Histone Deacetylase AtHDA7 Is Required for Female Gametophyte and Embryo Development in Arabidopsis

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Histone modifications are involved in the regulation of many processes in eukaryotic development. In this work, we provide evidence that AtHDA7, a HISTONE DEACETYLASE (HDAC) of the Reduced Potassium Dependency 3 (RPD3) superfamily, is crucial for female gametophyte development and embryogenesis in Arabidopsis (Arabidopsis thaliana). Silencing of AtHDA7 causes degeneration of micropylar nuclei at the stage of four-nucleate embryo sac and delay in the progression of embryo development, thereby bringing the seed set down in the Athda7-2 mutant. Furthermore, AtHDA7 down-and up-regulation lead to a delay of growth in postgermination and later developmental stages. The Athda7-2 mutation that induces histone hyperacetylation significantly increases the transcription of other HDACs (AtHDA6 and AtHDA9). Moreover, silencing of AtHDA7 affects the expression of ARABIDOPSIS HOMOLOG OF SEPARASE (AIAESP), previously demonstrated to be involved in female gametophyte and embryo development. However, chromatin immunoprecipitation analysis with acetylated H3 antibody provided evidence that the acetylation levels of H3 at HDACs do not change in the mutant. Further investigations are essential to ascertain the mechanism by which AtHDA7 affects female gametophyte and embryo development.

DNA of eukaryotic cells is associated with nuclear proteins to form the chromatin. Its basic unit is the nucleosome, which is composed of about 147 bp of DNA bound to an octamer of the canonical histones H3, H4, H2A, and H2B (Berr et al., 2011). Histone posttranslational modifications contribute to define chromatin states that drive different chromatin-based nuclear processes. In fact, histone posttranslational modifications, including acetylation, methylation, phosphorylation, and ubiquitination, control fundamental processes such as transcription (Bell et al., 2011), DNA replication (Ehrenhofer-Murray, 2004), cell cycle (O’Sullivan et al., 2010), DNA repair (Soria et al., 2012), and recombination (Perrella et al., 2010). Histone acetylation is carried out by histone acetylases (HATs), while it is erased by histone deacetylases (HDACs). Plant HDACs are grouped into three families: the RPD3/HDA1 superfamily, SIR2, and HD2 (Pandey et al., 2002). Two of these families are homologous to the classes of HDACs found in yeast and animals, while the HD2 class appears to be unique to plants and unrelated to the other classes. In Arabidopsis (Arabidopsis thaliana), 18 putative HDACs have been identified (Pandey et al., 2002). Some HDACs are emerging as crucial players in plant growth and development processes, including embryogenesis, flowering, meiosis, senescence, as well as responses to environmental cues (Hollender and Liu, 2008; Berr et al., 2011). In particular, mutations in genes encoding HDACs as well as treatments with trichostatin A (TSA), an inhibitor of HDACs, highlighted a requirement of the HDACs in reproductive development. Indeed, the down-regulation of AtHD19 induced delayed flowering, flower abnormalities, embryonic defects, and seed set reduction (Tian et al., 2003). Silencing as well as overexpression of AtHD2A severely affected seed development (Wu et al., 2000; Zhou et al., 2004). The Athda6 mutant was reported to exhibit reduced fertility to some extent (Aufsatz et al., 2002), and mutation of AtHDA9 led to an early-flowering phenotype in short-day-grown plants (Kim et al., 2013). Some findings strongly suggest a role for AtHDA7 in Arabidopsis reproduction. Indeed, AtHDA7 (At5g35600), encoding a putative HDAC of the Reduced Potassium Dependency 3 (RPD3) superfamily, is preferentially
expressed in flower bud (Schmid et al., 2005) and is up-regulated in microdissected hyperacetylated microsporocytes (Barra et al., 2012). To investigate the unexplored function of AtHDA7, we analyzed overexpression and silenced mutants, thereby proving that AtHDA7 is required for female gametophyte development and embryogenesis progression. Moreover, AtHDA7 down- and up-regulation lead to a delay of growth in postgermination and later developmental stages.

RESULTS

In Silico Analysis of AtHDA7

AtHDA7 (At5g35600) encodes a putative histone deacetylase belonging to class I of the RPD3 superfamily characterized by a Histone_deacetylase domain (PF00850; Pandey et al., 2002). This superfamily includes the homologs of yeast RPD3 that preferentially deacetylate histones H3 and H4 (Rundlett et al., 1996). AtHDA7 has five exons and four introns (www.arabidopsis.org) spanning 1,593 bp. To identify putative cis-regulatory motifs within the AtHDA7 promoter, a region of 1,000 bp upstream of the translation start codon was analyzed in silico (Fig. 1). Eight motifs were identified between nucleotides −893 and −12. The motif at position −245 is similar to the binding site of SBF1, a transcriptional repressor (Lawton et al., 1991). On the other hand, motifs including ZC2 recognized by chromatin remodelers with zinc-finger domains (Ponte et al., 1994), telobox recognized by Athb homeodomain proteins (Sessa et al., 1993), W-box recognized by WRKY transcription factors (Eulgem et al., 2000), BOX1* (Tijaden et al., 1995), and GCCAAG motif (Leah et al., 1994) are reported to be involved in transcriptional activation. The predicted motif composition of the AtHDA7 promoter suggests that its transcription is regulated in a complex way.

Overexpression and Silencing of AtHDA7 Affect Growth

AtHDA7 was reported to be highly expressed in flower bud stage 9 (Schmid et al., 2005). In order to investigate AtHDA7 function, the insertion line Salk_002912C (Alonso et al., 2003) was analyzed in this work. The Transfer-DNA insertion was confirmed to be in the putative promoter 43 bp upstream of the start codon, thereby introducing a gap between the GCCAAG activation motif and other motifs including SBF1-like (Fig. 1). Interestingly, AtHDA7 was overexpressed in the mutant, with a change of 246-fold with respect to the wild type (Supplemental Fig. S1). Consequently, the mutant is herein designated AtHDA7ex.

To silence AtHDA7, we generated transgenic plants constitutively expressing an artificial microRNA. One heterozygous transgenic plant, showing a strong down-regulation of AtHDA7 (AtHDA7−2; Fig. 2A), was selected for further characterization. To ascertain whether alteration of AtHDA7 expression affected histone acetylation, immunoblotting with histone extracts from AtHDA7−2 and the wild type was performed using an antibody specific for H3 diacetylated at Lys-9 and Lys-14. AtHDA7−2 showed increased levels of H3 acetylation compared with the wild type (P < 0.05; Fig. 2B).

Seed germination and growth rate studies were carried out on AtHDA7ex and AtHDA7−2. While seed germination rate was not affected in AtHDA7ex, a significant reduction was exhibited by AtHDA7−2 as compared with the wild type (89% versus 99%; P < 0.001; Fig. 3). Interestingly, a significant retardation of growth rate, measured as the number of vegetative leaves produced over time, was observed in vitro and in soil for AtHDA7ex as well as AtHDA7−2 (Figs. 4 and 5). For instance, 8 d after transfer of the imbibed seeds to growing conditions, 62% of AtHDA7ex and 66% of AtHDA7−2 seedlings reached growth stage 1.04, corresponding to four rosette leaves (Boyes et al., 2001), as compared with 94% of the wild type (Fig. 4). In AtHDA7ex plants grown in soil, growth rate was significantly different from the wild type.
starting on day 11 after sowing, while Athda7-2 plants diverged significantly from the wild type 25 d after sowing (Fig. 5). Furthermore, flowering time, measured as the number of leaves at bolting, was significantly different ($P < 0.05$) between the wild type (11.2 ± 1.1 leaves) and Athda7oe (9 ± 0.9 leaves) but was not affected in Athda7-2 (9.6 ± 1.3 leaves). Collectively, these findings highlight that both up- and down-regulation of AtHDA7 lead to a delay of growth.

**Down-Regulation of AtHDA7 Affects Fertility**

Pollen viability in Athda7oe and Athda7-2 did not differ from that in the wild type. Silique fertility in Athda7oe was similar to the wild type, whereas the number of fully developed seeds per silique decreased in Athda7-2 due to unfertilized ovules and aborted seeds (Fig. 6). Accordingly, the length of mature siliques was significantly reduced by 14% in Athda7-2 (1.2 ± 0.1 cm) with respect to the wild type (1.4 ± 0.1 cm). In order to evaluate whether AtHDA7 is required for the development of the female gametophyte, we assessed the transmission efficiency of the mutated allele linked to the nptII gene, the selectable marker used to obtain Athda7-2. The progeny of reciprocal crosses between heterozygous Athda7-2 and the wild type were scored in vitro for kanamycin resistance and confirmed by genotyping. As shown in Table I, 50% of the progeny were resistant to kanamycin when the mutant was used as male, thereby indicating that transmission efficiency was 100%, as expected. When the progeny of the reciprocal cross were analyzed, we found that only 33% were
kanamycin resistant, showing a severe reduction in transmission efficiency to 51% of the expected 100% through the female gamete (Table I).

These findings highlight that AtHDA7 down-regulation leads to partial failure of ovule/female gametophyte development and seed abortion.

**AtHDA7 Is Required for Female Gametophyte and Embryo Development**

The above-described phenotype suggests that ovule/female gametophyte development and seed/embryo development are impaired in the *AtHda7-2* mutant. Megasporeogenesis and megagametogenesis were observed in *AtHda7-2* and the wild type by differential interference contrast (DIC) visualization of clarified carpels with length from 1 to 2 mm to identify the developmental stages affected by the *AtHda7-2* mutation. In wild-type Arabidopsis, one megaspore mother cell per ovule undergoes meiosis and a tetrad of megaspores is produced. Subsequently, one surviving functional megaspore at the chalazal position undergoes gametogenesis. The production of functional megaspores observed in *AtHda7-2* (Supplemental Fig. S2) indicates that

![Figure 5](image)

*Figure 5.* In soil growth of *AtHda7oe* (*n* = 37) and *AtHda7-2* (*n* = 40) compared with the wild type (*wt*; *n* = 35). The number of rosette leaves was recorded over a period of 32 d after sowing (DAS). Flowering time is indicated by arrows. *P* ≤ 0.05, **P** ≤ 0.01, ***P** ≤ 0.001.

![Figure 6](image)

*Figure 6.* Silique fertility in *AtHda7oe* and *AtHda7-2* compared with the wild type (*wt*). Average values estimated on 20 siliques are shown with SD.
female meiosis is apparently regular in **AtHda7-2**. However, in **AtHda7-2** carpels of 1.3 mm, which contained mostly ovules with two/four-nucleate embryo sacs (ES), 12% of ovules (n = 40) still harbor megaspore mother cells undergoing meiosis, thereby suggesting a delayed progression of female meiosis (Supplemental Fig. S3).

In wild-type Arabidopsis, the functional megaspore undergoes three rounds of mitosis, producing an eight-nucleate/seven-celled ES (Fig. 7, A–F). In **AtHda7-2** (Fig. 7, G–L), defects were observed during the progression of megagametogenesis, followed according to the stages described by Christensen et al. (1997). While no deviation was observed in female gametophyte (FG) stages FG2 to FG3 (corresponding to two-nucleate ES; Fig. 7H), at stage FG4 of four-nucleate ES, 10% of **AtHda7-2** ovules (n = 80) showed ES with the pair of micropylar nuclei in degeneration (Fig. 7; Supplemental Fig. S3). In FG5 to FG6, **AtHda7-2** carpels of 1.7 and 2.0 mm showed both ovules with regular ES (Fig. 7K) and ovules with the first degeneration signs (Supplemental Figs. S3 and S4).

Mature **AtHda7-2** ES undergoing a normal fate exhibited nuclear migration and cellularization. 4′,6-Diamidino-2-phenylindole (DAPI) staining of **AtHda7-2** ovules (n = 128) excised from pistils at the prefertilization stage revealed that 14% of the ES were collapsed (Fig. 7L), confirming the findings of DIC visualization. Ovules in degeneration were also observed in pistils after fertilization (Fig. 8B). Based on the cytological findings, AtHDA7 is required for ES development.

**Table 1.** Transmission efficiency of the Athda7-2 allele estimated in reciprocal crosses between the heterozygous mutant (**AtHDA7/Athda7-2**) and the wild type (**AtHDA7/AtHDA7**).

<table>
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<tr>
<th>Sample</th>
<th>Total Seeds</th>
<th><strong>nptII</strong></th>
<th><strong>nptII</strong></th>
<th>Transmission Efficiency&lt;sup&gt;a&lt;/sup&gt;</th>
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<td></td>
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<td>Observed</td>
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<td><strong>Reciprocal crosses</strong></td>
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<tr>
<td><strong>AtHDA7/Athda7-2 × AtHDA7/AtHDA7</strong></td>
<td>62</td>
<td>21</td>
<td>41</td>
<td>51</td>
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<tr>
<td><strong>AtHDA7/AthDA7 × AtHDA7/Athda7-2</strong></td>
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<td><strong>Selfings</strong></td>
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<tr>
<td><strong>AtHDA7/Athda7-2 × AtHDA7/Athda7-2</strong></td>
<td>50</td>
<td>25</td>
<td>25</td>
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<tr>
<td><strong>AtHDA7/AthDA7 × AtHDA7/AthDA7</strong></td>
<td>50</td>
<td>0</td>
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<sup>a</sup>Transmission efficiency = (number of **nptII** seedlings/number of **nptII** seedlings) × 100 through female or male gametes according to Brukhin et al. (2011).

**Figure 7.** Ovule and female gametophyte development in the wild type (A–F) and **AtHda7-2** (G–L). A and G, One-nucleate female gametophyte at stage FG1 (according to Christensen et al., 1997), indicated by the arrows. Degenerate megaspores are present. B and H, Two-nucleate female gametophyte at stage FG2, indicated by the arrows. C, D, and I, Four-nucleate female gametophyte at late stage FG4. Two pairs of nuclei at each pole are separated by a large central vacuole, and the chalazal nuclei are positioned along a line that is parallel to the chalazal-micropylar axis. J, Female gametophyte at late stage FG4 showing micropylar nuclei in degeneration, indicated by the arrow. E and K, Eight-nucleate/seven-celled mature female gametophyte. Observations were performed by DIC (C, E, I, and K) and phase-contrast microscopy. The female gametophyte in A, B, D, E, and G is oriented with its micropylar pole to the left, while the micropylar pole in C, H, I, J, and K is at right. F and L, Ovules excised from prefertilization pistils and observed by fluorescence microscopy through DAPI staining. In **AtHda7-2**, the ovule is degenerating. Bars = 20 μm. [See online article for color version of this figure.]
In order to identify the cause of seed abortion, Athda7-2 clarified pistils after fertilization were analyzed by DIC microscopy (Fig. 8). Embryonic stages observed within the silique were strongly asynchronous in Athda7-2, suggesting a delay in the progression of embryo development. At 10 d after pollination, only 67% of Athda7-2 embryos (n = 686) had reached linear or torpedo stages compared with 90% of wild-type embryos (Fig. 9; P < 0.001). The remaining 33% of Athda7-2 embryos were at heart or earlier stages.

The above-reported findings indicate that AtHDA7 is required for both female gametophyte and embryo development.

Other HDACs and AtAESP Are Misregulated in Athda7-2

In order to verify whether a misregulation occurred in RPD3 HDAC members, quantitative reverse transcription (RT)-PCR analysis was performed to monitor the expression of AtHDA6, AtHDA9, and AtHDA19 in Athda7-2. As shown in Figure 10A, AtHDA6 and AtHDA9 levels were significantly up-regulated in Athda7-2. On the contrary, AtHDA19 was down-regulated, with a change of 0.8-fold (P < 0.005). To test the possibility that the higher expression of AtHDA6 and AtHDA9 in Athda7-2 originate from a distinct chromatin configuration at these loci, a chromatin immunoprecipitation (ChIP) analysis with anti-acetyl-histone H3 antibody was performed. As shown in Supplemental Figure S5, neither AtHDA6 nor AtHDA9 displayed hyperacetylation of histone H3 in Athda7-2 compared with the wild-type.

To find additional genes misregulated in the Athda7-2 background, the Expression Angler tool (http://bar.utoronto.ca/ntools) was used to identify genes coregulated with AtHDA7 at developmental stages corresponding to gametogenesis. This analysis showed that 589 and 317 genes were positively coregulated with AtHDA7 in embryos and flower buds, respectively, whereas no negatively coregulated genes were identified. In embryos, the majority of AtHDA7 coregulated genes related to protein metabolism, response to stress, and developmental processes (Supplemental Fig. S6A; Supplemental Table S1), while in flowers and ovaries, coregulated genes were classified as involved in protein metabolism, developmental processes, cell organization, and biogenesis (Supplemental Fig. S6B; Supplemental Table S1). For experimental validation, ARABIDOPSIS HOMOLOG OF SEPARASE (AtAESP) was selected, given its role in ovule and embryo development (Liu and Makaroff, 2006; Yang et al., 2011). As shown in Figure 10B, AtAESP was significantly down-regulated in Athda7-2 flower buds, with a change of 0.64-fold (P < 0.002) compared with the wild type. We examined whether AtHDA7 down-regulation affected the histone acetylation status of AtAESP by ChIP analysis with anti-acetyl-histone H3 antibody. AtAESP did not demonstrate any acetylation variation of histone H3 in Athda7-2 compared with the wild type (Supplemental Fig. S5).

DISCUSSION

In this work, the functional analysis of AtHDA7 was performed by means of mutations inducing overexpression in Athda7oe and silencing and histone hyperacetylation in...
Both down- and up-regulation of _AtHDA7_ caused delays of growth at different developmental stages. An analogous phenomenon was reported in _AtHDA18_, with a similar altered cellular patterning in the root epidermis of overexpressing and knockdown mutants (Liu et al., 2013). The decreased seed germination phenotype of _Athda7-2_ demonstrates the importance of histone deacetylation in early development. The reduction of germination rates was observed in seeds treated with TSA (Tanaka et al., 2008). However, seed germination was unaffected in _Athda6_ as well as in the double _Athda6/Athda19_ mutant (Tanaka et al., 2008; Chen et al., 2010). In _Athd2a_ and _Athd2c_ mutants, Colville and colleagues (2011) reported defects in seed germination due to altered responses to sugar. They found that _AtHD2A_ prevents germination while _AtHD2C_ enhances germination in the presence of Glc. In contrast to _AtHD2A_ and _AtHD2C_, _AtHDA7_ is not induced by Glc (Colville et al., 2011), thereby making it unlikely that sugar sensing is involved in the seed germination defects observed in _Athda7-2_. The similarity between the _Athda7-2_ phenotype and TSA treatment (Tanaka et al., 2008) suggests a role for _AtHDA7_ in Arabidopsis seed germination. With regard to the defects in postgermination growth observed in _Athda7-2_, similar behavior was exhibited by the _Athd6/Athda19_ double mutant and TSA-treated seeds (Tanaka et al., 2008). _AtHDA7_ seems to be not required by itself for Arabidopsis growth, which, instead, is controlled by the concerted action of different HDACs in response to the environment.

Female gametophyte and embryo development were affected in _Athda7-2_ mutants, while _Athda7cox_ lacked unfertilized ovules and aborted seeds, indicating normal ES and embryo development. In the silenced mutant, homozygous individuals for _nptII_ gene coding for kanamycin resistance were not recovered in the settings of _Athda7-2_. A likely explanation is that a portion of the female gametophytes carrying the silenced allele and the embryos with both silenced alleles abort during their respective development. The gametophytic mutation is partially penetrant, as observed by the reduced transmission of the silenced allele through the female gametophyte and by microscopic analysis indicating 10% defective _Athda7-2_ ES at the four-nucleate stage. Micropylar nuclei were seen to degenerate, and ovules harboring nonfunctional female gametophytes failed to undergo seed development. Several studies have highlighted the importance of nuclei migration and positioning for female gametophyte development (Sprunck and Gross-Hardt, 2011). At the late four-nucleate stage, the pair of sister nuclei at both poles of the ES acquire the position, along the micropylar-chalazal axis (long axis), that critically determines their fate. In particular, micropylar nuclei are fated to become the egg apparatus. _AtHDA7_ is required specifically for female (not for male)
gametophyte development in Arabidopsis, particularly at the four-nucleate stage. Only HAM1 and HAM2, belonging to MYST HATs, were described as essential for female as well as male gametophyte development in Arabidopsis (Latrassé et al., 2008). In HAM1/ham1; ham2/ ham1 and ham1/ham1; HAM2/ham2 plants, half of the ovules aborted, since megagametogenesis failed to progress beyond the one-nucleate stage.

Besides ES collapse before fertilization, seed failures in Athda7-2 are likely due to a noticeable delay in embryogenesis progression. This indicates that AtHDA7 is required for embryogenesis. Studies concerning the relationships between histone deacetylation/acetylation and embryogenesis have been reported in Arabidopsis (Long et al., 2006) as well as in maize (Zea mays), where HATs and HDACs were first isolated in embryos (Kölle et al., 1999). Varotto and colleagues (2003) showed that ZmHDA108, the closest homolog to AthDA7, is particularly abundant in kernels at 3 to 8 d after pollination and in embryos. Although ZmHDA108 was not functionally analyzed, its expression profile suggests a role in embryo development.

Since HDACs are transcriptional regulators, we identified the genes coregulated with AtHDA7 by an in silico approach. Among these genes, the separase AtAESP that was down-regulated in Athda7-2 may provide some insight into the role of AtHDA7. AtAESP is required for ovule and embryo development in Arabidopsis (Liu and Makaroff, 2006; Yang et al., 2011). Loss of separase activity dramatically slows the progression of megagametogenesis and embryogenesis. It was speculated that separase could prevent cyclin destruction, as demonstrated in yeast (Tinker-Kulberg and Morgan, 1999). It might be hypothesized that AtHDA7 interacts with histones associated with AtAESP. However, based on ChIP-PCR analysis performed in this work, the histone acetylation level in the AtAESP locus does not change in Athda7-2.

In the silenced mutant, a relationship exists between the expression of AtHDA7 and the other HDACs, including AtHDA6, AtHDA9, and AtHDA19. The lack of enhancement of H3 acetylation at AtHDA6 and AtHDA9 loci rules out that these genes are direct targets of AtHDA7, suggesting that other factors are involved in their transcription regulation. HDAC misregulation could be triggered by a compensatory effect for histone deacetylation to remedy the down-regulation of AtHDA7. It has been reported that the expression of HATs/HDACs is affected in Arabidopsis and maize upon changes in the degree of acetylation (Bhat et al., 2003; Tian et al., 2005). In particular, in the HAT mutant Atmcc1, characterized by histone hyperacetylation in meiosis, AtHDA7 resulted in up-regulated male meiocytes (Perrella et al., 2010; Barra et al., 2012). The misregulation of the different HDACs in Athda7-2 likely affects the expression of different classes of genes. For example, AtHDA19 is down-regulated in Athda7-2 and is considered to be a global regulator of gene expression in Arabidopsis, controlling 11% and 8% of the transcriptome in leaf and flower, respectively (Tian et al., 2005). AtHDA19 as well as AtHDA6 and AtHDA9 are involved in several aspects of plant development (Aufsatz et al., 2002; Earley et al., 2006; Fong et al., 2006; Kim et al., 2013).

The findings reported in this study provide evidence that AtHDA7 is required for female gametophyte and embryo development as well as plant growth in Arabidopsis. However, further investigation is essential for elucidating the underlying mechanisms.

MATERIALS AND METHODS

Plant Materials and Phenotype Analyses

Arabidopsis (Arabidopsis thaliana) material includes Salk_002912C (herein hda7-1), amiHDA7 lines (herein Athda7-1 and Athda7-2), and Columbia as the wild type. Plants were grown in controlled conditions with 16 h/8 h of light/dark at 22°C/18°C. In vitro culture was performed as described by Weigel and Glazebrook (2002). For transformation, Agrobacterium tumefaciens GV3101 and the floral spray method were used (Chung et al., 2000). Transformants were selected on kanamycin (50 mg L\(^{-1}\)) and 14 d after 4-d stratification as described by Tanaka and colleagues (2008). The growth rate of seedlings (stages 0.5–1.0) was estimated according to Boyes et al. (2001). Soil-based analysis of growth was performed every 2 d by counting the number of rosette leaves more than 1 mm in length produced over 31 d according to Boyes et al. (2001).

In Silico Analysis of HDA7

The gene structure of HDA7 (At5g36000) was obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org). An area 1,000 bp upstream of the predicted start codon of HDA7 was analyzed with NSITE-PL (http://limus1.softberry.com) and Akiyama’s TFSEARCHv1.3 (http://molsun1.cbr.ist.ac.jp/research/db/TFSEARCH.html) with default setting to detect putative regulatory motifs.

Artificial MicroRNA

The specific artificial microRNA to silence AtHDA7 (amiHDA7) and the oligonucleotide sequences were designed using the WMD3 Web tool according to the procedures and criteria described by Schwab and colleagues (2010; http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). The predicted mature microRNA sequence was 5′-UUGACAGAGUAAUGUGCCA-3′. Primers used in the construction of amiHDA7 are listed in Supplemental Table S2. The cloning of amiHDA7 was performed using the mir319a precursor-containing plasmid pRS300 as a template (Schwab et al., 2010). Primers A and B were modified to allow the cloning of the final PCR product with Gateway technology into pKCIW7 binary vector (http://gateway.psb.ugent.be) using pDONR/ZEO (Invitrogen) as donor. Eleven transgenic lines with a single insertion were obtained (Supplemental Table S3).

Expression Analysis

Total RNA was extracted from young leaves and floral buds using the RNeasy Plant Mini Kit (Qiagen) and treated with DNase I (Invitrogen). To obtain complementary DNA, SuperScript III Two-Step RT-PCR and Oligo(dT)12-18 (Invitrogen) were used in the construction of amiHDA7 as described by Weigel and Glazebrook (2002). For transformation, Agrobacterium tumefaciens GV3101 and the floral spray method were used (Chung et al., 2000). Transformants were selected on kanamycin (50 mg L\(^{-1}\)) and 14 d after 4-d stratification as described by Tanaka and colleagues (2008). The growth rate of seedlings (stages 0.5–1.0) was estimated according to Boyes et al. (2001). Soil-based analysis of growth was performed every 2 d by counting the number of rosette leaves more than 1 mm in length produced over 31 d according to Boyes et al. (2001).

ChIP

For ChIP analysis, 3-week-old seedlings were used. For immunoprecipitation, antibodies against acetylated histone H3 (Millipore; catalog no. 06–599), histone H3 (Abcam; catalog no. 1791), and unspecific IgG (from rabbit; Diagenode) were used. Cross linking and immunoprecipitation were performed with the LowCell Number ChIP kit from Diagenode as described previously.
(Gutzat et al., 2011). DNA was analyzed by quantitative PCR on a Roche Light Cycler 96 instrument and with the FastStart SYBR Green Master mix (Roche) according to the manufacturer’s instructions. For quantification, results from two PCRs were used. All ChIP experiments were performed with two biological replicates. Primer sequences were chosen either in highly histone H3-acetylated regions at the 5’ end of the open reading frame of AtHDA6 (At4g06319), AtHDA9 (At2g44680), or AtAESP (At4g22970) or farther downstream in less acetylated regions. Primer sequences are listed in Supplemental Table S5.

Immunoblotting

Histone-enriched protein extracts were prepared according to Gendrel et al. (2002). The chromatin was treated twice with 0.4 M sulfuric acid, and the proteins were precipitated with 20% TCA. All the buffers were supplemented with 0.1 mM phenylmethylsulfonyl fluoride and protease inhibitor tablets (Complete Mini; Roche). For hybridization, H3K9Ac antibody (Dharmacon; pAb-005-044) was incubated overnight in a 1:1,000 dilution in 1% milk Tris-buffered saline plus Tween 20 buffer. To normalize histone levels, membrane was reprobed with H3 antibody (Abcam; Ab1791). Immunoblotting was replicated three times. The hybridizing bands were quantified using ImageJ software.

Analysis of Fertility-Associated Traits

Pollen viability was assessed by Alexander’s staining (Alexander, 1969). For megagametophyte analysis, ovules were excised from differentiated-pistils (1, 1.3, 1.5, 1.7, and 2 mm) previously fixed in ethanolacetic acid (3:1, v/v). They were cleared with a chloral hydrate solution (8 g of chloral hydrate, 1 mL of glycerol, and 2 mL of water) and examined with the Leica DM6000B microscope equipped with DIC and phase-contrast optics. In addition, ovules dissected from pistils in floral buds at stage 13 (Smyth et al., 2000), clarified in 85% solution of lactic acid and phenol (2:1, w/w) for 30 min, and stained with 10 μg mL⁻¹ DAPI were observed with a Leica DMR microscope equipped with fluorescence. For embryo development analysis, seeds within silques were examined by DIC microscopy according to Meinke et al. (1994). Finally, the length of mature silques, the seed set, and the unfertilized ovules were assessed at stages 17 and 18 (Smyth et al., 1990) by stereomicroscopy at a magnification of 20×.

Statistical Analyses

A χ² test was used in the segregation analysis and in the growth rate experiments. Student’s t test was used in real-time RT-PCR analysis.

Analysis of Gene Coregulation

The Expression Angler tool (http://bar.utoronto.ca) was used to identify genes coregulated with AtHDA7 during embryo and flower development. The expression data of the experiments selected for our analysis are listed in Supplemental Table S6. An r value cutoff ranging from 0.75 to 1.0 and from −0.75 to −1.0 was chosen.

Supplemental Data

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

Supplemental Figure S1. Expression analysis of AtHDA7 in Athda7™ leaf with respect to the wild type.

Supplemental Figure S2. Ovule and megasporogenesis in Athd7-2: megaspore mother cell (A) and functional megaspore with degenerate megaspores (B).

Supplemental Figure S3. Stages (FG) of female gametophyte (ES) development in Athd7-2 compared with the wild type according to Christensen et al. (1997).

Supplemental Figure S4. Degenerating ovules excised from pistils of 2 mm in Athda7-2 observed by phase contrast.

Supplemental Figure S5. ChIP analysis of acetylation levels of histone H3 at AtHDA6, AtHDA9, and AtAESP in 3-week-old wild-type and Athd7-2 seedlings.

Supplemental Figure S6. Functional annotation of AtHDA7 coregulated genes in embryos (from globular to torpedo stage) and in ovaries and flowers (from stage 9 to stage 15; Smyth et al., 1990).

Supplemental Table S1. List of genes that are coregulated with AtHDA7 in embryos (from globular to torpedo stage) and in ovaries and flowers (from stage 9 to stage 15; Smyth et al., 1990).

Supplemental Table S2. Primers used to clone amiHDA7.

Supplemental Table S3. Segregation analysis of T2 amiRNAi-HDA7 lines on selective media.

Supplemental Table S4. Primers used for real-time RT-PCR experiments.

Supplemental Table S5. Primers used for ChIP-PCR experiments.

Supplemental Table S6. List of the microarray experiments selected to identify AtHDA7 coregulated genes during embryo and flower development.

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