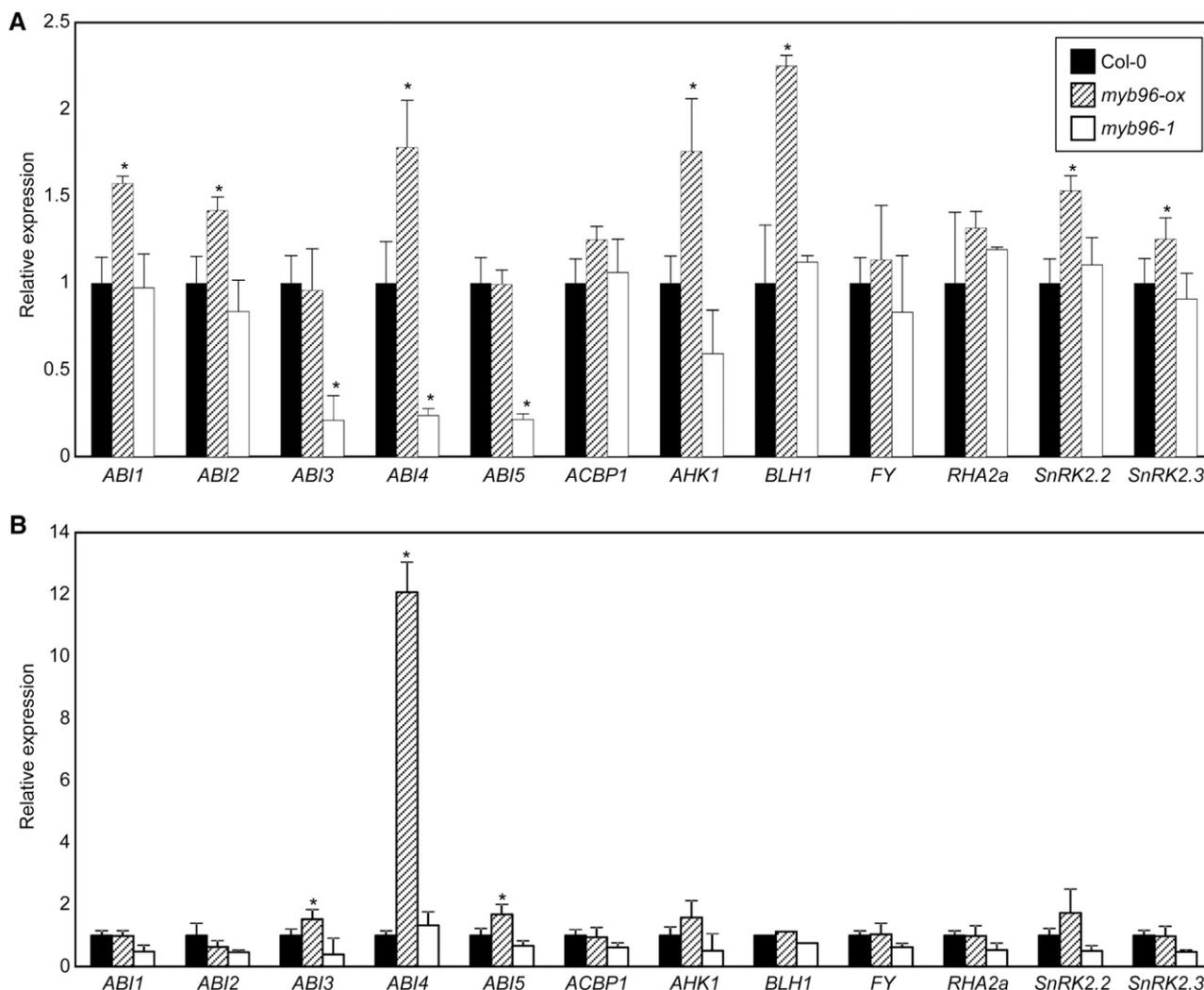
Figure 3. Expression of genes involved in ABA-dependent seed germination in *myb96-ox* and *myb96-1* mutants. Germinating seeds (A) and 10-d-old seedlings (B) grown under long-day conditions were harvested for total RNA isolation. Transcript accumulation was analyzed by qRT-PCR. The *eIF4a* gene was used as an internal control. Biological triplicates were averaged. Error bars indicate SE. Statistically significant differences between wild-type Columbia-0 (Col-0) and mutants are indicated by asterisks (Student's *t* test, *$P < 0.05$).

ABA-mediated seed germination. Consistent with this, the expression patterns of *ABI4* in seeds were similar to those of *MYB96*. The *ABI4* gene was highly expressed in dry seeds, particularly embryo (Söderman et al., 2000; Penfield et al., 2006; Bossi et al., 2009), and its expression was reduced after stratification and imbibition, like the transcript accumulation patterns of *MYB96* (Supplemental Fig. S8). Their spatial expression patterns were also similar (Supplemental Fig. S9), further supporting the positive regulation of *ABI4* by MYB96.

MYB96 Binds Directly to the *ABI4* Promoter

MYB96 is an R2R3-type MYB transcription factor (Seo et al., 2009). Plant R2R3 MYB-DNA-binding domain-containing proteins are known to bind directly to consensus DNA elements that are enriched in A and C residues (Seo et al., 2011; Prouse and Campbell, 2013). Therefore, we asked whether MYB96 binds directly to the consensus motifs on the *ABI4* promoter. Sequence analysis revealed that the *ABI4* promoter contains five conserved sequence motifs that are analogous to the R2R3-type MYB-binding consensus sequences (Fig. 5A). The presence of MYB-binding cis-elements led us to examine whether MYB96 is targeted to the *ABI4* promoter.

To perform chromatin immunoprecipitation (ChIP) assays, we generated *pMYB96:MYB96-MYC* transgenic plants. Total protein extracts from control pBA002 and *pMYB96:MYB96-MYC* transgenic seeds were immunoprecipitated with anti-MYC-antibody. DNA bound to epitope-tagged MYB96 proteins was analyzed by quantitative PCR assays. The ChIP analysis showed that the
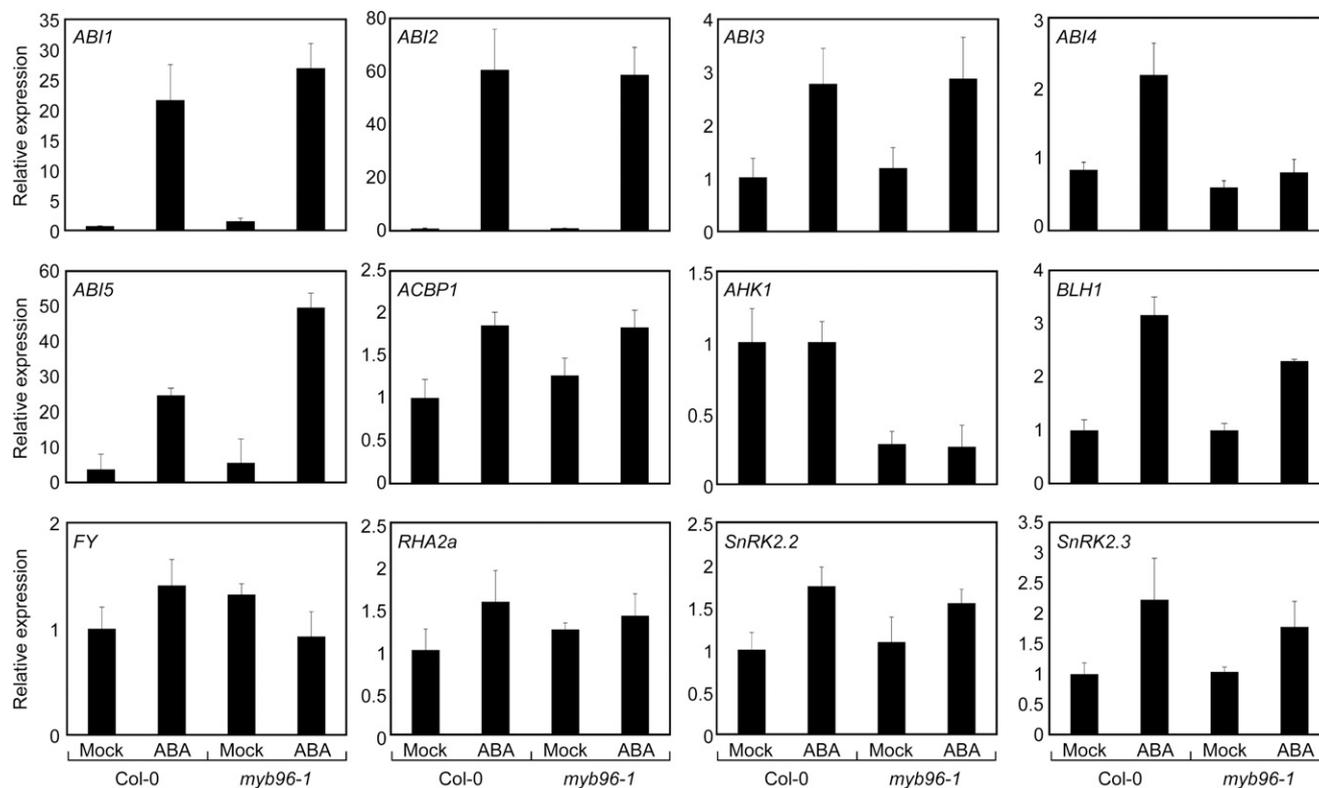
Figure 4. Compromised expression of *ABI4* in *myb96-1* upon exogenous ABA treatment. Germinating seeds were plated on MS medium supplemented with or without 5 μM ABA and incubated for 1 d. Transcript accumulation was analyzed by qRT-PCR. Biological triplicates were averaged. Error bars indicate SE. Col-0, wild-type Columbia-0.

A region of the *ABI4* promoter was enriched by MYB96 (Fig. 5B). In contrast, two other genomic fragments containing MYB-binding cis-elements, B and C, were not enriched (Fig. 5B), supporting the specific interaction of MYB96 with the A region of the *ABI4* promoter. In addition, control ChIP with resin alone did not enrich the A fragment (Fig. 5B). To confirm the direct binding of MYB96 to the *ABI4* promoter, we also generated transgenic plants expressing the R2R3-MYB DNA-binding domain (BD) of MYB96 fused with the MYC-coding sequences (35S:96BD-MYC). ChIP analysis revealed that the MYB DNA-binding domain was sufficient to bind to the *ABI4* promoter (Supplemental Fig. S10).

To confirm the MYB96 binding to core sequences of the *ABI4* promoter, we performed transient expression analysis using Arabidopsis protoplasts. Two core elements on the *ABI4* promoter, A-1 (−264; CAAGTAACTTATGA) and A-2 (−335; GCTATAGTTACCCA), were fused to the 35S minimal promoter. A recombinant reporter plasmid and an effector plasmid, p35S:MYB96, were cotransformed into Arabidopsis protoplasts. Cotransformation with the reporter A-1 increased the GUS activity by 2- to 3-fold, but cotransformation with A-2 did not affect reporter gene expression (Fig. 5C). These results indicate that MYB96 specifically targets proximal sequences upstream of the *ABI4* gene to activate its expression.

MYB96 Contributes to Inhibiting TAG Degradation during Germination

Seed germination is elaborately regulated by discrete actions of the embryo and endosperm (Lafon-Placette and Köhler, 2014). An intriguing example is ABA-controlled lipid breakdown and mobilization in the embryo under suboptimal growth conditions (Penfield et al., 2006). Seed TAG degradation is triggered to fuel embryonic growth during germination but is antagonized by ABA specifically in the embryo (Penfield et al., 2006). It is noteworthy that the ABI4 transcription factor is a well-known regulator of this process, which confers differential sensitivity of lipid catabolism to ABA in the embryo and endosperm (Penfield et al., 2006).

Since *ABI4* is a major regulatory target of the MYB96 transcription factor, we hypothesized that MYB96 may also be responsible for the ABA regulation of lipid breakdown during seed germination, similar to the action of ABI4 (Penfield et al., 2006; Wind et al., 2013). To examine this hypothesis, we measured the levels of total fatty acid and eicosenoic acid (20:1) in wild-type, *myb96-ox*, and *myb96-1* seeds for 3 d after cold imbibition in the absence or presence of exogenous ABA. In the absence of ABA, the levels of total fatty acid and eicosenoic acid were largely indistinguishable in *myb96-ox* and *myb96-1* compared with wild-type seeds
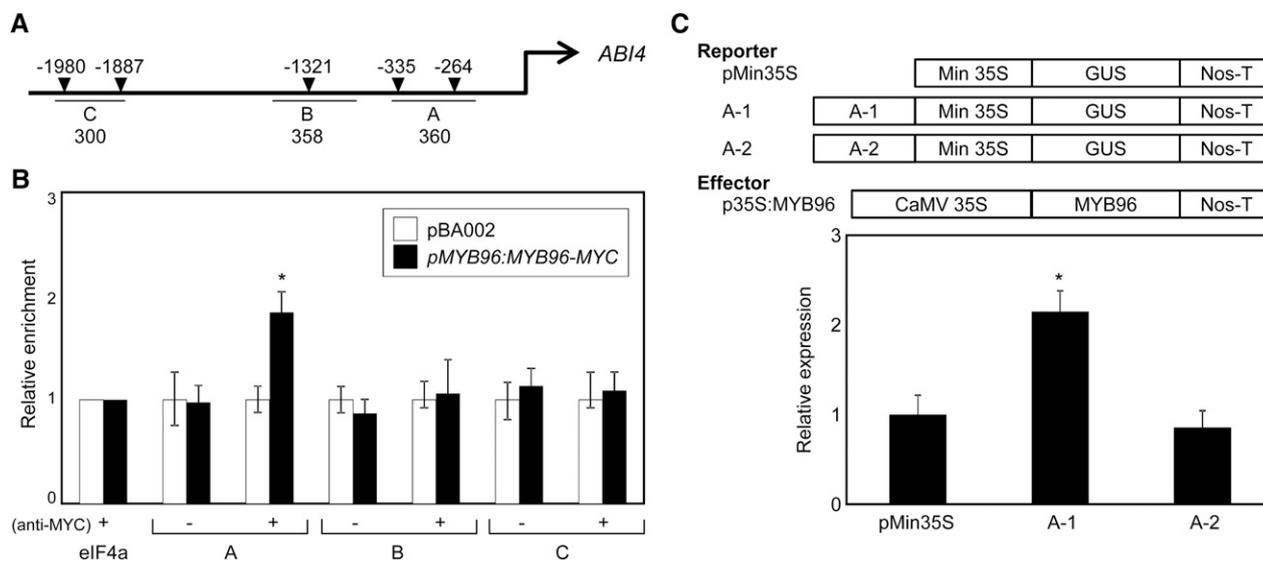
Figure 5. Binding of MYB96 to consensus sequences in the *ABI4* promoter. A, R2R3-MYB-binding consensus sequences on the *ABI4* promoter. Core binding sequences are marked with arrowheads. Underbars represent amplified genomic regions. The numbers below the underbars indicate amplification product size (bp). B, ChIP assays. Total protein extracts from *pMYB96:MYB96-MYC* transgenic seeds were immunoprecipitated with an anti-MYC antibody. Fragmented genomic DNA was eluted from the protein-DNA complexes and subjected to quantitative PCR analysis. Biological triplicates were averaged, and statistically significant differences of the measurements were determined using Student's *t* test (*P < 0.05). Error bars indicate SE. In each experiment, the measurement values in pBA002 were set to 1 after normalization against *eIF4a* for quantitative PCR analysis. C, Transient expression analysis using Arabidopsis protoplasts. The core promoter sequence elements of *ABI4*, A-1 (CAAG-TAACTTATGA) and A-2 (GCTATAGTTACCCA), were constructed in the reporter plasmid. The recombinant reporter and effector constructs were coexpressed transiently in Arabidopsis protoplasts, and GUS activities were determined fluorimetrically. Luciferase gene expression was used to normalize the GUS activities. The normalized values in control protoplasts were set to 1 and represented as relative activation. Three independent measurements were averaged. Statistically significant differences were determined by Student's *t* test (*P < 0.05). Error bars indicate SE. CaMV, Cauliflower mosaic virus; Nos-T, nopaline synthase terminator.

(Supplemental Fig. S11). However, in the presence of ABA, the levels of total fatty acid and eicosenoic acid were noticeably altered in *myb96-ox* and *myb96-1*. Total fatty acid levels were reduced by approximately 30% to 35% 3 d after cold imbibition in wild-type seeds. However, TAG breakdown was largely repressed in the *myb96-ox* seeds, whereas 43% of total TAG was catabolized in the *myb96-1* seeds (Fig. 6A). The levels of eicosenoic acid displayed similar degradation kinetics to total fatty acid (Fig. 6B). These observations demonstrate that MYB96 plays a role in regulating lipid breakdown during seed germination.

ABI4 Is Epistatic to MYB96

Our results indicated that the MYB96-ABI4 transcriptional cascade is crucial for the ABA regulation of seed germination, probably through lipid mobilization. To confirm the genetic relationship between *MYB96* and *ABI4*, we genetically crossed *myb96-ox* to the *abi4-1* mutant.

We investigated the ABA sensitivity of *myb96-ox/abi4-1* seeds during germination. Delayed germination was observed in *myb96-ox* seeds, whereas the *abi4-1* mutation led to accelerated seed germination, in the presence of ABA (Fig. 7A). The germination rate of *myb96-ox/abi4-1*

seeds was comparable to that of *abi4-1* (Fig. 7A), indicating that MYB96 is an upstream regulator of *ABI4* and that both genes act in the same pathway.

In agreement with the seed germination rate, the eicosenoic acid catabolism of *myb96-ox/abi4-1* was also indistinguishable from that of *abi4-1* (Fig. 7B). A significant amount of the stored TAG was degraded in both genotypes, even in the presence of ABA. Therefore, we conclude that *ABI4* is genetically epistatic to *MYB96* with respect to the control of ABA-dependent seed germination as well as TAG catabolism.

Taken together, our observations demonstrate that the MYB96 transcription factor serves as an ABA signaling mediator in modulating seed germination through ABI4-dependent embryonic lipid breakdown (Fig. 7C). MYB96 directly regulates *ABI4*, a key regulator of embryonic lipid mobilization during seed germination, by binding to its gene promoter. Genetic analyses further bolster this genetic hierarchy in the control of seed germination.

DISCUSSION

MYB96 Regulation of ABI4

ABA is perceived by receptor protein complexes that are composed of PYRABACTIN RESISTANCE
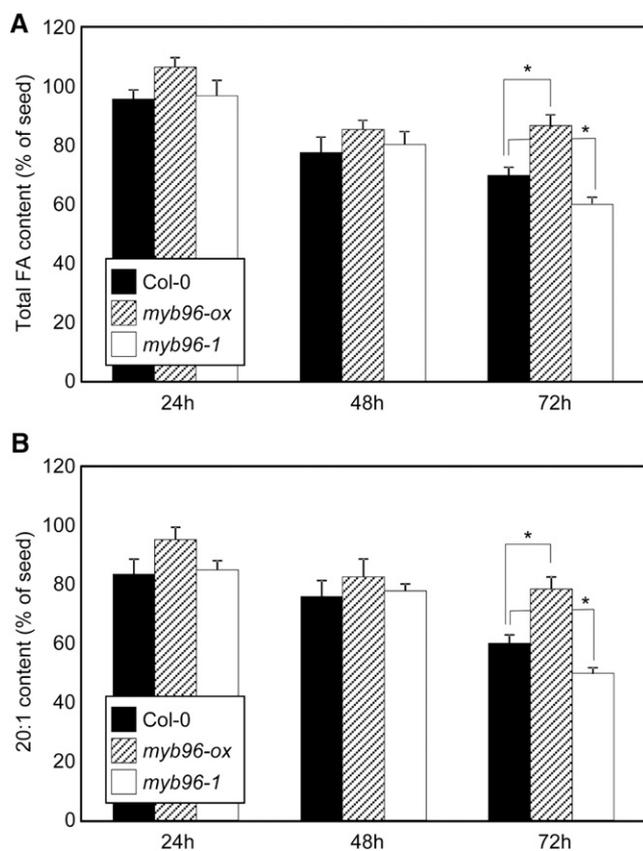
Figure 6. Lipid breakdown in wild-type Columbia-0 (Col-0), *myb96-ox*, and *myb96-1* seeds during seed germination in the presence of ABA. Seeds were germinated on MS plates supplemented with 1 μM ABA and incubated at 23°C under long-day conditions for 3 d. Lipid breakdown was determined by measuring the abundance of total fatty acid (FA; A) and eicosenoic acid (20:1; B). Fatty acid content was expressed as the percentage of the amount present in dry seeds of the indicated genotypes. At least 30 seeds per genotype were measured in each replicate. Biological triplicates were averaged. Error bars indicate SE.

(PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABSCISIC ACID RECEPTOR (RCAR), type 2C protein phosphatases (PP2Cs), and SnRK2s (Raghavendra et al., 2010; Weiner et al., 2010). In the presence of ABA, PYR/PYL/RCAR proteins lead to the suppression of PP2C activities, derepressing SnRK2 protein kinases to activate ABA-responsive binding factors/ABA-responsive element-binding proteins (Fujii et al., 2009; Nishimura et al., 2010; Gonzalez-Guzman et al., 2012). Subsequent ABA responses are mediated by a large number of transcription factors, indicating that transcriptional cascades are a fundamental framework in ABA signal transduction (Giraudat et al., 1992; Busk and Pagès, 1998; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Abe et al., 2003; Pandey et al., 2005; Seo et al., 2009).

In particular, the ABI3, ABI4, and ABI5 transcription factors all play a common role in the ABA regulation of seed germination by constituting combinatorial networks (Söderman et al., 2000; Nakamura et al.,

2001). However, despite their functional similarities, the regulatory mechanisms of ABI3, ABI4, and ABI5 differ markedly. The ABI3 protein is proteolytically degraded by a RING E3 ligase, ABI3-INTERACTING PROTEIN2, a negative regulator of ABA signaling (Zhang et al., 2005). Controlled protein degradation also underlies ABI5 activity regulation. The KEEP ON GOING (KEG) E3 ligase executes ABI5 degradation under normal growth conditions (Stone et al., 2006; Liu and Stone, 2010). In the presence of ABA, KEG is rapidly degraded, promoting the accumulation of high levels of ABI5. Additional E3 ligases, such as DWD HYPERSENSITIVE TO ABSCISIC ACID1 (DWA1), DWA2, and SIZ1 (for SAP [scaffold attachment factor, acinus, protein inhibitor of activated signal transducer and activator of transcription] and Miz1 [Msx2-interacting zinc finger]), further fine-tune the protein accumulation of ABI5 (Miura et al., 2009; Lee et al., 2010).

Compared with ABI3 and ABI5, relatively less is known regarding how ABI4 activity is regulated at the molecular level. This study demonstrates that the MYB96 transcription factor regulates *ABI4* expression by binding directly to the gene promoter and defines its spatial and temporal expression. Both *MYB96* and *ABI4* genes are strongly expressed in the embryo and are responsive to exogenous ABA (Penfield et al., 2006). Furthermore, the promotive effect of ABA on *ABI4* expression is largely impaired in the *myb96-1* mutant, indicating that MYB96 is an unequivocal transcriptional regulator required for the proper expression of *ABI4* in an ABA signaling pathway.

The question, then, is how MYB96 activity is regulated by ABA. Given the myriad targets of MYB96, it is likely that MYB96 acts upstream of ABA signaling pathways. It is probable that controlled protein turnover and/or posttranslational modifications mediated by ABA receptor-like complex might underlie the ABA regulation of MYB96 activity, and the ABA induction of *MYB96* transcription may be a result of feedback regulation. Further work is required to get a comprehensive view of the signaling network built up from MYB96.

TAG Degradation during Seed Germination

The utilization of seed storage reserves, such as carbohydrates, proteins, and lipids, is crucial to drive germination and postgerminative seedling growth (Penfield et al., 2004, 2006; Quettier and Eastmond, 2009), albeit controversial (Pritchard et al., 2002; Kelly et al., 2011). Arabidopsis seeds mainly store TAG as the major carbon reserve, which accumulate at the later stages of seed maturation (Lu and Hills, 2002; Maeo et al., 2009). Following seed imbibition, fatty acids are degraded through β-oxidation (Footitt et al., 2002) and primarily consumed as a form of soluble carbohydrates that are converted by the glyoxylate cycle and gluconeogenesis (Penfield et al., 2005; Pracharoenwattana et al., 2010). In agreement with this, mutants with defective TAG hydrolysis exhibit delayed germination and perturbed postgerminative
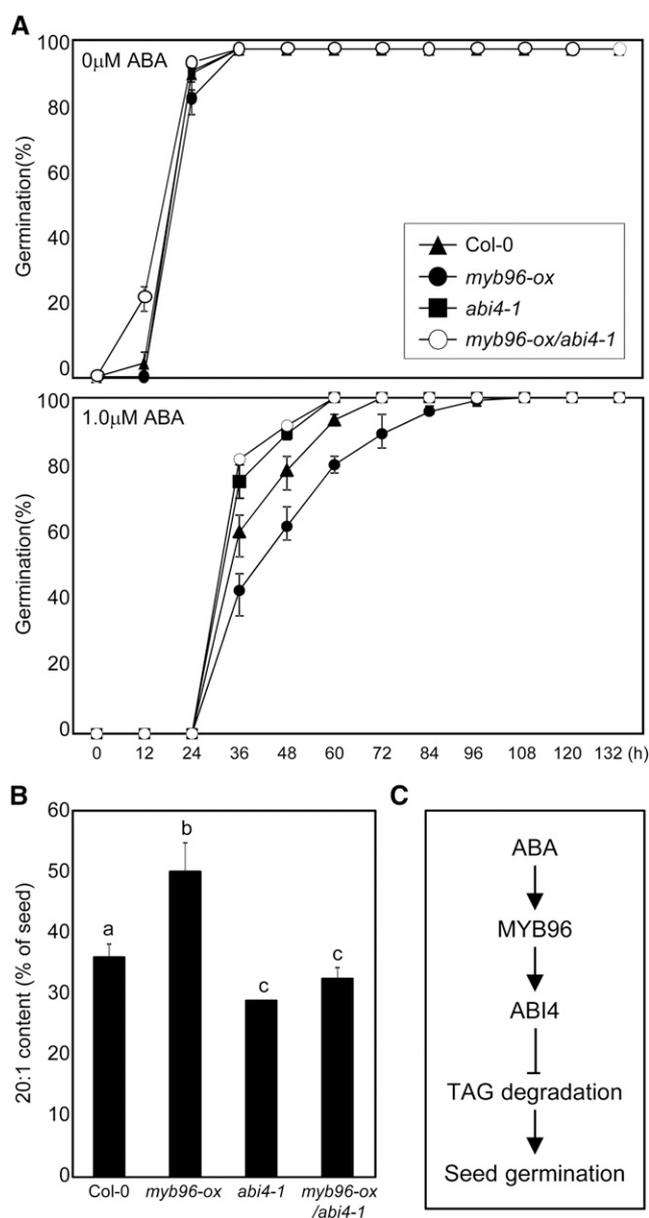
Figure 7. Genetic hierarchy of *ABI4* and *MYB96*. A, Germination rate of *myb96-ox/abi4-1*. The seed germination percentage of each genotype on 1 μM ABA was scored at the indicated time points after cold stratification. At least 50 seeds per genotype were measured in each replicate. Biological triplicates were averaged. Error bars indicate SE. B, Lipid breakdown of *myb96-ox/abi4-1*. Seeds were germinated on MS plates supplemented with 1 μM ABA and incubated at 23°C under long-day conditions for 3 d. Eicosenoic acid (20:1) content was expressed as a percentage of the amount in dry seeds of the indicated genotypes. At least 30 seeds per genotype were measured in each replicate. Biological triplicates were averaged. Error bars indicate SE. C, Proposed role of MYB96 during seed germination. The ABA-inducible R2R3-type MYB96 transcription factor regulates *ABI4* expression by binding directly to its gene promoter. The MYB96-ABI4 module inhibits lipid mobilization specifically in the embryo, thereby contributing to delaying seed germination under suboptimal conditions. Col-0, Wild-type Columbia-0.

seedling growth (Penfield et al., 2005, 2006; Quettier and Eastmond, 2009; Kelly et al., 2011).

Exogenous ABA provokes delayed germination in part by reducing lipid breakdown (Penfield et al., 2006). Genes involved in the conversion of TAG into soluble sugars are down-regulated in response to ABA (Pritchard et al., 2002); thus, the levels of fatty acid-derived sugars in ABA-treated seeds are markedly decreased (Pritchard et al., 2002). Notably, the ABA regulation of lipid breakdown is relevant particularly in embryos (Penfield et al., 2006), indicating the differential sensitivity of embryo and endosperm to ABA. The ABI4 transcription factor is a major regulator in this process. Embryo-expressed ABI4 negatively regulates lipid mobilization and thus seed germination in the presence of ABA (Penfield et al., 2006).

In this study, we discovered the MYB96-ABI4 signaling module that regulates the ABA-dependent seed germination process, possibly through the control of lipid mobilization. MYB96 not only confers ABA sensitivity to embryos but also participates in embryonic lipid mobilization to properly regulate seed germination, similar to the role of ABI4 in seed germination (Penfield et al., 2006). The *myb96-1* seeds degrade a significant content of TAG even in the presence of ABA, whereas TAG breakdown is largely impaired in *myb96-ox* seeds. Given that the MYB96-mediated physiological responses in seeds largely depend on *ABI4*, MYB96 appears to be an explicit transcriptional regulator of *ABI4* to define its expression pattern in seeds and to ensure proper seed germination.

Importance of MYB96-ABI4 Transcriptional Cascades in Seed Germination

The MYB96 transcription factor modulates a variety of plant developmental processes in response to ABA, including shoot development, root architecture remodeling, stomatal closure, cuticular wax accumulation, and hormone biosynthesis (Seo et al., 2009, 2011; Seo and Park, 2010). Based on this study, MYB96 also appears to play a role in ABA-dependent seed germination.

Multiple functions of MYB96 are achieved by regulating myriad transcriptional target genes that underlie a variety of signaling pathways. For example, ABA-induced cuticular wax biosynthesis is mediated by MYB96 regulation of *3-KETOACYL-COENZYME A SYNTHASE* genes encoding rate-limiting enzymes of the very-long-chain fatty acid elongation process (Seo et al., 2011). MYB96-GRETCHEN HAGEN3 transcriptional cascades regulate lateral root development under water-deficient conditions by reestablishing auxin homeostasis (Seo et al., 2009). The *RESPONSIVE TO DESSICATION22* gene is probably one of the regulatory targets of MYB96 that functions in the control of drought tolerance (Seo et al., 2009). LIPID TRANSFER PROTEIN3 is responsible in part for the MYB96 regulation of freezing and drought tolerance (Guo et al., 2013). In addition, the MYB96-ABI4 transcriptional module is crucial for ABA-dependent seed germination. Genetic analysis reveals that delayed germination

of *myb96-ox* is fully suppressed by the *abi4* mutation, indicating that *ABI4* is the principal target of MYB96 in the control of seed germination. Collectively, MYB96 is an important ABA signaling mediator that globally links multiple signaling pathways and thus helps plants to trigger efficient physiological and cellular reprogramming for environmental adaptation.

Meanwhile, ABI4 integrates a variety of external and internal signals, such as light, ABA, reactive oxygen species, and soluble sugars (Pesaresi et al., 2007; Shkolnik-Inbar and Bar-Zvi, 2010; Kerchev et al., 2011; Yang et al., 2011b; Foyer et al., 2012), and regulates hormone biosynthesis and sugar and lipid metabolism (Kerchev et al., 2011; Shu et al., 2013; Wind et al., 2013). Thus, ABI4 coordinates developmental programs with environmental signals to optimize plant growth and development processes. MYB96 is particularly relevant in ABA signal transduction to *ABI4*, and other signaling regulators are likely responsible for the transcriptional regulation of *ABI4* with respect to other input signals.

Taken together, the MYB96-ABI4 signaling module is important for the control of ABA-dependent seed germination. MYB96 precisely conveys ABA signaling to *ABI4* in embryos during seed germination, and ABI4 in turn integrates multitude environmental and developmental information to determine the proper timing for germination. Multiple transcriptional cascades not only allow for signal amplification but also enable plants to make integrative decisions.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Columbia-0 ecotype was used for all experiments described, unless specified otherwise. Plants were grown under long-day conditions (16-h-light/8-h-dark cycle) with cool-white fluorescent light (100 μmol photons m^{-2} s^{-1}) at 23°C. The *myb96-ox* and *myb96-1* mutants (GABI_120B05) were reported previously (Seo et al., 2009). The *abi4-1* mutant (CS8104) was obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/). The *myb96-ox/abi4-1* double mutant was generated by genetic cross of *myb96-ox* and *abi4-1* mutants. Identification of *abi4-1* was performed as described previously (Finkelstein et al., 1998).

qRT-PCR Analysis

Total RNA was extracted using TRI agent (Takara Bio) according to the manufacturer's recommendations. Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (Dr. Protein) with oligo(dT)$_{18}$ to synthesize first-strand complementary DNA (cDNA) from 2 μg of total RNA. Total RNA samples were pretreated with an RNase-free DNase. cDNAs were diluted to 100 μL with Tris-EDTA buffer, and 1 μL of diluted cDNA was used for PCR amplification.

qRT-PCR was performed in 96-well blocks using the Step-One Plus Real-Time PCR System (Applied Biosystems). The PCR primers used are listed in Supplemental Table S1. The value for each set of primers was normalized relative to *eIF4a* (At3g13920). All qRT-PCRs were performed in triplicate using total RNA samples extracted from three independent biological replicates. The comparative $\Delta\Delta C_T$ method, which is a calculation for the relative quantification of target DNA, was employed to evaluate relative quantities of each amplified product in the samples. The threshold cycle was automatically determined for each reaction by the instrument software using default parameters. The specificity of the qRT-PCR was determined by melt-curve analysis of the amplified products using the standard method installed in the system.

Measurement of GUS Activity

Seeds were dissected into embryo and endosperm surrounded with seed coat. For histochemical staining of GUS activity, plant materials were fixed by immersing in 90% (v/v) acetone for 20 min on ice, washed twice with rinsing solution [50 mM sodium phosphate, pH 7, 0.5 mM K$_3$Fe(CN)$_6$, and 0.5 mM K$_4$Fe(CN)$_6$], and subsequently incubated in staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Duchefa) at 37°C for 18 to 24 h, depending on staining intensity (Padmanaban et al., 2007).

Seed Germination Assays

All genotypes were grown at 23°C under long-day conditions, and seeds were collected at the same time. Harvested seeds were dried at room temperature at least 1 month before germination assays. For seed germination assays, 40 to 50 seeds for each line were sterilized and plated on MS medium (one-half-strength MS salts, 0.05% [w/v] MES, pH 5.7, and 0.7% [w/v] agar) supplemented with various concentrations of ABA (0, 0.5, 1, and 3 μM). Plates were stratified in darkness for 3 d at 4°C and transferred to a culture room set at 23°C with a 16-h-light/8-h-dark cycle. Germination was scored at the indicated time points by counting the frequency of radicle emergence from the seed coat and endosperm. For each germination assay, biological triplicates were performed.

To investigate the effects of ABA on cotyledon greening, the percentage of cotyledon greening was scored after the end of stratification. Cotyledon greening was defined as complete expansion of the cotyledon and greening.

ChIP Assays

ChIP assays were performed as described previously (Yang et al., 2011a). *pMYB96:MYC-MYB96* transgenic plants, anti-MYC antibodies (Millipore), and salmon sperm DNA/protein A agarose beads (Millipore) were used for ChIP. DNA was purified using phenol/chloroform/isoamyl alcohol and sodium acetate (pH 5.2). The level of precipitated DNA fragments was quantified by quantitative real-time PCR using specific primer sets (Supplemental Table S2). Values were normalized with the level of input DNA. The value in pBA002 control plants was set to 1 after normalization against *eIF4a* for quantitative PCR analysis.

Transient Gene Expression Assays

For transient expression assays using Arabidopsis protoplasts, reporter and effector plasmids were constructed. The reporter plasmid contains a minimal 35S promoter sequence and the *GUS* gene. The core elements on the *ABI4* promoter were inserted into the reporter plasmid. To construct the p35S:MYB96 effector plasmid, the MYB96 cDNA was inserted into the effector vector containing the cauliflower mosaic virus 35S promoter. Recombinant reporter and effector plasmids were cotransformed into Arabidopsis protoplasts by polyethylene glycol-mediated transformation (Yoo et al., 2007). GUS activities were measured by a fluorometric method. A cauliflower mosaic virus 35S promoter-luciferase construct was also cotransformed as an internal control. The luciferase assay was performed using the Luciferase Assay System kit (Promega).

Fatty Acid Determinations

The total fatty acid content and amount of eicosenoic acid (20:1 n-9) in seeds were measured by gas chromatographic analysis with a known amount of 15:0 fatty acid as an internal standard. Samples were transmethylated at 90°C for 90 min in 0.3 mL of toluene and 1 mL of 5% (v/v) H$_2$SO$_4$ (in methanol). After transmethylation, 1.5 mL of 0.9% (w/v) NaCl solution was added, and the fatty acid methyl esters were transferred to a new tube for three sequential extractions with 1.5 mL of *n*-hexane. Fatty acid methyl esters were analyzed by gas chromatography using a GC-2010 Plus instrument (Shimadzu) with a 30-m × 0.25-mm (i.d.) HP-FFAP column (Agilent), during which the oven temperature was increased from 170°C to 180°C at 1°C min^{-1}.

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ABI1* (At4g26080), *ABI2* (At5g57050), *ABI3* (At3g24650), *ABI4* (At2g40220), *ABI5* (At2g36270), *MYB96* (At5g62470), *ACBP1* (At5g53470), *AHK1* (At2g17820), *BLH1* (At2g35940), *FY* (At5g13480),

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RHA2a (At1g15100), *SnRK2.2* (At3g50500), *SnRK2.3* (At5g66880), and *eIF4a* (At3g13920).

Supplemental Data

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

Supplemental Figure S1. Temporal expression of *MYB96*.

Supplemental Figure S2. Expression of *MYB96* in embryo after cold imbibition.

Supplemental Figure S3. ABA induction of *MYB96* in embryo.

Supplemental Figure S4. Expression of *MYB96* in quadruple-DELLA and GA-deficient mutants.

Supplemental Figure S5. Cotyledon opening of *myb96-ox* and *myb96-1* in the presence of ABA.

Supplemental Figure S6. ABA dose-response assays.

Supplemental Figure S7. Expression of *ABI4* and *BLH1* in the *myb96-ox* mutant in the presence of ABA.

Supplemental Figure S8. *ABI4* expression in dry seeds and during seed germination.

Supplemental Figure S9. Spatial expression of *MYB96* and *ABI4*.

Supplemental Figure S10. ChIP using 35S:*96BD-MYC* transgenic plants.

Supplemental Figure S11. Lipid breakdown in wild-type, *myb96-ox*, and *myb96-1* seeds during seed germination in the absence of ABA.

Supplemental Table S1. Primers used in qRT-PCR.

Supplemental Table S2. Primers used in ChIP assays.

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