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HISTONE DEACETYLASE 9 represses seedling traits in *Arabidopsis thaliana* dry seeds

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SUMMARY

Plant life is characterized by major phase changes. We studied the role of histone deacetylase (HDAC) activity in the transition from seed to seedling in Arabidopsis. Pharmacological inhibition of HDAC stimulated germination of freshly harvested seeds. Subsequent analysis revealed that *histone deacetylase 9* (*hda9*) mutant alleles displayed reduced seed dormancy and faster germination than wild-type plants. Transcriptome meta-analysis comparisons between the *hda9* dry seed transcriptome and published datasets demonstrated that transcripts of genes that are induced during imbibition in wild-type prematurely accumulated in *hda9-1* dry seeds. This included several genes associated with photosynthesis and photoautotrophic growth such as *RuBisCO* and *RuBisCO activase* (*RCA*). Chromatin immunoprecipitation experiments demonstrated enhanced histone acetylation levels at their loci in young *hda9-1* seedlings. Our observations suggest that HDA9 negatively influences germination and is involved in the suppression of seedling traits in dry seeds, probably by transcriptional repression via histone deacetylation. Accordingly, *HDA9* transcript is abundant in dry seeds and becomes reduced during imbibition in wild-type seeds. The proposed function of *HDA9* is opposite to that of its homologous genes *HDA6* and *HDA19*, which have been reported to repress embryonic properties in germinated seedlings.

Keywords: seed dormancy, germination, histone deacetylase, *Arabidopsis thaliana*, HDA9, RuBisCO.

INTRODUCTION

Seeds are essential for plant dispersal, conservation and agriculture and constitute a major food source. Their protective structures and low hydration status, together with a low metabolic activity, allow dry seeds to survive long periods of unfavourable environmental conditions. Timing of germination is a crucial life history trait as it largely determines the environmental conditions in which the plant will grow and reproduce. Seed dormancy is an important mechanism controlling the moment of germination. Dormancy is defined as the incapacity of a viable seed to germinate under favourable environmental conditions and evolved to survive temporal unfavourable conditions, for instance dry summers or cold winters (Holdsworth *et al.*, 2008; Graeber *et al.*, 2012). In the model plant *Arabidopsis thaliana*, seed development takes about 20 days under standard greenhouse conditions and can be divided in two distinct stages. The first ~10 days are dedicated to embryo development and the second half of seed development, defined as the seed maturation phase, is committed to accumulation of storage reserves, acquisition of desiccation tolerance and establishment of seed dormancy. Dry storage of ripe seeds leads to a reduction in dormancy.

This process is called after-ripening and can take a few weeks up to several months, depending on the Arabidopsis accession (Bentsink *et al.*, 2010). Moreover, dormancy can be broken by environmental signals such as cold stratification in the presence of water (Graeber *et al.*, 2012). Germination occurs when a non-dormant, mature seed meets permissive environmental conditions regarding humidity, light and temperature and is defined by the moment of radicle protrusion through the surrounding seed structures (Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008). Upon germination the young seedlings establish and switch from heterotrophic growth driven by seed storage reserves to photoautotrophic growth, driven by photosynthesis.

The phase transitions from dormancy to germination and from heterotrophic to photoautotrophic growth involve the activation of many genes controlling the 'new' state and simultaneously repression of genes controlling the preceding phase. Such processes are typically governed by chromatin remodelling processes and associated changes in epigenetic modifications, which determine the accessibility of the DNA to the transcription machinery. During seed maturation, the nuclei shrink and the chromatin becomes highly compact (Van Zanten *et al.*, 2011). Reduction of chromatin compaction and increase of nuclear size occur during seed imbibition and are associated with dispersion of heterochromatic repeat sequences (Van Zanten *et al.*, 2011). At the epigenetic level, loci of major dormancy genes switch from the active histone modification H3K4me3 to inactive H3K27me3 during the transition from dormant seed to germinated seedling (Müller *et al.*, 2012). In accordance, several histone modifying enzymes and histone variants are differentially expressed in dormant compared to non-dormant seeds (Cadman *et al.*, 2006; Müller *et al.*, 2012). Several of these are functionally associated with the control of dormancy/germination. For example, mutations in *HISTONE MONOUBIQUITINATION 1* (*HUB1*), involved in monoubiquitination of histone H2B, cause reduced dormancy. Transcription of various genes required for seed dormancy was affected in this mutant during seed maturation (Liu *et al.*, 2007). Similarly, REDUCED DORMANCY 2 (RDO2)/Transcription elongation Factor S-II (TFIIS) is required for seed dormancy, presumably by counteracting negative effects of increased chromatin compaction during seed maturation (Liu *et al.*, 2011). A direct role of histone methylation in dormancy and germination was revealed by a study of the histone methyltransferase *KRYPTONITE* (*KYP*)/*SU(VAR)3-9 HOMOLOG 4* (*SUVH4*), which is required for the establishment of H3K9me2. The *kyp* mutants show increased seed dormancy whereas the reverse was observed in overexpression lines (Zheng *et al.*, 2012).

Acetylation of lysine (K) residues in histones is a reversible process that plays vital roles in epigenetic transcriptional regulation. Positioning and removal of these marks are respectively catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Loidl, 2004; Hollender and Liu, 2008). Generally, acetylated histones are linked to transcriptionally active loci with an open chromatin configuration, whereas deacetylation is associated with gene repression and condensed chromatin. The HDAC superfamily in Arabidopsis consists of 18 members, which are divided in three subfamilies; RPD3/HDA1, SIR2 and the plant specific HD2 family (Pandey *et al.*, 2002; Alinsug *et al.*, 2009). It was recently shown that mutations in two members of the histone deacetylation complex in Arabidopsis, SIN3-LIKE 1 (SNL1) and SNL2, have increased acetylation levels and reduced seed dormancy (Wang *et al.*, 2013). This indicates that histone acetylation is involved in the phase transition from the dormant seed to the germinated seedling.

Histone acetylation affects both dormancy and germination genes and its manipulation can therefore have both positive and negative influences on germination. Application of the histone deacetylase inhibitor Trichostatin-A (TSA) inhibited germination in maize (*Zea mays*), probably by affecting H3 acetylation levels at the *VIVIPAROUS1* promoter (Zhang *et al.*, 2011). Tai *et al.* (2005) reported the occurrence of a transient deacetylation event during early seed germination in Arabidopsis and mutations in the *AtHD2* family members affect germination (Sridha and Wu, 2006; Colville *et al.*, 2011). The best studied HDACs in Arabidopsis belong to RPD3 Class 1. These include HDA6, HDA19, HDA7, HDA9, and the pseudogenes HDA10 and HDA17 (Pandey *et al.*, 2002). HDA6 and HDA19 act redundantly in the repression of embryonic properties after germination (Tanaka *et al.*, 2008), but little information is available about the function of HDA7 and HDA9. A recent paper described a role for HDA7 in female gametophyte development and embryogenesis (Cigliano *et al.*, 2013). Gu *et al.* (2013) demonstrated that HDA9 is part of Sin3-HDAC multi-protein complexes that mediate transcriptional co-repression. The AT-hook motif-containing protein 22 (AHL22) physically interacts with HDA9 to modulate expression of *FLOWERING LOCUS T* (*FT*) in flowering time control (Yun *et al.*, 2012). HDA9 represses flowering under short day conditions by affecting H3K9Ac and H3K27Ac on the floral promoting gene *AGAMOUS-LIKE19* (*AGL19*) (Kim *et al.*, 2012).

In this work we report that Arabidopsis HDA9 is a negative regulator of germination. *Hda9* mutant seeds have enhanced histone acetylation levels, reduced seed dormancy and germinate significantly faster than wild-type. Meta-analysis comparisons between the *hda9* dry seed transcriptome and publicly available datasets revealed high transcript levels in the *hda9* mutant of genes normally only induced during imbibition and germination. The upregulation of these genes in *hda9* dry seeds correlated

with enhanced histone acetylation levels at their loci in seedlings. Our data show that HDA9 is a repressor of seedling traits in dry seeds. The function of HDA9 is therefore opposite to that of its homologous genes HDA6 and HDA19, which repress embryonic properties in germinated seedlings (Tanaka *et al.*, 2008).

RESULTS

HISTONE DEACETYLASE 9 controls seed dormancy and germination

To test the involvement of histone deacetylase (HDAC) activity in Arabidopsis seed dormancy, we imbibed freshly harvested wild-type Columbia-0 (Col) seeds in the presence of various concentrations of the HDAC inhibitor Trichostatin-A (TSA; Yoshida *et al.*, 1995). The used seeds had a low level of dormancy, which made it possible to identify both positive and negative effects of TSA. TSA enhanced germination in a dose-dependent manner, up to a level comparable to 4 days cold stratification, a treatment well known to break seed dormancy (Figure 1a,b). Similar results were obtained using the HDAC inhibitor Butyric acid sodium salt (BUT; Chen and Pikaard, 1997) (Figure 1b). These results indicate that inhibition of HDAC activity causes germination of seeds that otherwise remain dormant.

Transcripts of the Class I RPD3 HDACs; *HDA6*, *HDA19* and *HDA9* have been detected in dry seeds, whereas no transcript of the fourth member, *HDA7*, was found (Alinsug *et al.*, 2009). HDA6 and HDA19 redundantly act in the repression of embryonic properties in germinating seeds (Tanaka *et al.*, 2008). Accordingly, these two genes were found to be upregulated during seed imbibition in the dark in most publicly available seed-related microarray studies (Figure S1; Nakabayashi *et al.*, 2005; Cadman *et al.*, 2006; Finch-Savage *et al.*, 2007; Alinsug *et al.*, 2009). In contrast, *HDA9* transcript levels are reduced during imbibition in darkness (Figure S1; Hollender and Liu, 2008; Alinsug *et al.*, 2009), although a temporary increase in *HDA9* transcript was noted at the onset of imbibition (Figure S1c). We verified these observations by qRT-PCR on imbibed non-dormant Col seeds in the light. *HDA9* expression remained unaltered throughout seed maturation (Figure 2a), but was reduced upon imbibition/germination after a temporary increase during early imbibition (Figure 2b). The reduction of *HDA9* transcription became significant after 48 h imbibition (Figure 2b).

To test whether HDA9 controls dormancy and germination, we analysed primary seed dormancy in *hda9-1* and *hda9-2* mutant lines bearing T-DNA insertions in respectively the fifth exon (SALK_001723) and the first intron (GABI_305G03) of *HDA9* (Figure 3a) (Kim *et al.*, 2012). Both mutant lines lacked full-length *HDA9* transcripts (Figure 3b) and exhibited significantly reduced seed dormancy compared to wild-type (Figure 3c). Moreover, after-ripened *hda9-1* seeds germinated significantly faster than those of wild-type (Figure 3d). A controlled deterioration experiment showed that *hda9-1* longevity is significantly enhanced (Figure 3e).

Constitutive *HDA9* overexpression (Figure S2) in the *hda9-1* genetic background complemented the seed dormancy phenotype (Figure 3f,g). This confirms that *HDA9* is a positive regulator of dormancy. However, constitutive overexpression of *HDA9* in the wild-type background did not alter seed dormancy levels (Figure S3). This suggests that native HDA9 levels are sufficient to maintain dormancy in wild-type plants.

HDA9 affects seed germination and dormancy largely independent of abscisic acid, gibberellin and dormancy-related factors

Seed dormancy and germination are controlled by the balance between the phytohormones abscisic acid (ABA),

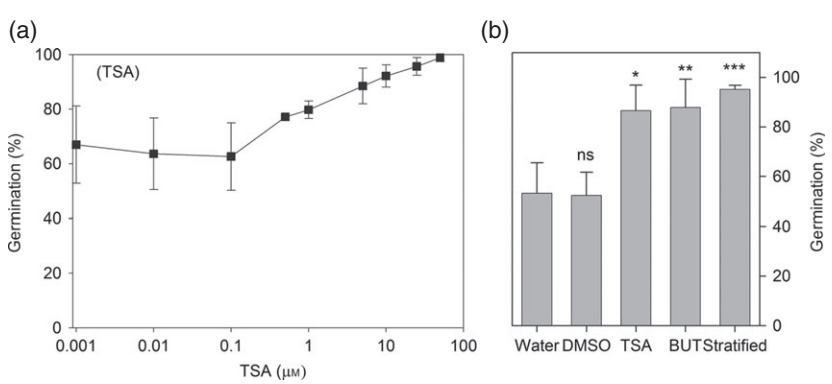

Figure 1. Inhibition of HDAC activity enhances germination.
(a) Dose–response effect of HDAC inhibitor trichostatin-A (TSA) on germination of freshly harvested wild-type seeds. Shown are averages ± standard error (SE) of $n = 3$–4 independent batches of seeds.
(b) Effect of TSA (50 μM), butyric acid sodium salt (BUT; 100 μM) and stratification (dark imbibition 3 days at 4°C) on germination of freshly harvested wild-type seeds (gray bars). Water treated and mock (DMSO solvent) are included as control. Significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns = non-significant; compared to water control (stratification) or DMSO MOCK (TSA and BUT) by Student's *t*-test. Shown are averages ± SE of $n = 3$–4 independent batches of seeds.

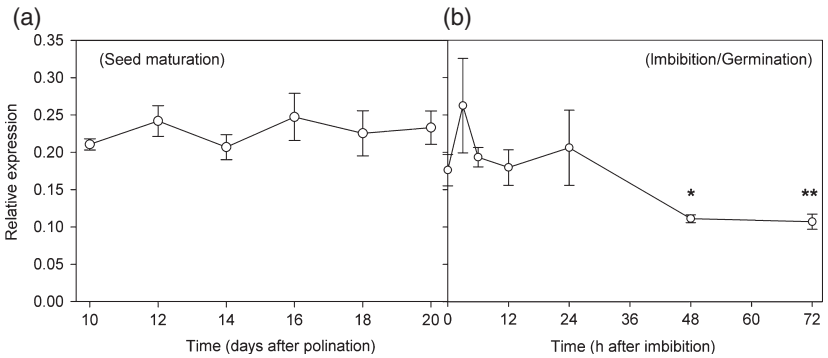

Figure 2. *HDA9* expression during imbibition and seed maturation.
(a, b) qRT-PCR analysis of *HDA9* expression in Col during (a) seed maturation; and (b) imbibition of after-ripened seeds. Expression values are relative to *ACTIN8*. $n = 3$–6 biological replicates; Error bars represent standard error (SE). Significance: $*P < 0.05$, $**P < 0.01$ by Student's *t*-test compared to dry seeds.

which enhances dormancy and suppresses germination, and gibberellin (GA), which stimulates germination (Finkelstein *et al.*, 2008; Holdsworth *et al.*, 2008; Graeber *et al.*, 2012). The sensitivity to ABA was similar in *hda9-1* and wild-type after-ripened seeds (Figure 4a). GA sensitivity appeared slightly enhanced in freshly harvested *hda9-1* mutant seeds (Figure 4b). Surprisingly, germination of after-ripened seeds showed a weakly enhanced sensitivity to the GA biosynthesis inhibitor Paclobutrazol (Figure 4c). This suggests that HDA9 has a marginal influence on GA sensitivity, which is opposite in freshly harvested and after-ripened seeds.

Mutants in *DELAY OF GERMINATION 1* (*DOG1*) (Bentsink *et al.*, 2006), *RDO2* (Grasser *et al.*, 2009; Liu *et al.*, 2011) and *HUB1* (Liu *et al.*, 2007) have very low dormancy levels. Altered expression of *DOG1* during seed maturation is correlated with changes in dormancy/germination behaviour (Nakabayashi *et al.*, 2012). Therefore, we followed expression of these genes during seed maturation in *hda9-1* (Figure 4d–f). As previously shown, *RDO2* and *HUB1* were gradually upregulated towards the end of seed maturation (Figure 4e, f) (Liu *et al.*, 2011) and *DOG1* expression peaked in the middle of seed maturation (Figure 4d) (Nakabayashi *et al.*, 2012). The expression of *RDO2* was similar in *hda9-1* and wild-type. However, *DOG1* and *HUB1* expression was slightly reduced in *hda9-1* between 12 and 18 days after pollination (DAP). This mild reduction could contribute to the reduced dormancy level of *hda9-1.*

HDA9 controls expression levels of photosynthesis related genes

To study which genes and biological processes are affected by *HDA9* in seeds, we compared the transcriptome of dry *hda9-1* seeds with wild-type seeds using microarrays. Evaluation of the data with RankProd (Hong *et al.*, 2006) revealed 576 significantly ($P < 0.05$) differentially expressed genes (209 upregulated; 367 downregulated). Using LIMMA (De Menezes *et al.*, 2004; Smyth, 2004), 869 significant differentially expressed genes were found (312 upregulated; 557 downregulated). Seventy upregulated and 141 significantly downregulated genes were identified by both methods, considering only those genes that changed more than two-fold compared to wild-type (Data S1). *HDA9* was among the most strongly downregulated genes as were its related pseudogenes *HDA10* (At3g44660) and *HDA17* (At3g44490) (Pandey *et al.*, 2002). In accordance with the qRT-PCR data (Figure 4d–f), *DOG1*, *RDO2* and *HUB1* were not identified as differentially regulated in dry seeds, neither were any GA and ABA-related genes. The microarray results were confirmed by qRT-PCR using five genes that were significantly downregulated and 11 that were upregulated in the transcriptome analysis (Figure 5a, b).

Because no differential expression of known dormancy or germination genes could be identified in the *hda9-1* mutant in the transcriptome experiment, a MapMan analysis (Thimm *et al.*, 2004) was performed to identify the biological processes that are affected by *HDA9.* Four bins were found to be significantly affected in the *hda9-1* mutant compared to wild-type after Benjamini–Hochberg correction. Most prominent among these is bin 1 (Photosynthesis; $P = 0.0004$), represented by 11 differentially regulated genes in *hda9-1* (Table 1). Of these, six belonged to bin 1.2 (Calvin cycle; $P = 0.047$). Furthermore, bin 16.5 (secondary metabolism, sulfur-containing; $P = 0.046$) and 16.5 (secondary metabolism, sulfur-containing glucosinolates; $P = 0.046$) were significantly differentially regulated in *hda9-1.*

HDA9 represses seedling traits in dry seeds

Interestingly, all 11 differentially regulated genes belonging to the MapMan photosynthesis bin (Table 1) were upregulated in *hda9-1* compared to wild-type. Among them was a gene encoding for the small subunit 2B of the key enzyme involved in carbon fixation; *Ribulose-1,5-bisphosphate carboxylase oxygenase* (*RuBisCO*; *RBCS*) and its regulatory factor; RuBisCO Activase (RCA). The latter is an ATPase driving conformational changes in RuBisCO that promotes release of inhibitory sugar phosphates from its catalytic site, resulting in activation of RuBisCO (Portis, 2003; Portis *et al.*, 2008). In accordance with the upregulation of RuBisCO (Table 1), *hda9-1* seeds that were 24 h

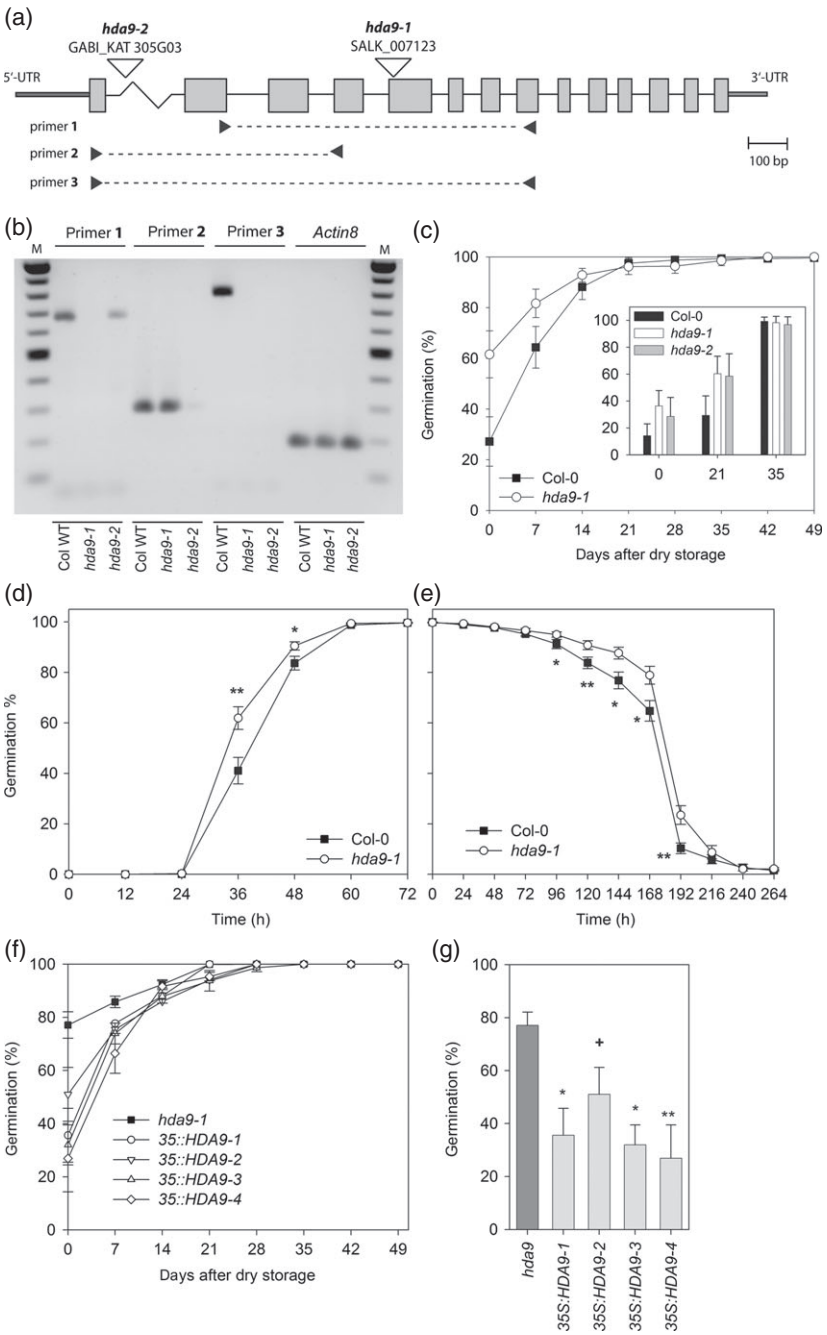

Figure 3. Identification of *HDA9* as a regulator of seed dormancy and germination.
(a) Schematic representation of the *HDA9* locus and T-DNA insertions (white triangles) of *hda9-1* (SALK_007123) and *hda9-2* (GABI_305G03). Light gray boxes indicate exons, dark gray bars represent the untranslated regions (UTRs) and the solid lines represents introns. Primers used for qRT-PCR are shown below the gene. Scale bar is 100 bp.
(b) qRT-PCR analysis using primers indicated in panel (a). *ACTIN8* (At1g49240) was used as control.
(c) Germination capacity of *hda9-1* (white circles) and wild-type Col (black squares) during dry storage. Inset represents germination at harvest and after 21 and 35 days storage of Col, *hda9-1* and *hda9-2* in an independent experiment; $n > 20$.
(d) Percentage of germinating seeds during imbibition of *hda9-1* (white circles) and wild-type (black squares).
(e) Germination (%) of *hda9-1* (white circles) and wild-type (black squares) after controlled deterioration; $n = 25$.
(f) Germination capacity of independent *HDA9* overexpression lines (in white) in the *hda9-1* genetic background (black squares) during dry storage.
(g) Germination capacity of the *HDA9* overexpression lines from panel (f), at fresh harvest ($t = 0$ days). Significance levels: $^{+}P < 0.1$; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to Col (panel [d, e] or *hda9-1* (panel [g]) by Student's *t*-test. Error bars represent standard error (SE), $n \geq 14$.

imbibed contained significantly higher protein abundance of the RuBisCO small subunit(s) (RBCS) per gram total protein, as well as the RuBisCO large subunit (RBCL) (Figure 5c), whereas seed weight itself was unaffected by the *hda9-1* mutation (*hda9-1*; 24.7 ± 0.93 versus wild-type; 24.6 ± 0.77 μg per seed). However, *hda9-1* does not affect all photosynthesis-related factors, since we could not detect major differences in initial Chlorophyll A and B levels in dry seeds, nor in the speed of their accumulation during imbibition/germination (Figure 5d,e).

Transcripts of genes associated with photoautotrophic growth, including *RuBisCO* subunits and *RCA* are not, or only at a very low level present in wild-type Arabidopsis dry seeds (Nakabayashi *et al.*, 2005; Fait *et al.*, 2006; Demarsy *et al.*, 2012). Publicly available microarray data (Figure S4a; Fait *et al.*, 2006; Demarsy *et al.*, 2012) and qRT-PCR experiments (Figure S4b) indicated that these genes are induced in the course of imbibition and at the radicle-protrusion stage of germination in wild-type seeds. The presence of their transcripts in *hda9-1* dry seeds therefore

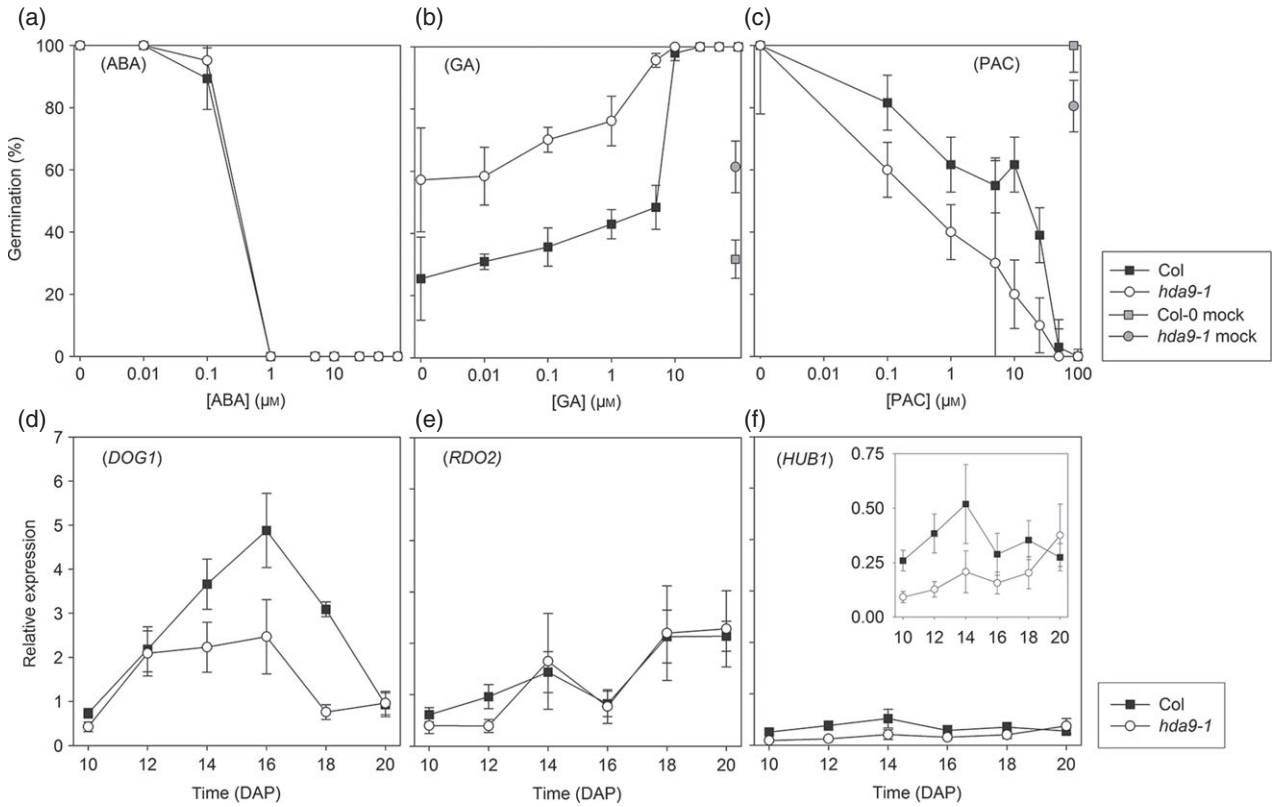

Figure 4. The relation of HDA9 with hormones and dormancy regulators.
Dose–response effects of (a) abscisic acid (ABA), (b) gibberellin A4 (GA), and (c) the GA biosynthesis inhibitor paclobutrazol (PAC), on germination behaviour of wild-type (black squares) and *hda9-1* (white circles).
(a, c) Seeds used for the ABA and PAC treatments had been dry-stored for 2 weeks to release dormancy. The GA treatment was performed on partly-dormant freshly harvested seeds. The effects of the mock treatment (0.01% ethanol for GA and 0.01% DMSO for PAC and ABA) are indicated as gray squares (Col) and gray circles (*hda9-1*). $n = 4$ independent batches of seeds. Error bars represent standard deviation (SD).
(d–f) Expression of (d) *DOG1*, (e), *RDO2* and (f) *HUB1* in Col (black squares) and *hda9-1* (white circles) seeds during seed maturation. Expression values are relative to *ACTIN8*. $n = 3$–6 biological replicates; error bars represent standard error (SE). Inset in (f) shows the expression of *HUB1* on a different scaling.

suggests that the imbibition program starts prematurely at the transcriptional level in this mutant. To verify this hypothesis we performed a meta-analysis in which our transcriptome data of *hda9-1* dry seeds were compared with published datasets during different times of seed imbibition (Goda *et al.*, 2008; Preston *et al.*, 2009) and ABA and GA treatments (Goda *et al.*, 2008), using the R statistical package *OrderedList* (Lottaz *et al.*, 2006; Yang *et al.*, 2006). A comparison of the lists of differentially regulated genes from seeds imbibed for 3 and 24 h with the differentially regulated genes in dry *hda9-1* seeds displayed significant greater similarities with respect to their orderings than expected for randomly selected genes (Table 2). We therefore concluded that the set of differentially regulated genes in dry *hda9-1* mutant seeds is enriched for those that are normally regulated in the course of imbibition/early germination in wild-type. No significant similarities were detected in the comparisons with hormone treatments (Table 2). This underlines our observations that HDA9 functions independently of ABA and that GA sensitivity was only marginally affected in *hda9-1* mutant seeds (Figure 4).

The *hda9* mutant shows enhanced H3K9 acetylation levels at photosynthesis genes

Kim *et al.* (2012) demonstrated that histone H3 acetylation levels are enhanced in *hda9* mutants. Accordingly, we found increased H3K9Ac levels in dry seeds of *hda9-1* compared to wild-type Col (Figure 6a). This increase was associated with the premature start of the imbibition program at the transcriptional level in dry *hda9-1* seeds. We were interested to find out whether this association also occurs at the individual gene level. Because chromatin immunoprecipitation in dry seeds is not feasible we used seedlings for these experiments. A selection of key photosynthesis-associated genes, found to be transcriptionally upregulated in *hda9-1* dry seeds (Figures 5a,b and S5), was assayed (*RBCS2B* (At5g38420/30), *RCA* (At2g39730), *PRK* (At1g32060), and *GLDP1* (At4g33010). As negative control *2OG* (At5g54000) was used, which is transcriptionally

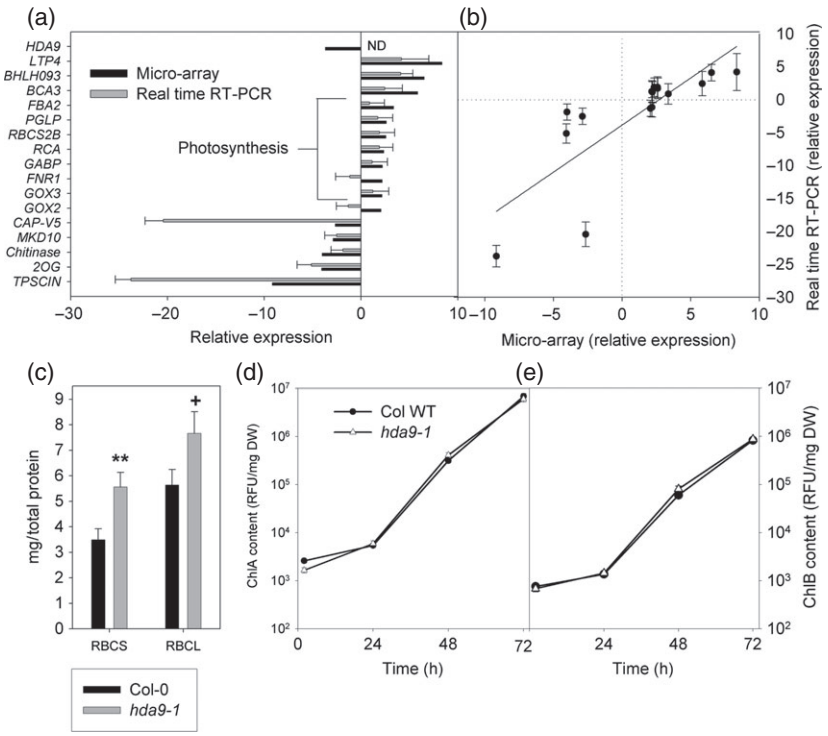

Figure 5. HDA9 represses vegetative properties in dry seeds.

(a, b) Confirmation of differentially expressed genes from the transcriptome analysis.

(a) Relative expression of a subset of genes detected as differentially regulated in *hda9-1* compared to wild-type by microarray analysis (black) and their confirmation by real-time qRT-PCR (gray). Genes belonging to the MapMan photosynthesis bin are indicated.

(b) Correlation analysis of the figure presented in panel (a). *LTP4, LIPID TRANSFER PROTEIN 4*; *BHLH093, BASIC HELIX-LOOP-HELIX 93, DNA binding/transcription factor*; *BCA3, BETA CARBONIC ANHYDRASE3*; *FBA2, FRUCTOSE-BISPHOSPHATE ALDOLASE2*; *PGLP, PHOSPHOGLYCOLATE PHOSPHATASE*; *RBCS2B, RuBisCO small subunit 2B*; *RCA, RUBISCO ACTIVASE*; *GABP, GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*; *FNR1, FERREDOXIN-NADP REDUCTASE1/NADPH dehydrogenase/electron transporter*; *GOX2, GLYCOLATE OXIDASE2*; *GOX3, GLYCOLATE OXIDASE 3*; *CAP-V5, allergen V5/Tpx-1-related family protein*; *MKD10, S-adenosyl-L-methionine:carboxyl methyltransferase family protein*; *2OG, oxidoreductase, 2OG-Fe(II) oxygenase family protein; TPSCIN, Terpene Synthase-Like Sequence-1,8-Cineole.* $n = 5$ (qRT-PCR) and $n = 3$ (microarray), both consisting of independent biological replicates of several pooled plants.

(c) Protein abundance of RuBisCO small (RBCS) and large (RBCL) subunits in 24 h imbibed seeds of wild-type (black bars) and *hda9-1* mutants (gray bars); $n = 14$.

(d, e) Chlorophyll A (d) and Chlorophyll B (e) accumulation as measured by fluorometric assays in imbibed/germinating seeds in wild-type (black circles) and *hda9-1* mutants (white triangles) indicated as relative fluorescence units (RFU) per gram dry weight. $n = 12$. Significance; $^{**}P < 0.01$, $^{+}P < 0.1$. Error bars represent standard error (SE) in all panels.

downregulated in *hda9-1* dry seeds (Figure 5a). In general, H3K9 acetylation is enriched at the promoter, shows a peak downstream of the translation start site and decreases in the coding region (Zhou *et al.*, 2010). Therefore, we examined acetylation levels in the promoter region (−500 bp from the start codon) and within the 5′ region of the gene body (+500 bp), as well as more downstream in the gene (towards the 3′ end). H3K9Ac levels of all four tested genes were clearly enhanced in the 5′ (+500 bp) region in *hda9-1* compared to wild-type (Figures 6b and S6). Generally, also the promoter region demonstrated enhanced levels of H3K9Ac, although the results varied moderately among the independent biological replicates (Figures 6b and S6). No, or only weak differences in acetylation levels were noted in the 3′ region of the genes. As expected, H3K9Ac levels were largely unaltered in the negative control locus (*2OG*).

Taken together, our data indicate that dry seeds of *hda9-1* mutants can be considered as being in an imbibed state at the transcriptional level and, at least for RuBisCO, also at the protein level. Loss-of-*HDA9* leads to premature accumulation of transcripts of photosynthesis genes in dry seeds, which correlates with enhanced acetylation levels at their corresponding loci in seedlings and a general enhancement of H3K9 acetylation in dry seeds. Although the changes at these loci cannot be directly connected with each other, it seems likely that HDA9 activity is required to repress transcription of specific genes in dry seeds to prevent premature expression of vegetative properties.

DISCUSSION

Plant life is characterized by major phase transitions including those from dormant seed to germinated seedling and the switch from heterotrophic to photoautotrophic

Table 1 Genes differentially expressed in *hda9-1* dry seeds compared to wild-type Col, belonging to MapMan Bin 1 (photosynthesis) and their qualitative response (upregulated [UP] or not altered [=]) upon imbibition based on the datasets of Nakabayashi *et al.* (2005) and Preston *et al.* (2009)

AGI code	Upregulation in *hda9-1*	Transcriptional response upon imbibition	Abbreviation	Gene name
AT5g38420/30	2.57	UP	*RBCS2B*	*Rubisco small subunit 2B*
AT2g39730	2.34	UP	*RCA*	*RUBISCO ACTIVASE*
AT4g28750	1.91	UP	*PSA-E1*	*Subunit E of Photosystem I*
AT1g32060	3.19	UP	*PRK*	*PHOSPHORIBULOKINASE*
AT3g14415/20	2.16	UP	*GOX2*	*GLYCOLATE OXIDASE 2*
AT4g18360	2.06	=	*GOX3*	*GLYCOLATE OXIDASE 3*
AT5g66190	2.17	UP	*FNR1*	*NADPH DEHYDROGENASE*
AT1g42970	2.21	UP	*GAPB*	*GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE B SUBUNIT*
AT5g36700	2.60	=	*PGLP*	*PHOSPHOGLYCOLATE PHOSPHATASE, PUTATIVE*
AT4g33010	3.12	UP	*GLDP1*	*GLYCINE DECARBOXYLASE P-PROTEIN1*
AT4g38970	3.36	UP	*FBA2*	*FRUCTOSE-BISPHOSPHATE ALDOLASE*

Table 2 Meta-analysis comparisons between *hda9-1* dry seeds and published microarray datasets. The *P*-values specify the significance of similarity of ordered gene lists

Comparison *hda9-1* mutant versus wild-type	*P*-value	References
Imbibition 1 h versus dry seeds	1	Preston *et al.* (2009)
Imbibition 3 h versus dry seeds	0.000	
Imbibition 24 h versus dry seeds	0.000	
ABA 24 h; 3 μM versus water	0.933	Goda *et al.* (2008)
ABA 24 h; 30 μM versus water	0.928	
GA 3 h; 5 μM versus water	1	
GA 6 h; 5 μM versus water	1	
GA 9 h; 5 μM versus water	1	

growth. The timing of these transitions largely determines the success of an individual plant and underlies adaptation to the various seasons of the year. The genetic components that are part of the molecular mechanisms controlling phase transitions need to be continuously adjusted to changes in the environment and developmental state to optimise plant performance. For example, dry heterotrophic seeds do not require the presence of photosynthetic machinery. Accordingly, transcripts encoding for the photosystems and RuBisCO are not detectable in wild-type dry seeds, but accumulate during germination to prepare for photoautotrophic growth (Demarsy *et al.*, 2012).

Activation and repression of gene activity often involves covalent epigenetic modifications of DNA and associated histone proteins. DNA can be methylated (5-methylcytosine), whereas marks associated with histones include acetylation, methylation, phosphorylation, and ubiquitination. Together, these decorations determine the transcriptional activity of a locus by controlling accessibility of the DNA to the transcription machinery (Loidl, 2004; He *et al.*, 2011). Over 20 possible targets for lysine-residue acetylation have been identified in histones (Mutskov *et al.*, 1998; Loidl, 2004; Hollender and Liu, 2008). Lysine has a positively charged amino group, which can be neutralized by acetylation and reduces its potential for electrostatic interaction with the negatively charged DNA (Charron *et al.*, 2009). Thus, dynamic acetylation and deacetylation allows the chromatin structure to open and condense respectively.

The role of histone deacetylation in the transition from seed to seedling

In Arabidopsis, deacetylation is catalyzed by three subfamilies of histone deacetylases; RPD3/HDA1, HD2 and SIR2 (Pandey *et al.*, 2002; Alinsug *et al.*, 2009). Some members of these families have been implicated in aspects of seed biology (Van Zanten *et al.*, 2013). Enhanced expression of Arabidopsis *HD2C* resulted in early germination (Sridha and Wu, 2006). Mutant analysis comprising the four HD2 family members (AtHD2A-D) demonstrated specific roles in seed dormancy (Colville *et al.*, 2011). For example, germination was reduced in *hd2c* mutants, but *hd2a* null mutants showed enhanced germination. Yano *et al.* (2013) demonstrated the presence of natural variation in suppression of *HD2B* transcription during imbibition, which partially correlated with seed dormancy levels among Arabidopsis accessions.

In contrast to our observations in Arabidopsis, inhibition of HDAC activity by TSA application led to delayed germination in maize (Zhang *et al.*, 2011). During early germina-

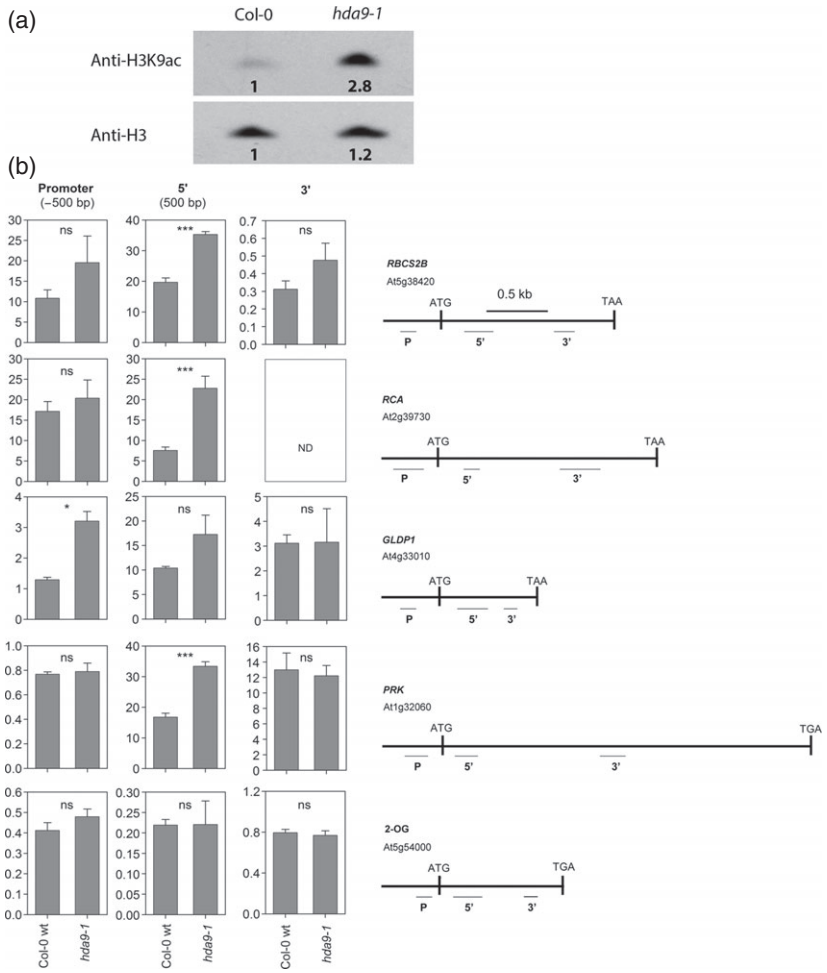

Figure 6. HDA9 affects histone acetylation levels of photosynthesis genes.
(a) Immunodetection of Histone H3K9 acetylation levels on histones isolated from freshly harvested Col wild-type and *hda9-1* dry seeds, using Anti-H3K9ac antibody. Anti-H3 was used as loading control. Values under the bands represent quantifications of the immunoblotting signal as measured by image analysis software, relative to Col wild-type.
(b) ChIP analysis of histone acetylation levels on *RBCS2B*, *RCA*, *PRK*, *GLDP1* and *2OG*. The accumulation of PCR product in wild-type and *hda9-1* after immunoprecipitation with Anti-H3K9ac antibody has been normalized to *ACTIN8*. A schematic diagram of each gene structure is depicted besides the panels including the ~500 bp promoter sequence. The black line between the vertical dashes indicates the open reading frame of the gene from start codon (ATG) to stop codon (TAA or TGA). The amplification sites used for each target gene are indicated as 'P' (promoter; −500 bp upstream start codon ATG; left column of graphs), '5'' (+500 bp downstream; middle column) and '3'' (within gene body, towards the 3′ end of the target gene; right column). The relative positions of the amplified fragments for each tested region are depicted below the gene structure. Scale bar represents 500 bp. A typical experiment is shown (out of three independent replicates; Figure S6). Immunoprecipitates were obtained from 10-day-old seedlings. Error bars represent standard deviation (SD). ND indicates non-detectable product. Significance levels (Student's *t*-test) are based on the variation within the experiment: $*P < 0.05$, $**P < 0.001$, $***P < 0.01$, ns = non-significant. For independent biological replicates see Figure S6.

tion of Arabidopsis seeds a transient deacetylation event was observed (Tai *et al.*, 2005). TSA application during the first day of imbibition resulted in a marked effect on gene expression. However, the influence of TSA on transcription was lost when it was applied 3 days after imbibition. It therefore appears that deacetylation events on the first day of imbibition are critical in the epigenetic control of gene expression during seed germination. Overall, the role of histone deacetylation in seed germination is complex, due to the presence of multiple histone deacetylases which likely have different target genes and expression patterns. Furthermore, histone deacetylation can affect genes that enhance and reduce germination capacity.

In this work, we showed that HDA9 is involved in the transition from seed to seedling and the switch from heterotrophic to photoautotrophic growth. HDA9 inhibits seed germination and is a repressor of seedling traits in seeds, most notably of core components of the photosynthetic machinery. HDA9 has been reported before to be ubiquitously expressed throughout most tissues including seeds. Its expression can be enhanced by GAs and Jasmonic Acid (Alinsug *et al.*, 2009), as well as by several days of cold stress (To *et al.*, 2011). We additionally showed that imbibition/germination of dry seeds resulted in downregulation of *HDA9* expression (Figure 2b). However, we found a transient increase in HDA9 expression during early seed imbibition. This may contribute to the above mentioned deacetylation event on the first day of imbibition, but it cannot be sufficient for induction of germination, as the *hda9-1* mutant was early germinating. Recently, HDA7 was shown to be crucial for female gametophyte development and embryogenesis (Cigliano *et al.*, 2013). Interestingly, *HDA9* expression was significantly upregulated in *hda7* mutants. Our meta-analysis, in which we compared transcriptomics data obtained from dry *hda9-1* seeds with various publicly available datasets, revealed striking and significant overlap in genes regulated by seed imbibition. This led us to conclude that dry seeds of *hda9-1* are in a partially imbibed state at the transcriptional level.

HDA9 control of seed germination

The role of HDA9 in seeds is evident by the various germination phenotypes of the *hda9-1* mutant. We observed reduced dormancy, enhanced germination speed and

increased resistance to controlled deterioration (artificial aging) (Figure 3c–e). Reduced dormancy is often associated with enhanced germination speed (Gardarin *et al.*, 2011) and it is therefore not surprising that the *hda9-1* mutant shows both phenotypes. The *hda9-1* mutant might germinate faster than wild-type because its transcriptome (and possibly its proteome) is already prepared for imbibition and germination in dry seeds. The presence of transcripts in dry *hda9-1* seeds that normally only become abundant during imbibition, suggests that these seeds are less adapted to dry storage, which could lead to reduced seed longevity during seed aging. Surprisingly, we observed an increased resistance to artificial aging in the *hda9-1* mutant. This seems counter-intuitive, but has recently also been shown by Nguyen *et al.* (2012), who observed a negative correlation between seed longevity and seed dormancy in a study of natural variation in Arabidopsis. The combination of better storability and faster germination with reduced seed dormancy in the *hda9-1* mutant could be interesting for agricultural and plant breeding applications.

HDA9 represses transcription of photosynthesis genes in dry seeds

The mRNA steady-state levels of several light-regulated genes corresponded with the H3K9Ac levels at their respective loci (Guo *et al.*, 2008). Moreover, a genome-wide assessment of H3K9Ac and H3K27Ac levels in young seedlings revealed that particularly photosynthesis-associated genes are prone to changes in acetylation levels. This was especially observed during the switch from darkness to light (Charron *et al.*, 2009) and it was suggested that acetylation could be a prerequisite for the transcriptional activation of photosynthetic genes. Photosynthesis-associated genes are downregulated in dry seeds and upregulated during imbibition (Fait *et al.*, 2006). We found a striking premature upregulation of photosynthesis-associated genes in *hda9-1* dry seeds (Figures 5a,b and S5 and Table 1), which correlated with enhanced general levels of H3K9 acetylation (Figure 6a). In addition, we showed that H3K9Ac levels were enhanced at the promoter sequences of these photosynthesis genes in *hda9-1* seedlings, and within their gene body close to the translation initiation site (Figures 6b and S6). This suggests that HDA9-mediated deacetylation is required to repress accumulation of photosynthesis-associated mRNAs in dry seeds. Accordingly, downregulation of *HDA9* transcription during imbibition/germination in wild-type seeds could contribute to the release of this repression (Figures 2b and S1), resulting in acetylation of the loci followed by the startup of photosynthesis and photoautotrophic growth. Hence, *hda9-1* mutants are considered to be in a partially imbibed state at the transcriptional level and HDA9 acts as a negative regulator of vegetative seedling traits in dry seeds.

ChIP assays cannot be performed on dry seeds (Van Zanten *et al.*, 2013), and therefore we used 10-day-old seedlings for these experiments. The analysed photosynthesis genes were selected because they have higher transcript levels in *hda9-1* dry seeds compared to wild-type (Figures 5 and S5). However, the opposite was observed in 10-day-old seedlings (Figure S7). Our ChIP data show nevertheless that the lower expression in *hda9-1* seedlings goes together with higher acetylation levels. This apparent contradiction might be explained by negative feedback regulation mechanisms on photosynthesis genes. When carbon metabolite levels/energy status are high, photosynthesis genes are repressed to balance carbon flow in the plant (Paul and Pellny, 2003). Because photosynthesis-associated gene transcripts and RuBisCO protein levels are more abundant in the early life of *hda9* mutants compared to wild-type plants (Figure 5), carbon gain will be earlier initiated in young *hda9* seedlings. This probably results in an enhanced nutritional status of *hda9* seedlings compared to wild-type, which might cause a differential negative feedback control of photosynthesis genes regardless of the acetylation state of the associated histones.

The RuBisCO large subunit, as well as other important proteins of the Calvin cycle, including RCA, are known to be modified at the posttranslational level by (de)acetylation of protein lysine residues in the cytoplasm/chloroplast (Finkemeier *et al.*, 2011; Wu *et al.*, 2011). The scenario that HDA9 directly affects RuBisCO protein acetylation is unlikely for several reasons. First, HDA9 is nuclear localized as it affects histone acetylation (Figure 6a; Kim *et al.*, 2012) and it interacts with the recently identified SAP30 FUNCTION RELATED 1 (AFR1) and AFR2 core structural components of the Arabidopsis histone deacetylase complexes in the nucleus (Gu *et al.*, 2013). In addition, Kim *et al.* (2012) showed increased H3K9 and H3K27 acetylation levels and increased expression of the *AGL19* locus in the *hda9* mutant, and we found a correlation between transcript abundance of HDA9 target genes and acetylation of the associated loci in *hda9-1* mutant seeds. Together, this suggests a direct role for HDA9 in histone acetylation. Nevertheless, the influence of the *hda9-1* mutant on RuBisCO seems to stretch beyond its direct regulation on histone acetylation as we observed enhanced protein levels of the large subunit of RuBisCO in *hda9-1* seeds. Unlike the small subunits that are encoded by four genes (*RbcS1A*, *RbcS1B*, *RbcS2B*, *RbcS3B*), there is only a single gene coding for the large subunit, which is located on the chloroplast genome (Bedbrook *et al.*, 1979; Sawchuk *et al.*, 2008). Plastid-encoded genes cannot be affected by histone deacetylation activity because they lack histones. However, expression of the large subunit is fine-tuned by the expression of the small subunits in Arabidopsis and the increased expression of the large subunit in dry *hda9-1* seeds may be a

consequence of the enhanced expression of the small subunit 2B (Izumi *et al.*, 2012).

Tanaka *et al.* (2008) reported that seeds treated with TSA arrested growth immediately after germination without any visible cotyledon expansion or greening. This indicates HDAC requirement for the switch from heterotrophic to the photoautotrophic program. This arrested phenotype was mimicked in the *hda6 hda19* double mutant, which also showed expression of embryo-like structures (Tanaka *et al.*, 2008). The effect of mutants in the seed maturation regulator-genes; *LEC1*, *FUS3* and *ABI3* could overcome the growth arrest phenotype. In conclusion, *HDA6* and *HDA19* synergistically act as repressors of embryonic properties shortly after germination when a transcriptional switch is required from the embryonic program into vegetative growth (Tanaka *et al.*, 2008). In accordance with this, we show here that *HDA6* and *HDA19* have the tendency to be transcriptionally upregulated during imbibition/germination (Figure S1). Interestingly, the *hda9* mutant revealed the opposite trend; *i.e.* seedling traits were prematurely induced in seed tissue where embryonic properties should prevail, and expression of *HDA9* is repressed during imbibition/germination in wild-type plants. The observation that HDAC activity is required to repress vegetative properties in seeds before germination implies that the actual phase transition from the seed transcriptional program to germination takes place during the process of imbibition/early germination, when *HDA9* transcript levels gradually drop and *HDA6* and *HDA19* expression increases. This is in accordance with the proposition made over two decades ago by Comai and Harada (1990), which was based on the observation of transcriptional timing of germination specific genes in *Brassica napus* L. It also matches previous observations that the physical chromatin structure strongly decondenses upon imbibition, just before germination occurs (Mathieu *et al.*, 2003; Van Zanten *et al.*, 2011).

EXPERIMENTAL PROCEDURES

Plant materials, growth conditions and construction of transgenic lines

Plant materials used are in the Col genetic background, unless indicated otherwise. Mutant *hda9-1* (SALK_007123) was obtained from the Nottingham Arabidopsis Stock Centre and *hda9-2* (Kim *et al.*, 2012; GABI_305G03) was a gift from Dao-Xiu Zhou. Semi-quantitative RT-PCR on 10-day-old seedlings confirmed the absence of full-length *HDA9* transcripts in the *hda9-1* and *hda9-2* mutants (Figure 3b). Plants were grown on soil (mixture of substrate and vermiculite 3:1) in growth cabinets (Elbanton, Kerkdriel, the Netherlands, Elbaton.nl) in 16 h light (22°C) and 8 h dark (16°C), unless described otherwise. In all cases where genotypes or treatments were compared, plants were grown and harvested in parallel to exclude environmental effects other than the tested variables on the observed phenotypes. In addition, all used seed materials were <1 year old when used, to circumvent detrimental effects of seed aging. Construction of *35S:HDA9* is described in Data S2.

Germination tests and controlled deterioration

Germination tests, germination-speed assays and imbibition of seeds used for qRT-PCRs, were performed by incubating seeds in Petri dishes (16 h light; 25°C/8 h darkness; 20°C) in moisturized transparent boxes. For germination tests, after 7-day germination percentages were analysed using a 'Germinator' setup (Joosen *et al.*, 2010) or were counted manually. After-ripening of dry seeds occurred in darkness (21°C; 50% relative humidity [RH]) in a controlled cabinet (MMM Medcenter, Brno, Czech Republic). For germination-speed assays and qRT-PCRs, after-ripened seeds were used (>6 weeks) treated in light.

For seed weight measurements batches of seeds were weighed on a microbalance, sown on moisturized filter paper and the number of seeds was determined using the 'Germinator' setup. Siliques used for experiments during seed maturation were tagged with coloured threads on the day of flower opening (0 DAP). Controlled deterioration experiments were performed by storing seed aliquots at 37°C and 83% RH. At indicated times seed batches were removed, dried and germination was tested using the 'Germinator' setup. For details see Data S2.

Pharmacological essays

Seeds were imbibed on small Petri dishes imbibed with a solution containing trichostatin-A (TSA), butyric acid sodium salt (BUT), ABA, or paclobutrazol (PAC) (all Sigma-Aldrich, Sigmaaldrich.com) dissolved in dimethyl sulfoxide (DMSO) or GA4 (GA) (Sigma-Aldrich) in ethanol. All solutions were diluted to the intended concentrations with water. Mock controls contained solvent, lacking the active component. The highest concentrations contained 0.1% solvent. In TSA and BUT assays, the testa (seed coat) of each individual seed was ruptured manually using a dissection needle to aid penetration of the chemical. Subsequently, the dishes were transferred to a moisturized transparent box and incubated for 7 days as described above. Germination was scored manually. Stratification of seeds comprised cold storage (4°C) on imbibed filter paper for 3 days.

Transcriptomics and quantitative RT-PCR

RNA was extracted from tagged dry 18–19 DAP siliques of wild-type and the *hda9-1* mutant as described in Data S2. Affymetrix GeneChip Arabidopsis ATH1 Genome-Micro (Affymetrix, Santa Clara, CA, USA) hybridization was performed at the Max Planck Genome Centre Cologne. Three independent hybridizations of independent biological replicates, obtained from several siliques of at least five plants per replica, were performed. Normalization, processing and statistical analysis of the raw data were performed using AFFY, LIMMA (De Menezes *et al.*, 2004; Smyth, 2004) and RankProd (Hong *et al.*, 2006) in R freeware. Genes were only considered differentially expressed between mutant and wild-type if both LIMMA and RankProd indicated a significant expression difference of at least two-fold. MAPMAN (Thimm *et al.*, 2004) was used for Gene Ontology (GO) analysis. All microarray data are MIAME compliant and have been deposited at the Gene Expression Omnibus database (GEO Accession number: GSE50786).

qRT-PCR was performed as described earlier (Liu *et al.*, 2011; Van Zanten *et al.*, 2011) and is described in Data S2.

Meta-analysis of publicly available Arabidopsis microarray datasets

Transcriptomics-derived data on *HDA6*, *HDA9* and *HDA19* transcription during imbibition was extracted from the Arabidopsis eFP browser (www.bar.utoronto.ca/efp) (Winter *et al.*, 2007). Meta-analysis between our microarray dataset on dry *hda9-1* seeds and published datasets was performed using the R package *OrderedList* (Lottaz *et al.*, 2006; Yang *et al.*, 2006). This method aims to detect significant overlap among the top-scoring genes in two ranked (ordered) gene lists by making a comparison of comparisons. For details see Data S2.

RuBisCO protein analysis and chlorophyll quantification

For RuBisCO protein measurements, proteins were extracted from 24 h-imbibed seeds and 40 μg of total extracted protein was separated on 13% polyacrylamide gels. The protein bands corresponding to the large (55 kDa) and the small (16 kDa) subunit of RuBisCO were cut from the gel with a razorblade. The Coomassie dye of each fragment was extracted in 50% isopropanol, 3% SDS overnight in a water bath (37°C). Subsequently, the absorbance was measured at λ595 nm on a Synergy4 multi-mode microplate reader (Biotek Instruments, biotek.com) and corrected against absorbance of the background. For details see Data S2.

Immunodetection of H3 acetylation and chromatin immunoprecipitation

For immunodetection of H3 acetylation levels, histones were isolated from dry seeds. Seeds were harvested in liquid nitrogen and ground in a buffer containing 0.4 M sucrose, 10 mM Tris pH 8.0, 10 mM $MgCl_2$, 0.1 mM PMSF, 5 mM β-mercaptoethanol. After filtering through a mesh and centrifugation, the pellet was washed with the buffer as above but containing 0.25 M sucrose and 1% Triton X-100 and thereafter with a buffer containing 1.7 M sucrose and 0.15% Triton X-100. The pellet was resuspended in 0.4 M H_2SO_4 and after centrifugation protein was precipitated from the supernatant using 15% trichloroacetic acid. Thereafter, the pellet was washed with cold aceton, air dried and resuspended in 10 mM Tris–HCl, pH 8.0. For immunodetection of H3 acetylation equimolar levels of histones isolated from fresh wild-type and *hda9-1* seeds were loaded and separated electrophoretically on a 4–12.5% polyacrylamide gel, and transferred to Immobilon PVDF membranes (Amersham, gelifesciences.com) for Western blot. Histone modifications were detected using H3K9Ac-specific antibody (Upstate 07-352, Merck Millipore, merckmillipore.com) and anti-H3 antibody (Upstate 05-499) and visualized with X-film using a horseradish peroxidase–conjugated anti-rabbit or anti- mouse IgG secondary antibody and the Super ECL plus detection system (Applygen Technologies Inc, ccne.mofcom.gov.cn). Quantification of the western blotting signal was performed using Gel-Pro analyser software (Media-Cybernetics, mediacy.com) and calculated relative to Col wild-type.

Chromatin preparation and immunoprecipitation (ChIP) were performed as described before (Bowler *et al.*, 2004; Wang *et al.*, 2013) using H3K9Ac-specific antibody (Upstate 07-352). The ChIP assay was repeated with three biological replicates. qPCR to detect pulled-down chromatin was performed with SYBR Premix Ex Taq (TaKaRa, takara.co.kr) on a real-time system (Eppendorf, eppendorf.com). *ACTIN8* (At1g49240) (He *et al.*, 2003; Wang *et al.*, 2013) was used as internal control for H3K9Ac and accumulative abundance of PCR product was calculated and normalized to this gene. Additionally, the acetylation levels in the negative control locus *2OG* (At5g54000) and internal control *ACTIN8* between Col and *hda9-1* were calculated using the comparative $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), expressing IP as percentage input (Figure S8a,b). This control experiment indicated that *ACTIN8* acetylation levels are unaltered in *hda9-1* compared to wild-type. For details see Data S2, primers are listed in Table S1.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Relative expression levels of Class 1 RPD3 HDACs during imbibition in the dark.

Figure S2. RT-PCR analysis of *HDA9* expression in independent *35S:HDA9* transformants.

Figure S3. Germination behaviour of *35S:HDA9* lines transformed in Col and L*er.*

Figure S4. Relative expression levels in dry and imbibed Col wild-type seeds of the 11 photosynthesis genes that were differentially regulated in *hda9-1* dry seeds.

Figure S5. Relative expression levels of *GLDP1* and *PRK* in Col wild-type, *hda9-1* and *hda9-2* dry seeds.

Figure S6. Independent replicates (replicate 1 left columns and replicate 2 right columns) of the chromatin immunoprecipitation analysis of histone acetylation levels in Col and *hda9-1.*

Figure S7. Relative expression levels of *RBCS2B*, *RCA*, *GLDP1* and *PRK* in 10 day-old seedlings of Col wild-type, *hda9-1* and *hda9-2.*

Figure S8. Chromatin immunoprecipitation analysis of histone acetylation levels.

Table S1. Primers used in this study.

Data S1. Differentially regulated genes in *hda9-1* mutant dry seeds compared to wild-type.

Data S2. Methods.

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