

# ARABIDOPSIS THALIANA HOMEBOX25 Uncovers a Role for Gibberellins in Seed Longevity<sup>1[C][W]</sup>

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Seed longevity is crucial for agriculture and plant genetic diversity, but it is limited by cellular damage during storage. Seeds are protected against aging by cellular defenses and by structures such as the seed coat. We have screened an activation-tagging mutant collection of *Arabidopsis* (*Arabidopsis thaliana*) and selected four dominant mutants with improved seed longevity (*isl1-1D* to *isl4-1D*) under both natural and accelerated aging conditions. In the *isl1-1D* mutant, characterized in this work, overexpression of the transcription factor ARABIDOPSIS THALIANA HOMEBOX25 (*ATHB25*; At5g65410) increases the expression of *GIBBERELLIC ACID3-OXIDASE2*, encoding a gibberellin (GA) biosynthetic enzyme, and the levels of GA<sub>1</sub> and GA<sub>4</sub> are higher (3.2- and 1.4-fold, respectively) in the mutant than in the wild type. The morphological and seed longevity phenotypes of the *athb25-1D* mutant were recapitulated in transgenic plants with moderate (4- to 6-fold) overexpression of *ATHB25*. Simultaneous knockdown of *ATHB25*, *ATHB22*, and *ATHB31* expression decreases seed longevity, as does loss of *ATHB25* and *ATHB22* function in a double mutant line. Seeds from wild-type plants treated with GA and from a quintuple *DELLA* mutant (with constitutive GA signaling) are more tolerant to aging, providing additional evidence for a role of GA in seed longevity. A correlation was observed in several genotypes between seed longevity and mucilage formation at the seed surface, suggesting that GA may act by reinforcing the seed coat. This mechanism was supported by the observation of a maternal effect in reciprocal crosses between the wild type and the *athb25-1D* mutant.

Seed longevity is an important trait for maintaining plant genetic diversity in nature and seed banks and for providing reliable crop seeds to farmers. Seeds gradually lose their viability during storage, and the rate of aging is strongly influenced by environmental and genetic factors (Rajjou and Debeaujon, 2008). The accumulation of reactive oxygen species and free radicals has often been considered as one of the most important factors influencing seed aging (Bailly, 2004). Accordingly, *ferric-chelate reductase1*, a mutant in mitochondrial NADH dehydrogenase showing constitutive reactive oxygen species accumulation, produces seeds very

sensitive to aging (Clerkx et al., 2004). Reactive oxygen species induce lipid oxidation, and *Arabidopsis* (*Arabidopsis thaliana*) mutants (*vitamin E deficient1* and *vitamin E deficient2*) affected in the biosynthesis of vitamin E (tocopherol, a lipophilic antioxidant) produce seeds with reduced longevity (Sattler et al., 2004). Repair of oxidized proteins by Met sulfoxide reductase (Châtelain et al., 2013), of oxidized DNA by DNA glycosylase/Apurinic/Apyrimidinic lyase (Chen et al., 2012), and of chemically degraded proteins by L-isoaspartyl methyltransferase (Ogé et al., 2008) enhances seed longevity. Other important factors are chaperones or heat shock proteins. Transgenic tobacco (*Nicotiana tabacum*) plants overexpressing the seed-specific transcription factor Heat Shock FactorA9 (Kotak et al., 2007) produce seeds with increased amounts of heat shock proteins that have improved longevity (Prieto-Dapena et al., 2006). On the other hand, a double mutant in two redundant peptidyl-prolyl cis-trans-isomerases, *rotamase fkbp1 rotamase fkbp2*, has seeds more sensitive to aging than the wild type (Bissoli et al., 2012). In addition to cellular stress defenses and repair systems, the protective effects of the seed coat or testa are very important for seed longevity (Rajjou and Debeaujon, 2008). Seeds from both structural and pigmentation mutants in the seed coat lose viability during aging faster than wild-type seeds (Debeaujon et al., 2000; Clerkx et al., 2004).

The differentiation of the seed coat, including mucilage production, occurs during the morphogenesis phase of seed development, while the accumulation

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of storage compounds and stress defenses and the acquisition of dormancy and desiccation tolerance occur during the second maturation phase (Holdsworth et al., 1999; Braybrook and Harada, 2008; Western, 2012). These processes are orchestrated by the LEAFY COTYLEDON (LEC) transcription factors (LEC1, LEC2, and FUSCA3 in Arabidopsis) and mediated by the growth hormones auxin and gibberellin (GA), the stress hormone abscisic acid (ABA), and the seed-specific, ABA-activated transcription factor ABSCISIC ACID INSENSITIVE3 (ABI3) (Holdsworth et al., 1999; Gutierrez et al., 2007; Braybrook and Harada, 2008; Suzuki and McCarty, 2008). ABI3 activates the previously mentioned transcription factor HSF1A9 (Kotak et al., 2007). Mutants affected in seed development, such as *lec1*, *lec2*, *fus3*, and *abi3*, have seeds of poor desiccation tolerance and longevity (Holdsworth et al., 1999; Clerkx et al., 2003, 2004).

In the maturation phase of seed development, a progressive increase in ABA levels prepares the seeds to resist desiccation and aging in a dormant state. The levels of GAs are reduced during the latest stages of maturation, but an increase in GA is required to break the ABA-induced dormancy at the time of germination (Holdsworth et al., 1999; Braybrook and Harada, 2008). No connection between GA and seed longevity has been found in previous genetic analyses (Clerkx et al., 2004).

Blind genetic screens to identify crucial genes for seed longevity have never been made. A genetic analysis to map quantitative trait loci for seed longevity has been performed (Nguyen et al., 2012), but no genes were identified so far. Also, all mutants previously described were obtained from known genes of stress defenses or involved in seed development. We have screened an "activation-tagging" mutant collection of Arabidopsis (Weigel et al., 2000), selecting for tolerance of seeds to natural and accelerated aging (Tesnier et al., 2002; Tejedor-Cano et al., 2010). As a first result, we report that the transcription factor ARABIDOPSIS THALIANA HOMEODOMAIN2/ZINC FINGER HOMEODOMAIN2/ZINC FINGER HOMEODOMAIN1 (ATHB25/ZFHD2/ZHD1; At5g65410) is an important determinant of seed longevity by positively regulating the GIBBERELLIC ACID3-OXIDASE2 (*GA3OX2*) gene and, therefore, GA biosynthesis. Thus, GA has a novel and unexpected role during seed development, increasing longevity by a mechanism unrelated to ABA and stress defenses. This may involve reinforcement of the seed coat, because the *athb25-1D* mutation (overexpressing *ATHB25*) exhibits a maternal effect.

## RESULTS

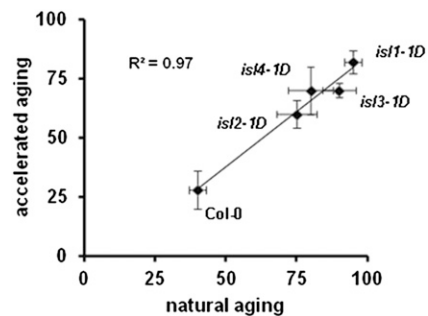
### Isolation of Arabidopsis Mutants with Increased Seed Longevity

We screened 30,000 lines of an Arabidopsis activation-tagging mutant collection (approximately 150,000 seeds) for increased seed longevity as determined by an accelerated aging procedure of imbibed seeds (24 h at 42°C in water; see "Materials and Methods"). In order to discern

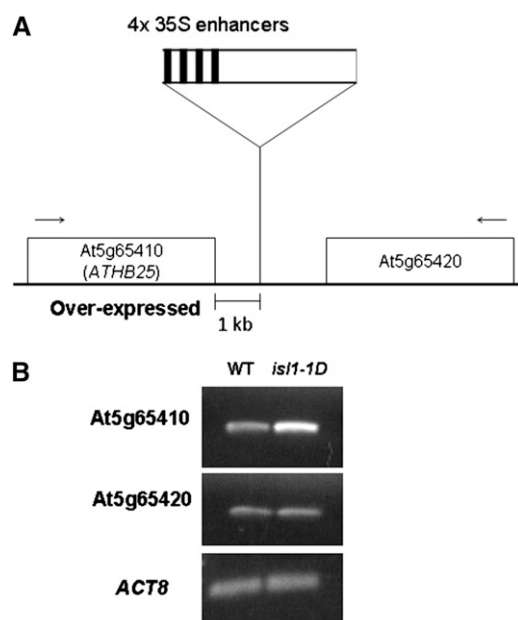
if our aging procedure induces secondary dormancy, we tested if there is an increase of ABA (Holdsworth et al., 1999; Braybrook and Harada, 2008), because it has been reported that high temperatures induce ABA biosynthesis during germination (Toh et al., 2008). Under our experimental conditions (high temperature during accelerated aging but normal temperature during germination), ABA levels are not increased (Supplemental Fig. S1); therefore, our mutants are not related to the breaking of secondary dormancy. Four dominant mutants were identified and named *isl1-1D* (for increased seed longevity, allele 1, dominant) to *isl4-1D*, and we could demonstrate a good correlation between the germination of wild-type and mutant seeds after our accelerated aging procedure and after natural aging in the dry state during 30 months at 20°C to 22°C and 40% to 50% humidity (Fig. 1). The characterization of the *isl1-1D* mutant is the subject of this work. The other mutants are not allelic to *isl1-1D* and will be described elsewhere.

### Overexpression of *ATHB25* Increases Seed Longevity

We detected in the *isl1-1D* mutant the presence of only one transfer DNA (T-DNA) insertion by Southern blot (Supplemental Fig. S2). The insertion was located by plasmid rescue and sequencing to a region between the At5g65410 and At5g65420 genes (Fig. 2A). The 35S transcriptional enhancer was close to the promoter of the At5g65410 gene, encoding transcription factor *ATHB25/ZFHD2/ZHD1*. This gene, but not At5g65420, was overexpressed in the mutant (Fig. 2B). In order to test whether the tolerance to aging of this mutant was linked to the T-DNA insertion, homozygous mutant plants were crossed to Columbia wild-type plants. From the segregating F2 generation, 30 plants from seeds tolerant to accelerated aging were selected, and the T-DNA was detected by PCR in all of them. Accordingly,



**Figure 1.** Correlation between germination after accelerated aging and after natural aging in the mutants with increased seed longevity. Seeds from wild-type Columbia (Col-0) and from four *isl* mutants were subjected to natural aging (dry seeds incubated during 30 months at room temperature and humidity) or to accelerated aging (seeds imbibed in water and incubated for 24 h at 42°C). The percentage of germination (mean of three experiments with 100 seeds each with  $\pm$  in both coordinates) is shown for the five genotypes as well as the correlation coefficient.



**Figure 2.** Molecular characterization of the *isl1-1D* mutant. A, Location of the T-DNA in the intergenic region between At5g65410 and At5g65420 as determined by plasmid rescue. B, RT-PCR semiquantitative analysis of At5g65410, At5g65420, and *ACTIN8* (*ACT8*) mRNAs from the wild type (WT) and the *isl1-1D* mutant.

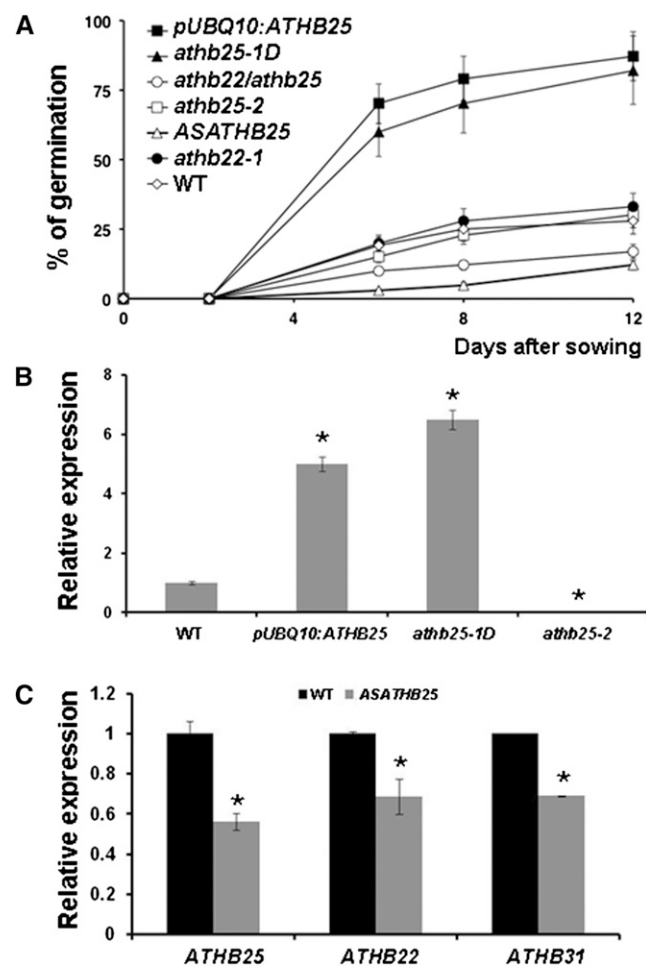
we conclude that the phenotype of this mutant is linked to the T-DNA ( $\chi^2 = 10$  and  $P < 0.0025$  for the hypothesis that it follows a 3:1 segregation, which must be rejected).

As indicated above, the At5g65410 gene has received three different names: *ATHB25* (Tan and Irish, 2006), *ZFHD2* (Tran et al., 2007), and *ZHD1* (Hu et al., 2008). We respect the first naming (*ATHB25*), and the over-expression mutant *isl1-1D* will be renamed *athb25-1D*.

To confirm the relationship between *ATHB25* and seed longevity, we generated transgenic plants in which the complementary DNA (cDNA) of the gene was cloned in sense and antisense orientations under the control of two promoters expressed in nearly all tissues: the strong 35S promoter (Benfey and Chua, 1990) and the moderate *UBIQUITIN10* promoter (Grefen et al., 2010). Transgenic lines overexpressing *ATHB25* from the *UBQ10* promoter expressed *ATHB25* at levels similar to those of the *athb25-1D* mutant (5- to 6-fold wild-type levels; Fig. 3B) and exhibited improved germination after accelerated aging (Fig. 3A). It must be remarked that all lines overexpressing *ATHB25* exhibited normal germination in the absence of the aging treatment (data not shown), and the expression of *ATHB25* was not significantly affected by the accelerated aging treatment (Supplemental Fig. S3). Five tested transgenic lines containing the 35S promoter had very high expression levels of *ATHB25* (40- to 50-fold wild-type levels) and did not show any improvement in seed longevity. These 35S promoter lines did not exhibit the GA-dependent phenotypes of the original mutant and the *UBQ10*

promoter lines (see below). These results suggest that the increased expression of *ATHB25* should be tightly regulated to improve seed longevity.

Further evidence supporting the role of *ATHB25* in seed longevity is the poor seed viability observed after



**Figure 3.** *ATHB25* (At5g65410) plays an important role in the seed longevity of Arabidopsis. A, Seeds from *pUBQ10:ATHB25*, activation-tagging mutant *isl1-1D* (referred to as *athb25-1D*), double null mutant *athb22-1 athb25-2*, single null mutant *athb25-2*, *ASATHB25*, single null mutant *athb22-1*, and the wild type (WT) were subjected to accelerated aging for 24 h and sown on MS plates. The percentage of germination was recorded at the indicated times. The results are averages of six experiments with 100 seeds per line, and the SE is indicated by error bars. Untreated lines germinated more than 99% after 3 d. B, Expression levels of *ATHB25* in the wild type and mutants with gain (*pUBQ10:ATHB25* and *athb25-1D*) and loss (*athb25-2*) of function of *ATHB25* determined by quantitative RT-PCR. C, Expression levels of *ATHB25* (At5g65410) and related genes *ATHB22* (At4g24660) and *ATHB31* (At1g14440) in the wild type and *ASATHB25* (three independent lines were tested with similar results). In B and C, results are averages of three determinations, and error bars correspond to SE. Asterisks indicate significant differences with controls (the wild type) at  $P < 0.05$ . In the case of *pUBQ10:ATHB25* and *ASATHB25*, three independent transgenic lines were tested with similar results. In B and C, plants (10-d-old seedlings grown in solid MS medium) were grown under long-day conditions before RNA extraction.

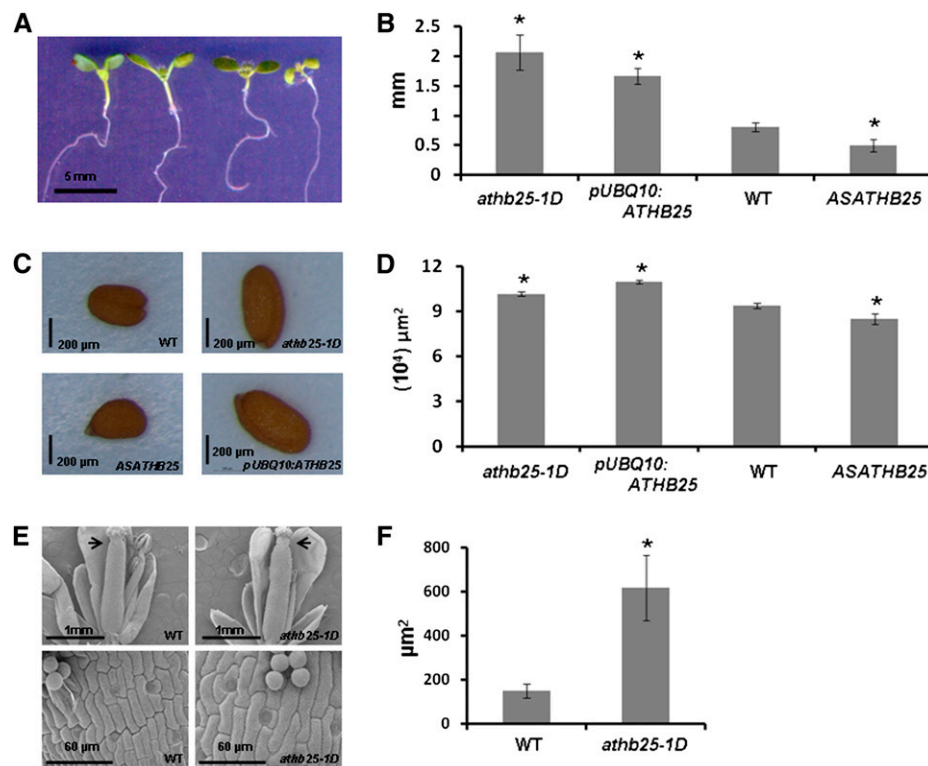
accelerated aging treatment of the lines expressing an antisense construct of this gene with the 35S promoter (Fig. 3A), where the expression level of *ATHB25* is compromised (Fig. 3C). However, a loss-of-function mutant (*athb25-2*) did not show sensitivity to aging despite greatly reduced expression of this gene (Fig. 3B). This can be explained by the high sequence similarity (more than 75% sequence identity at the nucleotide level) and genetic redundancy of the Arabidopsis zinc finger-homeobox gene family, consisting of 14 members with overlapping regulatory roles in Arabidopsis floral development (Tan and Irish, 2006). As indicated in Figure 3C, the antisense construct of *ATHB25* inhibits the expression of two other members of this family, the closely related genes At4g24660 (*ATHB22*) and At1g14440 (*ATHB31*), and this may explain the observed phenotype. This cross reaction of antisense constructs has been observed in other gene families with high sequence identity (Tieman et al., 2001).

*ATHB22* is the zinc finger homeodomain most similar to *ATHB25* (Tan and Irish, 2006). Additional evidence for a redundant role of the corresponding genes (*ATHB25* and

*ATHB22*) in seed longevity has been provided by determining the tolerance to accelerated aging of a double mutant in both genes. As indicated in Figure 3A, the double mutant exhibits a reduced tolerance to accelerated aging, a phenotype similar to that of the antisense line.

#### GA-Dependent Phenotypes in *ATHB25*-Overexpressing Lines

*ATHB25* is significantly expressed in the shoot apex, vegetative rosette, flowers, and seed embryo (Arabidopsis electronic Fluorescent Pictograph browser; <http://bbc.botany.utoronto.ca>); therefore, we carried out a phenotypic characterization in shoots of the *athb25-1D* mutant and transgenic plants overexpressing *ATHB25* from the *UBQ10* promoter. At first glance, both lines displayed pleiotropic phenotypes that may be associated with typical responses to growth-promoting hormones such as GAs and auxins. These include elongated petioles and hypocotyls (Fig. 4, A and B), earlier flowering,



**Figure 4.** *ATHB25* regulates GA-dependent phenotypes. A, Hypocotyl length of representative seedlings of *athb25-1D*, *pUBQ10:ATHB25*, the wild type, and *ASATHB25* (from left to right, with one representative plant for every line) on MS medium under long-day conditions for 7 d. B, Means from three independent experiments as in A with 10 plants from each line. C, Representative dry seeds of *athb25-1D*, *pUBQ10:ATHB25*, the wild type (WT), and *ASATHB25*. D, Means from three independent experiments of the projected area of 50 dry seeds from each line as in C. E, Scanning electron micrographs of flowers (top) and cells of the style (bottom) of the wild type and *athb25-1D*. F, Means from 10 cells of the style areas from the lines shown in E. Arrows indicate the locations of the cells measured in the flower; error bars indicate *se*, and asterisks indicate significant differences from controls (the wild type) at  $P < 0.05$ . In the case of *pUBQ10:ATHB25* and *ASATHB25*, three independent transgenic lines were tested with similar results. [See online article for color version of this figure.]

larger seeds (Fig. 4, C and D), wider siliques, and increased cell size (Fig. 4, E and F). Plants expressing an antisense construct of *ATHB25* from the 35S promoter have shorter petioles and hypocotyls (Fig. 4, A and B) and smaller seeds (Fig. 4, C and D).

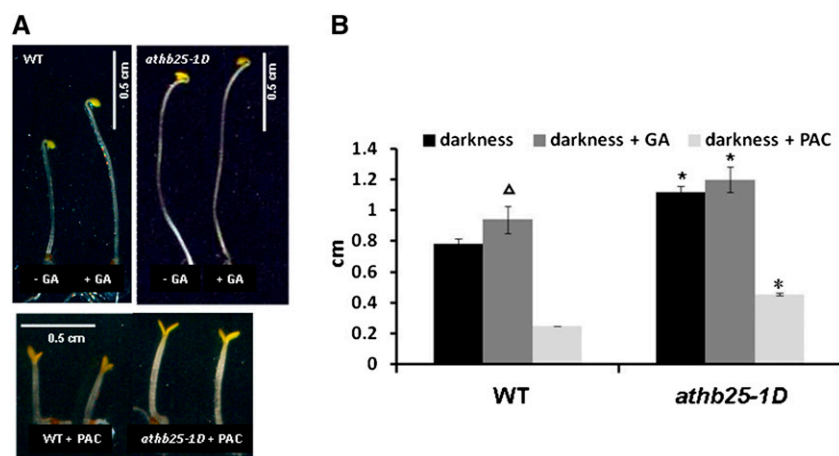
In order to determine which growth-promoting hormone(s) could explain these phenotypes, other auxin-related phenotypes, such as apical dominance or epinastic cotyledons (Kim et al., 2007), were analyzed, but no changes with respect to the wild type were observed (data not shown). Next, we tested whether GA signaling is activated in *athb25-1D* by monitoring hypocotyl length in the presence of paclobutrazol, an inhibitor of GA biosynthesis (Cowling and Harberd, 1999). In darkness and after paclobutrazol treatment, the reduction in the hypocotyl elongation of *athb25-1D* (58%) was less effective than in the wild type (70%; Fig. 5). This result and the failure of the already elongated hypocotyls of *athb25-1D* to respond to exogenous GA for elongation (Fig. 5A) reveal that *ATHB25* is somehow related to the synthesis or the signal transduction of this important phytohormone.

In order to determine whether GA-dependent phenotypes exhibited by *ATHB25*-overexpressing lines are due to the overproduction of this hormone, as observed in lines overexpressing *GA20OX1* (Huang et al., 1998), or due to the constitutive activation of the GA signaling pathway, as in *spindly* mutants (Jacobsen and Olszewski, 1993), the level of the endogenous bioactive GA was measured in 10-d-old seedlings. Wild-type seedlings accumulated the expected levels of both  $GA_4$  and  $GA_1$  (Huang et al., 1998). However, *athb25-1D* seedlings accumulated 1.4 times more  $GA_4$  and showed a 3.2-fold increase in the level of  $GA_1$  (Fig. 6A). Then, we used quantitative reverse transcription (RT)-PCR to measure the expression levels of the four genes of GA 3-oxidases and the five genes of GA 20-oxidases in Arabidopsis, whose induction could explain the high level of GA accumulated in the *athb25-1D* mutant (Hedden and Thomas, 2012). The expression levels of *GA3OX1* and *GA3OX3* were below detection, but we found a 2.5-fold induction of *GA3OX2* in this mutant (Fig. 6B). As

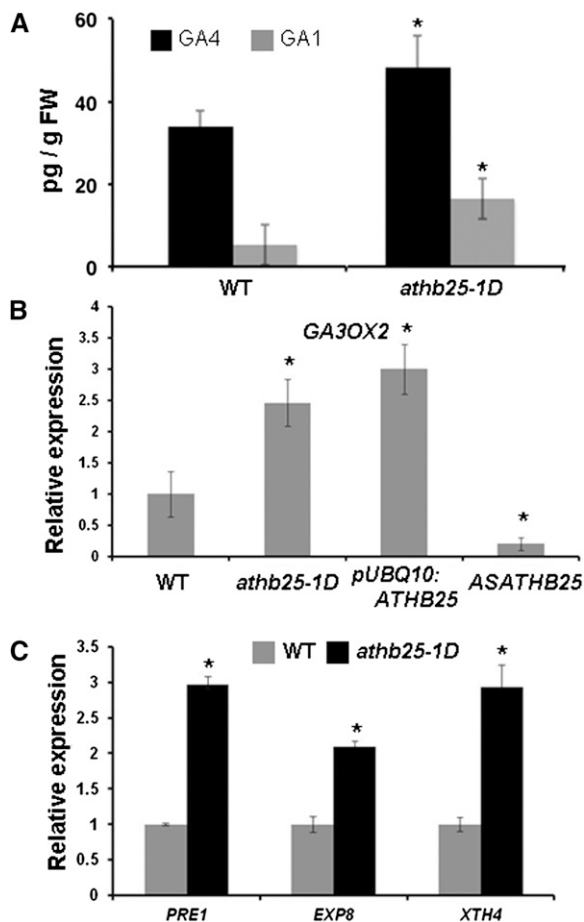
indicated in Supplemental Figure S4, *GA3OX4* and some GA 20-oxidase genes (*GA20OX1* and *GA20OX3*) were only slightly induced (less than 1.5-fold) in the *athb25-1D* mutant, while *GA20OX4* and *GA20OX5* were repressed in this mutant. *GA3OX2* is a key gene responsible for GA biosynthesis (Curaba et al., 2004) and is mainly expressed in germinating seeds (Yamaguchi et al., 2001), predominantly in the hypocotyls (Mitchum et al., 2006). The regulation of the expression of this GA 3-oxidase by *ATHB25* was confirmed by the induction of *GA3OX2* in the *PUBQ10:ATHB25* lines and by the repression in the antisense (*ASATHB25*) lines (Fig. 6B). In agreement with the described results, we observed that several GA response genes implicated in the control of cell elongation, such as *PACLOBUTRAZOL RESISTANCE1 (PRE1)*, *EXPANSIN8 (EXP8)*, *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE4 (XTH4)* (Ogawa et al., 2003; Bai et al., 2012), have increased expression in the *athb25-1D* mutant with respect to the wild type (Fig. 6C). This may explain why the *athb25-1D* mutant presents larger cells in the style of the flower (Fig. 4, E and F).

#### Global Gene Expression of the *ATHB25* Overexpression Mutant Reveals an Induction of GA-Responsive Genes

In order to further characterize the *athb25-1D* mutant, we studied its differential gene expression with respect to the wild type by microarray experiments. A 2-fold change threshold was used to identify 598 differentially expressed genes; 208 genes were up-regulated and 390 genes were down-regulated in *athb25-1D* as compared with wild-type plants (Table I; Supplemental Table S1). We failed to identify any overrepresented functional categories among the up-regulated genes using Gene Ontology. However, by comparing this group of genes with those induced after  $GA_3$  treatment (Nottingham Arabidopsis Stock Centre [NASC] array-177), we found a significant overlap ( $P = 0.037$ ) between these two sets of genes: 24 genes (11.5%) induced in *athb25-1D* were also induced after  $GA_3$  treatment (Tables I and II).



**Figure 5.** Effects of GA and paclobutrazol (PAC) in the *athb25-1D* mutant. A, Hypocotyls of representative 5-d-old seedlings of the wild type (WT) and *athb25-1D* grown in darkness with or without 50  $\mu$ M GA (top) and with 0.5  $\mu$ M PAC (bottom). B, Means from three independent experiments quantifying the hypocotyl length in 10 plants of lines as in A. Error bars indicate se, and asterisks indicate significant differences from controls (the wild type) at  $P < 0.05$ ; the triangle indicates a significant difference ( $P < 0.05$ ) between darkness and darkness + GA in the wild type. [See online article for color version of this figure.]



**Figure 6.** *ATHB25* increases GA levels and the expression of *GA3OX2*. A, Endogenous GA<sub>4</sub> and GA<sub>1</sub> levels in 10-d-old *athb25-1D* and wild-type (WT) seedlings. FW, Fresh weight. B, Quantitative RT-PCR analyses were performed in triplicate on RNA samples from 10-d-old seedlings. Relative values refer to the expression levels of the *GA3OX2* gene in the *athb25-1D* mutant, *pUBQ10:ATHB25*, and *ASATHB25* with respect to the wild type, which was taken as 1. Error bars indicate *se*, and asterisks indicate significant differences from controls (the wild-type) at  $P < 0.05$ . C, Quantitative RT-PCR analyses were performed in triplicate on RNA samples from 10-d-old seedlings. Relative values refer to the expression levels of *PRE1*, *EXP8*, and *XTH4* genes in the *athb25-1D* mutant with respect to the wild type, which was taken as 1. Error bars indicate *se*, and asterisks indicate significant differences from controls (the wild type) at  $P < 0.05$ .

Analyzing down-regulated genes, we found several overrepresented Gene Ontology categories involved in “response to stimulus.” The dominant categories for repressed genes were “defense response” ( $P = 1.06e^{-10}$ ), including “response to water deprivation” ( $P = 3.94e^{-5}$ ), “response to biotic stimulus” ( $P = 2.41e^{-3}$ ), and “response to ABA” ( $P = 3.7e^{-3}$ ; Table I).

Determining the expression of several genes by quantitative RT-PCR validated the microarray results. Among induced genes, three of the GA-responsive genes, *PRE1*, *EXP8*, and *XTH4*, were already analyzed (Fig. 6C). In addition, the expression of the gene encoding the epithiospecifier protein was tested. In the

group of repressed genes, *LEUCINE RICH REPEAT PROTEIN1* (Choi et al., 2012) and *MITOGEN ACTIVATED PROTEIN KINASE/EXTRACELLULAR SIGNAL REGULATED KINASE KINASE KINASE1* (Suarez-Rodriguez et al., 2007) were analyzed as representative kinases involved in defense signaling. All of them confirmed the microarray results (Supplemental Fig. S5).

We carried out a “preferentially located motif” study (Bernard et al., 2010) in the two sets of differentially regulated genes (Table I). This approach allows the identification of overrepresented motifs in a specific location relative to the transcription start site. Among the genes up-regulated in the *athb25-1D* samples, we observed, at positions  $-150$  to  $-250$  from the transcription start site, the TAATTA motif (Fig. 7). This is directly bound by Arabidopsis *ATHB33* and includes the sequence ATTA, the canonical binding site for homeodomain proteins (Tan and Irish, 2006). Also enriched in the promoters of up-regulated genes were AAAG sites (the core site required for the binding of DNA binding with one finger [Dof] transcription factors and for the induction of some genes by GA; Sun and Gubler, 2004) and TGTCTC sites (the binding site for auxin response transcription factors; Guilfoyle and Hagen, 2007). The motif most represented for down-regulated genes is ABRE (YACGTGKC; International Union of Biochemistry coding: Y = T or C; K = G or T; Yamaguchi-Shinozaki and Shinozaki, 2005), a motif for ABA-induced genes included in the promoters of many GA down-regulated genes (Ogawa et al., 2003).

### Seed Longevity in Arabidopsis Is Regulated by GAs

GAs have been recognized as plant growth regulators that act as endogenous hormones controlling various aspects of growth and development, such as seed germination, stem elongation, fruit growth, leaf shape, and flower development (Swain and Singh, 2005). However, we are unaware of any published study linking GA and seed longevity. Therefore, the key question suggested by our results is whether the ability of *ATHB25*-overexpressing lines to resist seed aging is due to the overaccumulation of bioactive GA. We carried out an accelerated aging experiment of seeds collected from plants either treated with GA<sub>3</sub> or subjected to drought stress. We also included the quintuple DELLA mutant (*gibberellic acid insensitive-t6* [*gai-t6*] repressor of *gibberellic acid-t2* [*rga-t2*] repressor of *gibberellic acid like1-1* [*rgl1-1*] *rgl2-1* *rgl3-1*) and *pre1* mutant lines in this analysis. While all of these lines exhibited normal germination in the absence of the aging treatment (data not shown), all demonstrated a correlation between GA and seed longevity. GA<sub>3</sub>-treated plants and the quintuple DELLA mutant, which shows constitutive activation of the GA signaling pathway, produced seeds that are more resistant to accelerated aging, as compared with their respective controls. However, plants subjected to drought stress, with an expected decrease in GA content (Wang et al., 2008), and the *pre1* mutant, with a compromised

**Table I.** Summary of microarray data of genes differentially expressed in the *athb25-1D* mutant with respect to the wild type

Regulation	No. of Genes	Functional Category Overrepresented	Motifs Overrepresented			
			Sequence	Window	Percentage in Microarray	Percentage in Arabidopsis
Up-regulated	208	GA-responsive genes (24)	TATAWA	(-44, -18)	38.97	25.24
			AATAAA	(-106, -85)	15.44	9.44
			AAAG	(-140, -90)	65.44	58.47
			TGAC	(-93, -68)	22.79	16.85
			AMCWAMC	(-141, -107)	11.76	5.26
			TGTCTC	(-29, 52)	11.76	6.85
			TAATTA	(-250, -150)	17.5	14
Down-regulated	390	Response to water deprivation (15)	TATAWA	(-38, -23)	34.74	22.67
		Response to biotic stimulus (22)	ACGT	(-241, -12)	53.78	46.1
		Response to ABA (15)	ACGTG	(-227, -2)	35.95	25.9
		Programmed cell death (15)	CACGTG	(-222, -8)	12.69	8.68
		Transmembrane receptor activity (13)	ACGTGKC	(-188, -11)	12.08	8.28
			BACGTGKM	(-244, -11)	16.92	11.97
			GATA	(-148, -142)	12.08	6.21

response to GA (Bai et al., 2012), produced seeds less resistant to accelerated aging, as compared with their respective controls (Fig. 8). Finally, the addition of GA<sub>3</sub> to the germination plates did not rescue aged seeds to germinate (data not shown), as expected if these seeds were irreversibly damaged and not just dormant.

#### Seed Longevity Dependent on GA Correlates with Mucilage Formation and Shows a Maternal Effect

The mechanism of GA action on seed longevity cannot be related to cellular stress defenses because

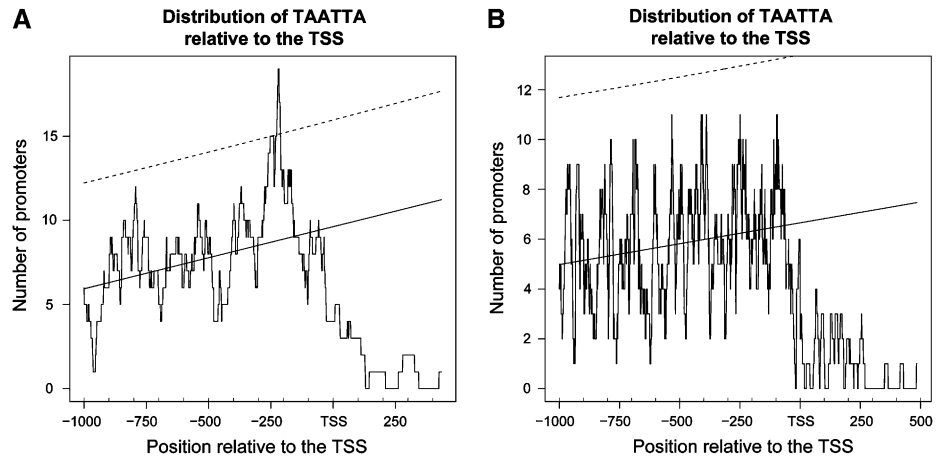
these hormones do not induce them as ABA does and, in addition, GAs repress ABA-induced genes (Ogawa et al., 2003). As GAs regulate growth and development (Swain and Singh, 2005), the alternative mechanism could be that GAs promote the formation of some parts of the seed coat, an important structure for seed longevity (Debeaujon et al., 2000; Clercx et al., 2004; Rajjou and Debeaujon, 2008).

Kim et al. (2005) have reported that the seed coat of a knockout mutant in the *GA3OX4* gene (encoding a seed-specific enzyme of GA biosynthesis) is distorted, with abnormal cell shape and reduced mucilage. The

**Table II.** Genes induced in the *athb25-1D* mutant with respect to the wild type and that are responsive to GAs according to NASC array-177 (2- to 9-fold induction)

GAs Response Genes	Fold Change	Description
At3g46480	6.1	2-Oxoglutarate and Fe(II)-dependent oxygenase superfamily protein
At2g44570	5.5	GLYCOSYL HYDROLASE9B12
At5g15360	5.4	Unknown protein
At1g18220	4.6	PURINE PERMEASE9
At4g29200	4.1	$\beta$ -Galactosidase-related protein
At1g43880	3.9	Transposable element gene
At5g49250	3.8	$\beta$ -Galactosidase-related protein
At4g08840	3.7	PUMILIO11
At1g29090	3.6	Cys proteinase superfamily protein
At1g10420	3.6	Protein coding
At5g48850	3.4	SULFUR DEFICIENCY INDUCED1
At2g01200	3.4	INDOLE-3-ACETIC ACID INDUCIBLE32
At3g56890	3.4	F-box-associated ubiquitination effector family protein
At2g17430	3.0	MILDEW RESISTANCE LOCUS O7
At1g26530	2.8	PIN domain-like family protein
At1g59640	2.8	Basic helix-loop-helix-encoding gene (BIGPETAL)
At3g15130	2.8	Tetratricopeptide repeat-like superfamily protein
At1g14970	2.4	O-Fucosyltransferase family protein
At5g28680	2.2	Receptor-like kinase (ANXUR2)
At1g64290	2.2	F-box protein related
At1g62940	2.2	ACYL-COENZYME A SYNTHETASE5
At4g37770	2.1	1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE8
At5g38070	2.1	RING/FYVE/PHD zinc finger superfamily protein
At1g04645	2.0	Plant self-incompatibility protein S1 family

**Figure 7.** Distribution of the TAATTA motif in promoters of genes modulated by overexpression of *ATHB25*. A, The set of 136 promoters for induced genes, where a preferential position of this motif has been detected in the region [−250, −150] before the transcription start site (TSS). B, The set of 331 promoters for repressed genes, where no significant peak was detected. In both graphs, the solid line is the linear mean and the dashed line is the 95% confidence interval.



latter is a pectin complex secreted by specialized epidermal cells of the seed coat (Arsovski et al., 2010). We have investigated the mucilage of *ATHB25* and DELLA mutants and, as indicated in Figure 9, the increased expression of *ATHB25* in *athb25-1D* and *pUBQ10:ATHB25* mutants is accompanied by increased mucilage, while the reduced expression in the antisense mutant (*ASATHB25*) correlates with decreased mucilage. Increased GA signaling in the DELLA mutant is also accompanied by increased mucilage. Therefore, one feature of the seed coat, mucilage formation, correlates with seed longevity in the GA mutants investigated in this work.

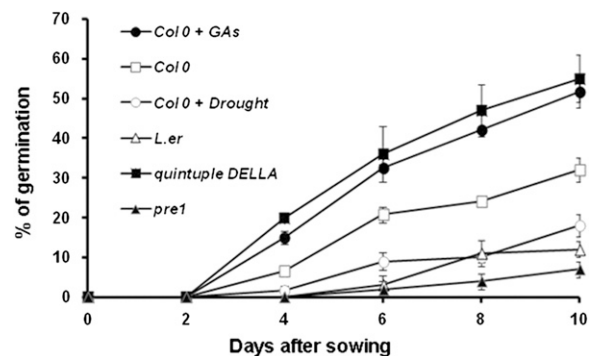
Any seed coat mechanism should have a maternal effect, because this structure is of maternal origin (Haughn and Chaudhury, 2005; Rajjou and Debeaujon, 2008). We made reciprocal crosses between the wild type and the *athb25-1D* mutant and found that the F1 seeds are tolerant to accelerated aging if the mother plant is a mutant but not when the mother plant is the wild type (Supplemental Fig. S6). These results are in agreement with a role of the seed coat in the improved seed longevity of the *ATHB25*-overexpressing mutants.

## DISCUSSION

Many protocols for accelerated aging of plant seeds (sometimes referred to as “controlled deterioration tests”) have been developed to speed up studies in seed longevity. Seeds are partially or completely rehydrated and exposed to high temperatures, leading to rapid loss of seed viability (e.g. days instead of years, as in natural aging; Woodstock and Taylorson, 1981; Tesnier et al., 2002; Prieto-Dapena et al., 2006; Tejedor-Cano et al., 2010). Accelerated aging mimics natural aging (Tesnier et al., 2002; Rajjou et al., 2008), and similar results are obtained with controlled hydration or with seeds imbibed in water (Tejedor-Cano et al., 2010). Our aging procedure with imbibed seeds incubated at 42°C for 1 to 2 d has been validated by the observed correlation

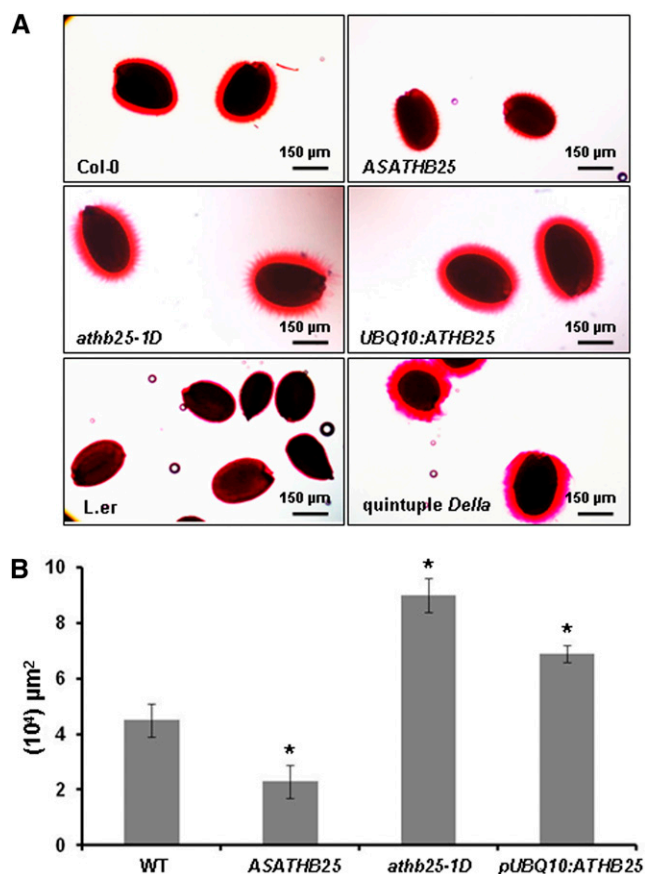
between tolerance to natural aging and to accelerated aging in four different mutants obtained in our screening (Fig. 1).

Most mutants in seed longevity are of two types: recessive mutants, with poor longevity affected in genes required for seed development (such as *lec1*, *lec2*, *fus3*, and *abi3*; Holdsworth et al., 1999; Clercx et al., 2003, 2004), and dominant mutants, with improved longevity obtained by reverse genetics overexpressing stress defenses and repair systems such as small heat shock proteins (Prieto-Dapena et al., 2006), Met sulfoxide reductase (Châtelain et al., 2013), DNA glycosylase/AP lyase (Chen et al., 2012), and L-isoadipyl methyltransferase (Ogé et al., 2008). To our knowledge, our *athb25-1D* mutant is the first mutant obtained by forward genetics and the first dominant mutant not related



**Figure 8.** Seed longevity in Arabidopsis is regulated by GA. Seeds from control Columbia (Col 0), from Columbia treated with 50  $\mu\text{M}$  GA (Col 0 + GA) or subjected to drought stress (Col 0 + drought), the *pre1* mutant (in the Columbia background), control Landsberg *erecta* (*L.er*), and the quintuple DELLA mutant (in the Landsberg *erecta* background) were subjected to accelerated aging treatment for 24 h and sown on MS plates. The percentage of germination was recorded after 4, 6, 8, and 10 d. The results are means of six experiments with 100 seeds per line, and error bars indicate SE. Untreated lines germinated 100% after 3 d.





**Figure 9.** Mucilage staining in seeds from genotypes with differences in seed longevity. A, Seeds stained with ruthenium red after first shaking in water. B, Measure of the surrounded mucilage area. Each value is the mean of 20 seeds chosen at random from two different batches, 10 each. Error bars indicate SE, and asterisks indicate significant differences from the control (the wild type [WT]) at  $P < 0.05$ . [See online article for color version of this figure.]

to stress defenses or repair systems. Gain-of-function mutants such as those obtained by overexpression obviate genetic redundancy and can identify bottlenecks in biological pathways (Weigel et al., 2000; Alejandro et al., 2007).

Analyses of mutants with gain and loss of function of the *ATHB25* gene, encoding a zinc finger homeobox transcription factor, suggest a novel mechanism of seed longevity based on the action of GA during seed development. That *ATHB25* acts by increasing GA biosynthesis is demonstrated by the observations that the expression of *ATHB25* correlates with hypocotyl length, seed and ovary cell size (Fig. 4), sensitivity to paclobutrazol (Fig. 5), concentrations of the active GAs  $GA_4$  and  $GA_1$ , and expression of *GA3OX2*, encoding a crucial enzyme in GA synthesis (Fig. 6). Further evidence is provided by transcriptomic analysis of the *athb25-1D* mutant, which reveals increased expression of many GA-induced genes, decreased expression of many GA-repressed genes, enrichment of the AAAG motif

(present in many GA-induced genes) in the promoter of induced genes, and enrichment of the ABRE motif (present in many ABA-induced genes, typically repressed by GA) in repressed genes (Tables I and II). Finally, treatment of plants with GA produces seeds with improved longevity, and mutants with increased (quintuple DELLA mutant) or decreased (*pre1* mutant) response to GA have seeds with increased or decreased longevity, respectively.

Our results suggest that, in addition to the accumulation of cellular stress defenses by the action of ABA, the promotion of some aspects of seed development by GA during seed maturation improves longevity. A plausible mechanism for the latter effect may consist of the formation of the seed coat or testa. GAs are required for the normal formation of this structure through starch degradation (Kim et al., 2005), and the seed coat is very important to protect the embryo from stresses during storage (Clerkx et al., 2004; Rajjou and Debeaujon, 2008). The maternal effect observed during reciprocal crosses between wild-type and *ATHB25*-overexpressing plants suggests that a plausible mechanism for this transcription factor to increase seed longevity is that, by increasing GA synthesis, it reinforces the seed coat. The observed correlation between expression levels of *ATHB25* and seed mucilage and the increased mucilage in the quintuple DELLA mutant (Fig. 8) supports this hypothesis. Several transcription factors (AP2, TRANSPARENT TESTA GLABRA1 [TTG1], TTG2, ENHANCER OF GLABRA3, TRANSPARENT TESTA2 [TT2], TT8, MYB DOMAIN PROTEIN5 [MYB5], MYB61, and GLABRA2) are required for the differentiation of the outer layers of the seed coat and the secretion of mucilage (Arsovski et al., 2010), but the expression of none of them is altered in the *athb25-1D* mutant (Supplemental Table S1).

It must be noted that the observed correlation between GA levels, seed longevity, and the amount of mucilage does not imply that the increased mucilage is specifically or solely responsible for the observed phenotype of seed longevity. As a hormone, GA could be acting on many aspects of the seed coat differentiation. Other portions of the seed coat that could play a role in seed coat reinforcement and seed longevity are the secondary cell walls of the epidermal mucilage cells (columellae) and the subepidermal palisade cells (Debeaujon et al., 2000; Rajjou and Debeaujon, 2008). It is plausible that during starch degradation promoted by GA, these walls are also made thicker, in addition to the increased mucilage production (Kim et al., 2005). It is also possible that altered GA production could increase proanthocyanidin synthesis in the endothelial layer of the seed coat, which has also been implicated in seed longevity (Rajjou and Debeaujon, 2008).

We do not expect *ATHB25* to directly regulate *GA3OX2*, because it was not possible to detect binding of this transcription factor to the promoter of this gene in chromatin immunoprecipitation experiments (data not shown). The transcription factors that directly regulate the expression of *GA3OX* genes in Arabidopsis are only partially known (Hedden and Thomas,

2012). Mutation of genes encoding some transcription factors, such as *AT-HOOK PROTEIN OF GA FEEDBACK1* (Matsushita et al., 2007), *SCARECROW-LIKE13* (Zhang et al., 2011), and *SOMNUS* (Kim et al., 2008), affects *GA3OX1* and *GA3OX2* expression, but there is no evidence for their binding to the promoter of the genes. Only *FUS3* (Curaba et al., 2004) and *DOF AFFECTING GERMINATION1* (Gabriele et al., 2010) have been shown to repress *GA3OX1* and *GA3OX2*, respectively, by binding to the promoter of the regulated gene. None of the genes encoding all these transcription factors are regulated by *ATHB25*, according to our microarray experiments (Supplemental Table S1).

## CONCLUSION

The identification of the *ATHB25* gene in a screening of activation-tagging mutants with selection for increased seed longevity has uncovered a novel role for GAs in seed longevity. The *ATHB25* transcription factor induces the expression of the GA biosynthetic enzyme *GA3OX2* and increases the levels of bioactive GAs. The mechanism of GA-dependent longevity may be based on reinforcement of the seed coat, and these findings may be of relevance to improve seed longevity, with important benefits for biodiversity and agriculture.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

*Arabidopsis* (*Arabidopsis thaliana*) plants were grown and germinated on Murashige and Skoog (MS) plates with Suc as described (Alejandro et al., 2007). Plants treated with GA<sub>3</sub> were sprayed twice per week with a 50 μM solution. Plants subjected to drought were grown with irrigation for 3 weeks, and then watering was stopped. We used the following *Arabidopsis* strains: wild-type Columbia, wild-type Landsberg *erecta*, activation-tagging mutant overexpressing *ATHB25* (*athb25-1D*), transgenic lines overexpressing *ATHB25* under the control of the *UBQ10* promoter (*pUBQ10:ATHB25*), transgenic lines overexpressing *ATHB25* under the control of the 35S promoter (*35S:ATHB25*), lines overexpressing antisense At5g65410 (*ASATHB25*), loss of function of *ATHB25* (NASC line N665321; *athb25-2*), loss of function of *ATHB22* (NASC line N667869), quintuple DELLA mutant (NASC line N16298; *gai-t6 rga-t2 rgl1-1 rgl2-1 rgl3-1*), and paclobutrazol-resistant mutant *pre1* (Bai et al., 2012).

### Accelerated Aging

This procedure was a modification of the "basal thermotolerance assay" of Tejedor-Cano et al. (2010). Seeds were imbibed in water, stratified for 2 d at 4°C, and incubated at 42°C for 1 to 2 d (relative humidity of 100%). In the initial screening for mutants, the seeds were exposed to 42°C for 48 h because, after this time, the germination of wild-type seeds was completely inhibited. In other experiments, incubation at 42°C was for 24 h.

### Molecular Techniques

Plasmid rescue from mutant plants, RNA extraction, RT-PCR (qualitative and quantitative or real time) to measure gene expression, PCR to detect T-DNA, and Southern-blot analysis to determine the number of T-DNA insertions were performed as described (Alejandro et al., 2007). Plants (10-d-old seedlings grown in solid MS medium) were grown under long-day conditions. The sequences of the primers are shown in Supplemental Table S2. The

constructs for overexpression of the *ATHB25* (At5g65410) gene in plants under the control of the cauliflower mosaic virus 35S promoter were made as follows. The cDNA containing the complete open reading frame was obtained in plasmid pUNI51 from the Genomics Science Center at RIKEN. The cDNA was released by digestion with *SfiI*, blunted, subcloned into plasmid pBluescript SK+ (Stratagene), digested with *XbaI* and *NotI*, and blunted. Recombinant plasmids with both orientations of the cDNA were obtained. For expression from the cauliflower mosaic virus 35S promoter, a *SacI*-*BamHI* fragment including the cDNA was subcloned into pBI121 by replacing the GUS coding region between the *SacI* and *BamHI* sites. For expression from the *UBQ10* promoter, the pBluescript SK+ recombinant plasmid was digested with *SacI*, blunted, and then digested with *ApaI*. The fragment including the cDNA was subcloned into plasmid pUBQ10:MCS (Grefen et al., 2010), digested with *SpeI*, blunted, and then digested with *ApaI*.

## Global Gene Expression Using Long Oligonucleotide Microarrays

Plants (10-d-old seedlings grown in solid MS medium) were grown under long-day conditions, RNA extraction, amplification, labeling, and hybridization were performed as described previously (Bueso et al., 2007), and the analysis of microarray data was as described by Bissoli et al. (2012). Long oligonucleotide microarrays were provided by Dr. David Galbraith (University of Arizona; <http://www.ag.arizona.edu/microarray/>). Oligonucleotides were from the Operon Arabidopsis Genome Oligo Set version 3.0 (<https://www.operon.com/>), which contains 29,110 70-mer probes, representing 26,173 protein-coding genes, 28,964 protein-coding gene transcripts, and 87 microRNAs. The design is based on ATH1 release 5.0 of The Institute for Genomic Research Arabidopsis genome annotation database (<http://www.tigr.org/tldb/e2k1/ath1/>) and release 4.0 of the microRNA registry at the Sanger Institute (<http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml>).

The microarray data from this article have been submitted to the ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress/>) and assigned the identifier E-MEXP-3857.

### Measure of Hypocotyl Length and Seed Area

Photographs were taken with the manual fluorescence stereomicroscope Leica MZ16 F, and hypocotyl length and seed area were measured with the Java-based image-processing program ImageJ (<http://rsb.info.nih.gov/ij/>).

### Measure of Cell Area

For scanning electron microscopy, samples were fixed in 50% ethanol, 5% acetic acid, and 3.7% formaldehyde for 16 h at 4°C. Samples were dehydrated in an ethanol series and critical point dried in liquid CO<sub>2</sub> (Polaron E300 apparatus). Dried samples were mounted on slides with activated carbon. Then, samples were coated with gold palladium (4:1) in a Sputter Coater SCD005 (BALTEC). Scanning electron microscopy was performed with a JEOL JSM-5410 microscope (10 kV).

### GA Extraction and Quantification

Aliquots of 100 mg fresh weight from 10-d-old seedlings were homogenized in cold 80% (v/v) methanol-water, stirred overnight at 4°C, and reextracted twice for 30 min with one-half volume of methanol using [<sup>17</sup>,<sup>17</sup>-<sup>2</sup>H]GA<sub>1</sub>, [<sup>17</sup>,<sup>17</sup>-<sup>2</sup>H]GA<sub>4</sub>, [<sup>17</sup>,<sup>17</sup>-<sup>2</sup>H]GA<sub>8</sub>, [<sup>17</sup>,<sup>17</sup>-<sup>2</sup>H]GA<sub>9</sub>, [<sup>17</sup>,<sup>17</sup>-<sup>2</sup>H]GA<sub>19</sub>, [<sup>17</sup>,<sup>17</sup>-<sup>2</sup>H]GA<sub>20</sub>, [<sup>17</sup>,<sup>17</sup>-<sup>2</sup>H]GA<sub>29</sub>, [<sup>17</sup>,<sup>17</sup>-<sup>2</sup>H]GA<sub>34</sub>, and [<sup>17</sup>,<sup>17</sup>-<sup>2</sup>H]GA<sub>44</sub> as internal standards. The methanolic extracts were combined, taken to dryness in vacuo, and dissolved in 0.5 mL of 10% methanol solution. This extract was then applied to a pre-equilibrated 500-mg SAX column (BondElut SS-SAX; Varian Scharlau). The column was washed with water, pH 8.0, before being eluted with 4 mL of 0.2 M formic acid solution. The formic acid eluate was run directly onto a pre-equilibrated 500-mg C18 column (BondElut C18; Varian Scharlau) that was then washed with water, pH 3.0, and finally eluted with 5 mL of 80% methanol. The methanol eluate was dried, dissolved in 0.2 mL of 2-propanol, and then methylated with trimethylsilyl-diazomethane in hexane. After methylation, the residue was dried, and the samples were trimethylsilylated using 7.5 μL of pyridine and 7.5 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide solution containing 1% trimethyl chlorosilane. For gas chromatography-mass

spectrometry analysis, the samples were injected dissolved in the mix of pyridine/*N,O*-bis(trimethylsilyl)trifluoroacetamide into a gas chromatograph (7890A; Agilent Technologies) coupled to a mass spectrometer (7000 Triple Quad; Agilent Technologies). Samples were injected in splitless mode into an HP 5890 gas chromatograph (Hewlett-Packard), fitted with a fused silica glass capillary column (30 m long, 0.25 mm i.d.) with a chemically bonded 0.25- $\mu$ m DB-5MS stationary phase (J&W Scientific). The injector temperature was 270°C. The column temperature program varied depending on which GA was being analyzed. The column effluent was introduced into the ion source of a JMS-SX/SX102A mass spectrometer (JEOL). The interface temperature was 280°C, and the ion source temperature was 250°C. The amount of GA in the samples was quantified by isotopic dilution, and we used the MassHunter Quantitative software provided by Agilent.

### Identification of Preferentially Located Motifs

We studied a set of 199 known motifs coming from the AGRIS database (Yilmaz et al., 2011), and for each motif, we extracted all occurrences in a promoter set. The promoter sequences were then divided into two regions. First, the [−1,000, −300] region was used to learn the distribution model using a simple linear regression and determine a 95% confidence interval. Second, within the [−300, untranslated region (UTR)] region, we searched for non-evenly distributed motifs (i.e. those exhibiting a peak above the confidence interval). When a motif is considered, like an overrepresented motif in a set of promoters, we performed a binomial probability to compare percentages between this considered set and the whole promoter set of *Arabidopsis*. Each motif with a probability of less than 5% was considered significant. We then applied the same methodology to study all the four- and six-base motif lengths. This methodology to detect if a motif had a preferential location has been described in detail (Bernard et al., 2010). We used FLAGdb<sup>++</sup> (Derozier et al., 2011), an integrative database of plant model genomes, to define the transcriptional units by aligning all the available transcripts to gene models (gene models, ESTs, and full-length cDNAs are defined in The Arabidopsis Information Resource [http://www.arabidopsis.org/]). The promoter was defined by 1 kb upstream of the predicted transcription start site and contained the whole 5' UTR. We excluded promoters with a 5' UTR smaller than 10 bases in order to avoid false promoter regions. Thus, we defined promoters for 19,921 genes of *Arabidopsis* as well as 136 and 331 promoters, respectively, for the induced and repressed gene sets studied.

### Staining of the Mucilage with Ruthenium Red

Seeds were imbibed in water for 3 min before the application of 0.2% (w/v) aqueous ruthenium red solution for 15 min. Photographs were taken with Nikon's Eclipse E600 research microscope, and mucilage area was measured with the Java-based image-processing program ImageJ.

### Extraction and Quantification of ABA

ABA was extracted overnight at 4°C from 50 mg of seeds in 2.5 mL of extraction buffer containing 80% (v/v) acetone, 100 mg L<sup>−1</sup> butylated hydroxytoluene, and 500 mg L<sup>−1</sup> citric acid and quantified with the Phyto-detek-ABA kit (Agdia).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers BT002385.1 GI: 27311558 (At5g65410).

### Supplemental Data

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

**Supplemental Figure S1.** Endogenous ABA content of *Arabidopsis* seeds of the wild type and the *isl1-1D* mutant after accelerated aging.

**Supplemental Figure S2.** The *isl1-1D* mutant has a single insertion of T-DNA.

**Supplemental Figure S3.** *ATHB25* expression during accelerated aging treatment.

**Supplemental Figure S4.** Expression levels of *GA20OX* and *GA3OX* genes in the wild type and the *athb25-1D* mutant.

**Supplemental Figure S5.** Microarray validation.

**Supplemental Figure S6.** Maternal effect of increased seed longevity conferred by the *athb25-1D* mutation.

**Supplemental Table S1.** Genes induced more than 2-fold and genes repressed more than 2-fold in the *athb25-1D* mutant with respect to the wild type.

**Supplemental Table S2.** Primers used in this work.

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