Arabidopsis Histone Deacetylase HD2A and HD2B Regulate Seed Dormancy by Repressing DELAY OF GERMINATION 1

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Y.H. and C.L. conceived research plans; C.L., C.B., G.H. and R.K. supervised the experiments; Y.H., C.J.W., and P.H. performed the experiments; Y.H., C.L., E.G., G.H., and R.K. designed the experiments and analyzed the data; Y.H. and C.L. wrote the article with contributions of all the authors; E.G., C.B., and J.D. reviewed and edited the text. C.L. agrees to serve as the author responsible for contact and ensures communication.

Running title

HD2A/HD2B regulate *DOG1* expression.

Abstract

Seed dormancy is a crucial developmental transition that affects the adaption and survival of plants. Arabidopsis DELAY OF GERMINATION 1 (DOG1) is known as a master regulator of seed dormancy. However, the upstream regulation of DOG1 is largely unknown. Histone acetylation is an important regulatory layer, controlled by histone acetyltransferases and histone deacetylases. Histone acetylation strongly correlates with transcriptionally active chromatin, whereas heterochromatin is generally characterized by hypoacetylated histones. Here we describe that loss of function of two plant-specific histone deacetylase, HD2A and HD2B, resulted in enhanced seed dormancy in Arabidopsis. Interestingly, silencing of *HD2A* and *HD2B* caused hyperacetylation of the *DOG1* locus and promoted the expression of *DOG1* during seed maturation and imbibition. Knockout of *DOG1* could rescue the seed dormancy and the disturbed development phenotype of *hd2ahd2b*. Transcriptomic analysis of the *hd2ahd2b* line shows that many genes involved in seed development were impaired. Moreover, we demonstrated that HSI2 and HSL1 interact with HD2A and HD2B. In sum, these results suggest that HSI2 and HSL1 recruit HD2A and HD2B to *DOG1* to negatively regulating *DOG1* expression and to reducing seed dormancy, consequently, affecting seed development during seed maturation and promoting seed germination during imbibition.

Key words: *Arabidopsis thaliana*, delay of germination 1, histone acetylation, plant-specific histone deacetylases, seed dormancy, seed germination

Introduction

As the initial phase of a plant's life cycle, seed germination is essential for seedlings' establishment and growth. The proper timing of seed germination ensures plant development under suitable conditions and is determined by seed dormancy release. Seed dormancy is an evolutionary adaptive mechanism that can be simply defined as viable seeds that fail to germinate under favourable conditions (Finch-Savage & Leubner-Metzger, 2006). Dormancy is imposed by phytohormones and genetic factors, established during seed maturation, persists in mature seeds, and can be released by after-ripening and seed stratification (Gubler *et al.*, 2005). Abscisic acid (ABA) and gibberellin acid (GA) are recognized as essential endogenous phytohormones that play antagonistic roles in regulating seed dormancy. DELAY OF GERMINATION 1 (DOG1; At5g45830) was identified as a master regulator of primary dormancy in a QTL analysis for seed dormancy using a set of recombinant inbred lines derived from a cross between low dormant accession Landsberg erecta (Ler-0) and very dormant accession Cape Verde Islands (Cvi-0) (Alonso-Blanco *et al.*, 2003; Bentsink *et al.*, 2006). *DOG1* encodes a nuclear protein with unknown biochemical function and is mainly expressed in seed (Bentsink *et al.*, 2006; Nakabayashi *et al.*, 2012). The DOG1 protein accumulates during seed maturation and peaked in freshly harvested seeds. At this developmental stage the DOG1 level determines the seed dormancy level (Nakabayashi *et al.*, 2012). Although DOG1 protein still persists during dry storage and seed imbibition, the after ripened seeds lose the dormancy. This indicates a loss of DOG1 activity at the after-ripening stage, which might be caused by an altered protein structure (Nakabayashi *et al.*, 2012). Recent evidence suggests that multiple factors are involved in regulating *DOG1* expression. Nakabayashi et al. (2012) found that lower seed maturation temperature upregulated *DOG1* expression and increased seed dormancy. This might be triggered by increased expression of transcription factor (TF) bZIP67 which can bind to the *DOG1* promoter (Bryant *et al.*, 2019). Additionally, *DOG1* expression is regulated by epigenetic regulators. Histone demethylases LDL1/LDL2 and histone methyltransferases KRYPTONITE (KYP)/SUVH4 /SUVH5 repress *DOG1* during seed maturation (Zheng *et al.*, 2012; Zhao *et al.*, 2015). *DOG1* expression also can be regulated by alternative splicing, cis-acting antisense noncoding transcript (as*DOG1*), and histone acetylation (Nakabayashi *et al.*, 2015; Fedak *et al.*, 2016).

B3 domain-containing transcriptional repressors HIGH-LEVEL EXPRESSION OF SUGAR INDUCIBLE2 (HSI2) and HSI2-LIKE1 (HSL1) play also critical roles during plant reproduction and seed germination (Qüesta *et al.*, 2016; Schneider *et al.*, 2016; Yuan *et al.*,

2021). HSI2 and HSL1 can form dimers to bind on the *DOG1* promoter recruiting components of polycomb-group proteins for consequent deposition of H3K27me3 marks resulting in repression of *DOG1* (Li *et al.*, 2019). Additionally, HSI2 and HSL1 interact with histone deacetylase (HDA) 6 and HDA19 and participate in down-regulating seed maturation gene expression in Arabidopsis seedlings (Zhou *et al.*, 2013; Chhun *et al.*, 2016).

HDAs are enzymes that catalysis the deacetylation of histone and non-histone proteins (Zhao *et al.*, 2010). Histone deacetylation leads to chromatin compaction, which is usually transcriptionally inactive. Arabidopsis has 18 HDAs, which are grouped into 3 subfamilies type I RPD3-like HDAs, HD-tuins, and sirtuins. The RPD3-like HDAs have a conserved HDA domain that shares high homology with the yeast transcriptional regulator RPD3 (reduced potassium deficiency 3). HD-tuins (HD2-type HDAs) are plant-specific and containing 4 members, HD2A, HD2B, HD2C, and HD2D. These proteins are related to the FKBP family of *cis-trans* peptidyl-propyl isomerases (Aravind & Koonin, 1998; Dangl *et al.*, 2001). Although inhibition or loss of HD-tuin function resulted in accumulation of hyperacetylated histones (Bourque *et al.*, 2011; Ding *et al.*, 2012), it is more likely that HD-tuins interact with RPD3-like HDAs and recruit them to the DNA (Luo et al., 2012a; Luo et al., 2012b). Treatment of seeds with the RPD3-like HDAs inhibitor trichostatin A (TSA) results in to 90% inhibition of seed germination, concluding that histone deacetylation is required for processing seed germination (Tanaka et al., 2008). KO-mutant analysis revealed, that HD2A and HD2C play opposing functions in seed germination. While HD2A restrains germination, HD2C enhances germination (Colville et al., 2011). A combination of associated mapping and transcriptomics led to the identification of HD2B as a genetic factor associated with seed dormancy (Yano et al., 2013). However, little is known about the underlying precise mechanism of HD2B in seed germination.

In this study, we demonstrated that HD2A and HD2B were recruited by HSI2 and HSL1 and function redundantly in regulating seed dormancy by affecting the *DOG1* expression. Silencing of HD2A and HD2B leads to hyperacetylation of the *DOG1* locus, consequently, causing a strong accumulation of *DOG1* transcripts. *hd2ahd2b* seeds displayed abnormal phenotypes, but wild type phenotype could be restored by additional knock-out of *DOG1*. Transcriptome analysis revealed that the transcription of many seed storage-related genes is significantly changed in *hd2ahd2b*. Taken together, these data suggest that transcription repressors HSI2 and HSL1 recruited HD2A and HD2B to repress *DOG1* expression during seed maturation and

germination, contributing to seed normal development, and promoting seed germination in Arabidopsis.

Materials and Methods

Plant Materials and Growth Condition

The *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) or mutants in the Col-0 background were used in all experiments. The T-DNA insertion lines GABI_355H03 (*hd2a*), Sail_1247_A02 (*hd2b*), Salk_039784 (*hd2c*), GK_379G06 (*hd2d*), and SM_3_20886 (*dog1-4*) were described previously (Luo *et al.*, 2012a; Fedak *et al.*, 2016; Li *et al.*, 2017) and were verified by PCR on genomic DNA using gene-specific primers **(Supplementary Table S2)**. The double mutant *hd2ahd2b*, *hd2ahd2c*, *hd2ahd2d*, *hd2bhd2c*, *hd2bhd2d*, *hd2chd2d,* and the triple mutants *hd2ahd2bdog1-4* were produced by crossing. Homozygous lines were isolated by genotyping with gene-specific primers **(Supplementary Table S2)**. The HD2A and HD2B complementation lines were gifts from Ton Bisseling (Li *et al.*, 2017). Seeds were sown in moist soil mixed with sand in ratio 10:1 and cultivated in the growth chambers under long-day conditions (14 h light/10 h dark and 20°C/18°C, respectively) or short-day conditions (10 h light/14 h dark and 20°C/16°C, respectively). The Arabidopsis plants used for seed production were grown first under short-day conditions for 4 weeks before transfer to long-day conditions for flowering. The seeds were harvested and storage in the dark under room temperature.

HDA activity measurement of total protein extracts

Measurements of HDA activity of *Arabidopsis* tissue protein extracts were performed by a fluorescence-based method adapted from Wegener et al. (Wegener *et al.*, 2003a; Wegener *et al.*, 2003b) and (Nott *et al.*, 2008). 150 mg of grinded deep-frozen plant material per replicate was transferred to a pre-cooled Lysing Matrix D tube (MP Biomedicals, Santa Ana, California, USA) and homogenized for 1 min at full speed in a FastPrep®-24 homogeniser (MP Biomedicals). After placing the tubes on ice, 300 µl homogenization buffer (50 mM Tris-HCl pH 7.0, 1 M D-Glucose and 1x protease inhibitor cocktail) was added and the samples were again homogenized for 30 sec. The supernatant was transferred to a 1.5 ml microcentrifuge tubes and centrifuged for 10 min at 25,000*g* and 4°C to remove cell debris. Protein concentration of the supernatant was determined according to Bradford (Bradford, 1976) and adjusted to a concentration of 1.2 µg/µl with homogenization buffer. HDA activity of the supernatant was assayed in 30 µl fractions per replicate in a flat-bottom 96-well black microtiter

plate. 100 µM BOC-(acetyl) Lys-AMC (Bachem, Bubendorf, Switzerland) in 25 µl HDA reaction buffer (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl and 1 mM $MgCl_2$) was added. After incubation for 2 h at 37°C, 10 mg/ml trypsin and 1 µ M TSA in 60 µl HDA stopping buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl) was added per well and fluorescence output, representing HDA activity, was measured after 20 min incubation at 30 °C at λ $380nm_{Excitation}$ and $440nm_{Emission}$.

Co-IP Analysis

For Co-IP analysis, the *35Spro:HSI2-myc* and *35Spro:HSL1-myc* constructs were transiently expressed in *35Spro:HD2A-GFP* and *35Spro:HD2B-GFP* protoplast. After brief centrifugation (100*g*, RT, 3 min), the supernatant was removed and total protein was extracted by re-suspending and disrupting the protoplast with 1 ml of extraction buffer (50 mM Tris-HCl, pH 8, 1 mM PMSF, 5% glycerol, 150 mM NaCl, 0.1% Nonidet P-40, 5 mM $MgCl_2$, 1 mM DTT, and 1x protease inhibitor cocktail). After gentle shaking for 1 h at 4°C, the sample was centrifugated at 12,000*g* for 10 min. To purify GFP-tagged proteins, the supernatant was incubated with 25 µl GFP-Trap agarose beads (Nano tag; catalog no. N0510) at 4°C overnight by gentle rotation. After washing with extraction buffer for four times, proteins were eluted with 50 µl 2x SDS sample buffer and analyzed by immunoblotting using anti-Myc antibody.

ChIP-qPCR assays

The ChIP-qPCR assay was performed as previously described (Bowler *et al.*, 2004). The chromatin was extracted from 24 h imbibed WT and *hd2ahd2b* seeds and from 10 d old 35Spro:HD2B-GFP seedlings. The seeds were imbibed at room temperature under dark, and at that time point, no germination visible. About 1 g of imbibed seeds and 2 g of seedlings were cross-linked in cross-linking buffer (400 mM sucrose, 10 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol, 1 % formaldehyde) by vacuum infiltration for 1 h and 10 min, respectively. Cross-linking was stopped by adding glycine to an end concentration of 0.125 M and additional vacuum infiltration for 5 min. The cross-linked plant materials were washed two times with ice water, dried with paper towels, and ground in liquid nitrogen to fine powder. The chromatin was extracted with 20 ml extraction buffer (400 mM sucrose, 10 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol, 1x protease inhibitor cocktail) by gentle shaking for 20 min and pelleted by centrifugation at 4000g for 25 min at 4°C. The pellet was washed with nuclei washing buffer (20 mM Tris/HCl, pH 7.4, 25 % glycerol, 2.5 mM $MgCl_2$, 0.2 % Triton x-100), re-suspended

in 600 µl nuclei sonication buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1 % SDS, 1x protease inhibitor cocktail) and the chromatin was sheared to 200–1,000 bp by sonication. the nuclei were transferred into 1.5 ml Bioruptor Microtubes (Cat No. C30010016), and 15 cycles with 30 sec ON/OFF was used with Bioruptor® Pico ultrasonic bath and Covaris E220 Evolution. After centrifugation for 10 min at 12,000g and 4°C the supernatant was directly used for immunoprecipitation with specific antibodies. For H3K9ac, H4K5ac, and H4ac analysis, the antibodies (anti-H4ac, anti-H4K5ac, anti-H3K9ac) were coupled to the magnetic protein G beads by incubating at 4°C on a rotation platform overnight. Afterward, 100 µl sonicated chromatin was mixed with antibody-magnetic protein G beads and incubated overnight at 4°C on a rotating platform. The beads were sequential washed with low salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), high salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1 % NP-40, 1 % sodium deoxychlorate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0), and TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). And the beads were washed twice with each buffer. Finally, the chromatin was eluted with 400 µl of elution buffer (1 % SDS, 100 mM $NaHCO_3$) by incubation for 20 min at 65 °C, and the chromatin de-crosslinking was performed at 65°C for over 6 h after adding 16 µl of 5 M NaCl. After treated with proteinase K and RNase, DNA was purified by phenol-chloroform method, eluted with dH_2O, and quantified for qPCR. For anti-GFP analysis, the GFP-Trap agarose beads were used instead of antibody-coupled magnetic protein G beads, and analysis was performed with the same procedure as above.

Bimolecular fluorescence complementation assay

For bimolecular fluorescence complementation assays, the ORF of *HD2A*, *HD2B*, *HSI2*, and *HSL1* (without stop codon) were transferred into the pDONOR221 vector by Gateway Cloning and subsequently shifted into the pBiFCt-2in1-NN vector (LR reactions) according to the described (Grefen & Blatt, 2012). Then, the constructs were transferred into Arabidopsis protoplasts by PEG-transformation as described above. After incubation for 16 h to 24 h in the dark, the YFP fluorescence signal was monitored using a laser scanning confocal microscope (Leica TCS SP8 confocal).

Determination of ABA and GA3

The endogenous ABA and GA3 contents were measured with Agilent 1290 Infinity II-6470 triple quadrupole LC/MS /MS System according to the previously reported method (Liu *et al.*, 2021) with minor modifications. Briefly, 0.1 g dry seeds and 0.3 g 24 h imbibed seeds were ground into a fine powder with liquid nitrogen and were transferred into a 2 ml microtube containing 1 ml ethyl acetate. The samples were vortexed and incubated for 30 min at 4°C on a shaker. Afterwards, the samples were centrifuged at 12,000*g* for 10 min at 4°C and the supernatant was transferred into a 1.5 ml tube and dried (speedvac). The residue was re-dissolved in 200 µl of 50% methanol and filtered through a 0.22 µm filter for sample loading. For each sample, 100 µl methanol solution was subjected to LC-MS/MS analysis. ABA (yuan ye biotech, catalog no. B50724) and GA3 (yuan ye biotech, catalog no. B20187) were used as authentic reference standards. All determinations were performed in triplicate.

Result

Silencing of HD2A and HD2B caused deeper seed dormancy

To investigate the precise functions of HD2s in Arabidopsis germination, we analyzed seed germination of all four HD2s T-DNA insertion lines of Col-0 background under long day condition, designated as *hd2a*, *hd2b*, *hd2c*, and *hd2d* **(Figure 1A)**. Reduced transcript accumulation in 24h-imbibed seeds was confirmed by qRT-PCR. The results showed that nearly no transcripts of *HD2A*, *HD2C,* and *HD2D* were detectable **(Figure 1B)**, whereas transcription of HD2B is reduced by approx. 80% **(Figure 1B)**. Moreover, double mutants were generated by crossing the HD2 T-DNA insertion lines to determine, if functional redundancy exists among the different HD2s **(Figure 1C)**. Freshly harvested seeds were used for seed dormancy analysis. After three days between 80% and 85% of WT, *hd2a*, *hd2c*, and *hd2d* seeds germinated, whereas, only 70% of *hd2b* seeds germinated **(Figure 1C)**. Among the six double mutant lines, *hd2ahd2b* double mutant showed a strongly enhanced seed dormancy phenotype **(Figure 1C)**. Of freshly harvested *hd2ahd2b* seeds only around 15% germinated. These results suggest that HD2A is at least partly functional redundant to HD2B in repressing seed germination. After 4 weeks dry storage at 4°C, seeds of WT and *hd2a* and *hd2b* single mutant lines germinated almost 100%, whereas *hd2ahd2b* seeds germinated only to 25% and even after extended storage of 16 weeks only to 65% **(Figure 1D)**. These results indicated that HD2A and HD2B play an important role in promoting seed germination.

HD2A and *HD2B* expression pattern during seed maturation and imbibition

To unveil the underlying function of HD2A and HD2B in seed dormancy release, their temporal expression pattern during seed maturation and imbibition was examined by RT-qPCR. *HD2A* has an analogous expression pattern as *HD2B*, rapidly increasing from 12 d after pollination (DAP) and reaching the highest expression level in 12 h imbibed seeds **(Figure 2)**. In stored seeds, the expression level of *HD2B* is significantly higher than that of *HD2A*. In general, imbibed seeds displayed significantly higher expression levels of both genes than maturating seeds **(Figure 2)**. These results imply a function of HD2A and HD2B in seed dormancy establishment as well as dormancy release.

HD2A and HD2B regulate seed dormancy via a ABA signal transduction pathway

To analyse, if HD2A and HD2B regulate seed germination via ABA and/or GA biosynthesis and signal transduction pathways, transcripts of genes involved in ABA and GA3 biosynthesis/catabolism/signaling have been quantified **(Figure 3A, B)**. The transcription of *ABA1* and *CYP707A2*, involved in ABA biosynthesis and catabolism, respectively, was increased in *hd2ahd2b* **(Figure 3A)**, whereas the expression of *NCED3*, *SnRK2.3*, and *ABI2*, which are related to ABA synthesis and ABA signal transduction, was not significantly different between *hd2ahd2b* and WT **(Figure 3A)**. In contrast, expression of *ABI5*, another ABA signal transduction-related gene, was enhanced in *hd2ahd2b* **(Figure 3A)**.

Regarding genes involved in GA biosynthesis, the expression of *GA3OX1* but not that of *GA3OX2* was upregulated in the *hd2ahd2b* seeds in comparison of WT. Moreover, the expression of *GA2OX2*, a gene required for GA catabolism, was not changed **(Figure 3B)**. Furthermore, the analysis of the GA repressed gene *WRI1* and the gibberellin receptors encoding genes *GID1b* and *GID1c* revealed that *GID1b* was downregulated in *hd2ahd2b*, whereas the expression of *WRI1* and *GID1c* was not significant different in WT and *hd2ahd2b* seeds (**Figure 3B**).

Since the expression of at least a few genes related to ABA and GA3 biosynthesis/catabolism/signalling is affected in *hd2ahd2b*, we determined the content of ABA and GA3 in WT and *hd2ahd2b* seeds. ABA and GA3 content is lower in 24 h imbibed seeds in comparison to dry seeds of WT and *hd2ahd2b* plants **(Figure 3C, D)**. Surprisingly, the amount of ABA and GA3 was not significant different neither in dry nor in imbibed seeds of *hd2ahd2b* and WT **(Figure 3C, D)**.

In conclusion, although ABA and GA3 content is not significantly affected in *hd2ahd2b* dry and imbibed seeds, the upregulation of ABI5 indicated that HD2A and HD2B function somehow in the ABA signalling pathway to induce seed dormancy.

Since mutants with a seed dormancy phenotype are usually hypersensitive to ABA (Zhao *et al.*, 2015; Née *et al.*, 2017), we analysed the germination of fully after-ripened *hd2ahd2b* and WT seeds in presence of different concentrations of ABA. *hd2ahd2b* displayed significantly reduced seed germination with increasing concentrations of ABA, whereas no effect was observed in seed germination of WT and the single mutant lines, demonstrating that only the double mutant *hd2ahd2b* is hypersensitive to ABA **(Figure 4A)**. A time course experiment demonstrated that *hd2ahd2b* seeds are 100% viable, but showed delayed germination already in absence of ABA (**Supplementary Figure 1**). Moreover, we tested germination of *hd2ahd2b* in presence of 100 µM of GA3 and after stratification at 4°C for 3 days. Both treatments slightly promoted germination of freshly harvested and completely ripened *hd2ahd2b* seeds **(Figure 4B)**.

HD2A and HD2B promote seed germination via repressing *DOG1*

Besides ABA, the protein DELAY OF GERMINATION 1 (DOG1) is an essential regulator of seed dormancy (Nakabayashi et al., 2012; Bentsink et al., 2006; Zhao et al., 2012). The gradually elevated expression of *HD2A* and *HD2B* during seed maturation and the fact that *hd2ahd2b* double mutant line displays a "hypersensitive to ABA" germination phenotype, led us assume that HD2A and HD2B affect expression of *DOG1*. Therefore, we analyse the relative expression level of *DOG1* in imbibed seeds of WT, the single mutant lines *hd2a* and *hd2b*, the double mutant line *hd2ahd2b,* the double mutant line either complemented with *HD2A-GFP* (*pHD2A:HD2A-GFP)* or *HD2B-GFP* (*pHD2B:HD2B-GFP)* and the two *HD2B* overexpression lines *HD2B-OE9*, *HD2B-OE14*. *DOG1* transcription level in the *pHD2B:HD2B-GFP* line was comparable to that of WT seeds **(Figure 5A)**. In contrast, the *DOG1* expression level was significantly decreased in the *HD2B* overexpression lines and elevated in the line with a lower *HD2B* transcription level **(Figure 5A)**. Notably, in seeds of the *hd2ahd2b* double mutant, the *DOG1* expression level was increased more than 20 times in comparison to WT, while in *hd2a* and *hd2b* expression of *DOG1* was only two- and six-fold increased, respectively **(Figure 5A)**. Surprisingly, the expression level of *DOG1* in the *pHD2A:HD2A-GFP* complementation line is significantly higher than in WT **(Figure 5A)**. In conclusion, higher *HD2B* expression correlates with lower *DOG1* expression indicating that HD2B represses *DOG1* expression

during seed germination. Moreover, both, HD2A and HD2B functions are essential for regulating the expression of *DOG1* during seed germination.

We further analysed the dynamics of *DOG1* expression in *hd2ahd2b* during seed maturation and imbibition. We observed that the *DOG1* mRNA accumulation decreased from 12 DAP until seed maturation, which is consistent with the reported data in Col-0 background (Zhao *et al.*, 2015). Then, *DOG1* expression increased rapidly in dry seeds and quickly vanished after seed imbibition **(Figure 5B)**. Different from the expression pattern in WT, the *DOG1* transcript level in the *hd2ahd2b* double mutant increased during seed maturation, peaked in dry seed, and decreased during imbibition. Interestingly, after an initial significant decrease at beginning of imbibition, *DOG1* expression in *hd2ahd2b* seeds did not vanish as observed in WT seeds but remained at a relatively stable level over at least 24 h **(Figure 5B)**. In general, the *DOG1* expression level in *hd2ahd2b* is significantly higher during seed development and imbibition in comparison to WT **(Figure 5B)**, concluding that freshly harvested seeds of *hd2ahd2b* might accumulate more DOG1 than WT seeds. To further get evidence for a coordinated function of HD2A/HD2B and DOG1 in seed germination, we analysed seed germination of Arabidopsis lines with different *HD2A/HD2B* and *DOG1* expression levels. Compared to all other lines analysed, the seeds of the *hd2ahd2b* double mutant with the highest *DOG1* mRNA level showed significantly lower (delayed) germination. Interestingly, this reduced germination phenotype is restored in the complementation line *pHD2B:HD2B-GFP* and partially in *pHD2A:HD2A-GFP*. Although the *dog1* mutant and the *HD2B* overexpression lines *HD2B-OX9* and *HD2B-OX14* showed reduced expression of *DOG1* and a similar percentage of germination as WT seeds after 2 days of incubation, the percentage of germination of WT is slightly delayed in comparison to that of *dog1* and both *HD2B* overexpression lines **(Figure 5C)**.

To demonstrate the functional relationship between HD2A, HD2B, and DOG1, we crossed *hd2ahd2b* and *dog1-3* and *dog1-4* to generate *hd2ahd2bdog1-3* and *hd2ahd2bdog1-4* homozygous plants. Almost all triple mutants' seeds germinated after incubation for 48 h, indicating a none dormancy phenotype similar to *dog1* mutants **(Figure 5C)**. In conclusion, DOG1 function is responsible for *hd2ahd2b* mediated seed dormancy. All these results suggested that HD2A and HD2B promote seed germination by inhibiting *DOG1* expression.

HD2A/HD2B deacetylate *DOG1*

HD2A and HD2B are annotated as HDAs, however, their exact biochemical functions in Arabidopsis are unknown. To confirm that HD2A and HD2B are required for histone

deacetylation, the relative HDA activity in 10 days old seedlings of *hd2ahd2b* and WT plant was measured. *hd2ahd2b* displayed a 45% reduction in total HDA activity compared to WT **(Figure 6A)**, suggesting that HD2A and HD2B are required for HDA activity in Arabidopsis. Furthermore, the global acetylation levels of H4, H4K5, and H3K9 in germinated seeds of WT and *hd2ahd2b* double mutant were analysed. In *hd2ahd2b* a 1.4-fold and 2.2-fold enhanced H4ac and H4K5ac level, respectively, was detected, but no significant difference was observed in the H3K9ac level **(Figure 6B, C)**. Subsequently, we analysed whether the acetylation level at the *DOG1* promoter and the *DOG1* coding region is altered in the *hd2ahd2b* line in comparison to WT. We performed chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) on 24 h imbibed seeds of WT and *hd2ahd2b* using specific anti-H3K9ac, anti-H4ac, and anti-H4K5ac antibodies. A 103 bp promoter region P1, 1064 bp upstream of the transcription start site (TSS), and a 189 bp coding region P2, 157 bp downstream of TSS, was amplified with specific primers **(Figure 7A)**. Loss of HD2A and HD2B function enhanced the acetylation level of H4ac and H4K5ac at the coding region P2 of *DOG1*, but not at the promoter region P1 **(Figure 7B)**. In contrast, H3K9ac level did not significantly change in *hd2ahd2b* in comparison to WT, neither in the *DOG1* promoter region P1 nor in the *DOG1* coding region P2 **(Figure 7B)**. To check, whether HD2B directly binds to the *DOG1* locus, we performed ChIP-qPCR on 24 h imbibed seeds of WT and *hd2ahd2b* complemented with *35Spro: HD2B-GFP* using an anti-GFP antibody. Arabidopsis intergenic region between AT5G43175 and AT5G43180 was selected as the negative control. Fragments corresponding to the *DOG1* coding region P2 were significantly enriched concluding that HD2B-GFP binds to *DOG1*. No enrichment was detected in the promoter region P1 and the negative control. In sum, these data indicate that the up-regulation of *DOG1* in *hd2ahd2b* is due to increased histone acetylation at the *DOG1* coding region P2 .

HD2A and HD2B form hetero-oligomers and are recruited by HSL1and HSI2 to regulate *DOG1* expression through deposition of histone acetylation

HD2A and HD2B need to be recruited to the DNA by different transcriptional regulators and form multi-protein complexes to modulate chromatin structure and consequently regulate gene expression in the different development stages. HSI2 and its homolog HSI2-like 1 (HSL1), also known as VAL1 and VAL2, respectively, repress *DOG1* expression by recruiting LIKE HETERCHROMATIN PROTEIN 1 (LHP1) and CURLY LEAF (CLF) for consequent deposition of H3K27me3 marks at *DOG1* locus (Chen *et al.*, 2020). The interaction between

HD2A and HD2B was shown by bimolecular fluorescence complementation (BIFC) **(Figure 8 A)**.

We assumed that HD2A and HD2B are also recruited to *DOG1* by HSL1 and HSI2. The interaction between HD2A and HD2B was demonstrated by bimolecular fluorescence complementation (BIFC) **(Figure 8 A)**. To analyse the interaction of HD2A/HD2B with HSL1/HSI2, HD2A and HD2B were fused to the N-terminus of YFP, and HSL1 and HSI2 were fused to the C-terminus of YFP. YFP signals were observed in the nucleus whenever HD2A or HD2B were co-expressed with HSL1 or HSI2 (**Figure 8B**). No signals were detected when HD2A and HD2B were co-transfected with the empty plasmid containing YC-YFP (negative control, **Figure 8B**). Co-IP confirmed the interactions observed in the BIFC assay, since both, HSI2 and HSL1, co-immunoprecipitated with HD2A-GFP and HD2B-GFP **(Figure 8C)**. These results indicate that both HD2A and HD2B interact with HSI2 and HSL1.

HD2A and HD2B regulate seed development via affecting expression of *DOG1*

Seed development comprises embryo morphogenesis and seed maturation (Baud *et al.*, 2008). The phenotype of Arabidopsis cotyledons was determined during embryo morphogenesis and the maturation process ensures the embryo accumulates enough storage reserves, which are important for seed dormancy and desiccation tolerance establishment (Baud *et al.*, 2008; Carrillo-Barral *et al.*, 2020). Embryo dormancy and coat-imposed dormancy are the two major types of seed dormancy mechanisms (Bewley, 1997).

It was reported that the DOG1 was involved in seed development by affecting multiple aspects of seed maturation via genetic interaction with ABI3 (Dekkers *et al.*, 2016). To untangle the underlying dormancy mechanisms caused by the up-regulation of *DOG1* in *hd2ahd2b* lines, the phenotype of *hd2ahd2b* seeds and seedlings was analysed. Arabidopsis WT and *hd2ahd2b* seeds were phenotyped using the *pheno*Seeder (Jahnke *et al.*, 2016), which consists of a pick-and-place robot and several sensors, enabling measurement of seed traits such as mass, volume, density, length, width, and size (i.e., projected area) for individual seeds. Although *hd2ahd2b* seeds have an irregular surface, there were no relevant differences between mean traits of the genotypes **(Figure 9A-G, Table 1)**, except that trait distributions of *hd2ahd2b* were wider and showed deviations from normal distributions for seed mass and volume **(Figure 9 E, F)** (Bourque *et al.*, 2011). We observed smooth testa, oval-shape and brown color for WT seeds, whereas *hd2ahd2b* seeds displayed abnormal seed phenotype, with wrinkled epidermis, irregular shape, and deeper testa color **(Figure 9G, H)**.

All those seed phenotypes are similar to the phenotype of mutants in seed maturation regulators and seed coat mutants, which could affect seed germination (Focks & Benning, 1998). Additionally, we found that *hd2ahd2b* displayed dysplastic cotyledons (tri-cotyledony, fused cotyledons, asymmetric cotyledons, and in the most extreme cases blurred border or junction between the petiole and the blade) **(Figure 9I)**, indicating abnormal embryo morphogenesis. These abnormal phenotypes were recovered in *hd2ahd2bdog1* triple mutants **(Figure 9I)**, further suggesting a genetic interaction between *DOG1* and *HD2A/HD2B*. These findings demonstrate that HD2A and HD2B is involved in seed and seedling development via regulating *DOG1* expression.

Transcriptome analysis of *hd2ahd2b* mutant seedlings

To get a general overview about the physiological processes, HD2A and HD2B are involved in, we performed an RNA-sequencing (RNA-seq) analysis of ten days old WT and *hd2ahd2b* seedlings.

Differentially expressed genes were defined based on a threshold of at least 2-fold change (P-value < 0.05). We found, that 1720 and 772 genes were up-and down-regulated in *hd2ahd2b*, respectively **(Figure 10A; Supplemental Table S1)**, demonstrating the repressive function of plant-specific histone deacetylases (Wu *et al.*, 2003; Zhou *et al.*, 2004; Li *et al.*, 2017; Chen *et al.*, 2018). Interestingly, approx. 45% of the up-regulated genes (~775), but only 15% of the down-regulated genes (~120) have a function related to "response to stimuli" **(Figure 10B)**. Moreover, significant more genes related to "localization" and "growth and development" are up-regulated in *hd2ahd2b* seedlings (**Figure 10B**). The Gene Ontology enrichment analyses of 2492 differentially expressed genes revealed that within the up-regulated genes, genes related to "response to different chemicals/stimuli", "seed dormancy" and "seed maturation" were highly enriched, whereas within the down-regulated genes, genes related to "sugar and sulfur metabolic processes" were enriched **(Figure 10C)**. Interestingly, DOG1-like 1 (At4g18660) and DOG1-like 3 (At4g18690) genes are up-regulated in *hd2ahd2b* seedlings, further confirming the repressive function of HD2A and HD2B on DOG1 gene family. Furthermore, transcription levels of genes responding to ABA and GA were also affected. These results demonstrate that HD2A and HD2B function is required for shutting down stimuli responses and genes involved in seed development and germination processes in ten days old seedlings.

Discussion

The plant-specific histone deacetylase subfamily HD2s plays multiple functions during plant development by acting as a transcription repressor (Wu *et al.*, 2000; Colville *et al.*, 2011). In this study, we showed that HD2A and HD2B were recruited by HSI2 and HSL1 to *DOG1* to promoting seed development and germination by repressing *DOG1*..

HD2A and HD2B regulate *DOG1* expression

A previous study showed that HD2A and HD2C have contrasting roles in seed germination through glucose signalling, where HD2A restrains germination and HD2C promotes germination (Colville *et al.*, 2011). In another study, HD2A function positively correlated with seed germination and negatively with dormancy-associated genes (Footitt *et al.*, 2015). Similar to the later report, our results provide evidence that HD2A positively affects seed germination since *HD2A* single ko-mutant has an elevated *DOG1* expression level **(Figure 5A)**. Surprisingly, we did not observe a significant difference in the seed germination phenotype between *hd2a* and WT **(Figure 1D)**. Probably two-fold upregulation of *DOG1* in *hd2a* is insufficient for a detectable delay of germination. In contrast, *HD2B* knock-down resulted in a seven-fold upregulation of *DOG1* and significantly enhanced dormancy **(Figure 1D, Figure 5A)**, which is consistent with previous reports (Yano *et al.*, 2013). Both HD2A and HD2B are essential and functional redundant for that process since the *hd2ahd2b* line has a significantly higher *DOG1* expression level (25-fold) and stronger dormancy phenotype in comparison to the corresponding single mutants **(Figure 1D, Figure 5A)**. Although both, HD2A and HD2B function, are important for controlling seed dormancy, HD2B function seems to be more dominant in this process in comparison to HD2A. This is supported by a stronger effect of *HD2B* knock-down on germination and *DOG1* expression than knock-out of *HD2A* **(Figure 1C, D, Figure 2)**.

DOG1 is a major genetic factor with a conserved function in controlling seed dormancy (Graeber *et al.*, 2014; Huo *et al.*, 2016). In mature and viable seeds, a higher *DOG1* transcript level is associated with stronger dormancy (Bentsink *et al.*, 2006; Nakabayashi *et al.*, 2012). Inter-accession variation of *DOG1* expression reflects the dormancy level of seeds of the different accessions. For example, Arabidopsis highly dormant accession Cvi has a higher *DOG1* expression level than the low-dormant accession Ler (Bentsink *et al.*, 2006). In the highly dormant accession Cvi, *DOG1* expression level is upregulated during seed development and peaked at 16 DAP, and decreased until seed maturation (Nakabayashi *et al.*, 2012). In

494 contrast, in low-dormant accession Col, *DOG1* expression level peaked at 9 DAP and
495 decreased until seed maturation (Zhao *et al.*, 2015), indicating a different regulation mechanism
496 of DOG1 in different dormant accessions. Our result confirmed the earlier decline of *DOG1*
497 expression in the low-dormant accession Col **(Figure 5B)**. The different *DOG1* expression
498 levels might be a consequence of different *HD2B* expression levels, since HD2B directly
499 represses *DOG1* by deacetylating the *DOG1* coding region **(Figure 7)**. At least, a natural
500 variation of the *HD2B* expression is described for different Arabidopsis accessions. Arabidopsis
501 highly dormant accession Cvi has a 25-fold lower *HD2B* expression level in comparison to the
502 low-dormant accession Col (Yano *et al.*, 2013).
503 We showed that HD2A and HD2B repress *DOG1* **(Figure 5A)**. The higher *HD2B* expression
504 during seed maturation contributes to the earlier decline of *DOG1* expression in low-dormant
505 accessions. The earlier decline of *DOG1* expression, in turn, leads to less DOG1 accumulation
506 in dry seeds and, subsequently, leads to a low-dormant phenotype, such as that of Col. In the
507 *hd2ahd2b* line, without the repressing function of HD2A and HD2B, *DOG1* expression is
508 continuously upregulated reaching the highest expression level in dry seeds **(Figure 5B)**.
509 Although *DOG1* expression dramatically decreased after imbibition in both, WT and *hd2ahd2b*,
510 *hd2ahd2b* seeds still display a significantly higher *DOG1* expression than WT **(Figure 5B)**.
511 Interestingly, the *DOG1* expression level was dramatically increased in fresh dry seeds both in
512 Col **(Figure 5C)** and Cvi accession (Bentsink *et al.*, 2006). The precise mechanism behind that
513 is still unknown. Probably unknown regulators with unique and independent functions from
514 HD2A and HD2B accumulated during that developmental stage or *DOG1* mRNA stability is
515 affected.
516
517 **HD2A and HD2B are interfering/interacting with ABA and GA3 signaling pathways**
518 High *DOG1* expression level results in deeper seed dormancy. It was reported that, DOG1
519 protein level positively correlates with the ABA level in freshly harvested dry and imbibed
520 seeds and negatively correlates with GA biosynthesis during imbibition (Nakabayashi *et al.*,
521 2012). In this context, enhanced expression of *DOG1* in *hd2ahd2b* **(Figure 5A, B)** would
522 indicate a higher ABA content and lower GA content. Surprisingly, we observed a comparable
523 amounts of ABA and GA3 in dry and imbibed seeds of WT and *hd2ahd2b* **(Figure 3C, D)**
524 concluding that higher *DOG1* expression level in *hd2ahd2b* does not affect ABA and GA3
525 levels. This is further supported by the unchanged expression of ABA and GA signal
526 transduction-related genes **(Figure 3A, B)**. Interestingly, key regulatory genes of ABA and GA

biosynthesis and ABA catabolism were upregulated in *hd2ahd2b* **(Figure 3A, B)**, suggesting that HD2A and HD2B are involved in regulating histone acetylation of these genes.

HD2A and HD2B were recruited by HSL1 and HSI2 to mediate deacetylation of H4K5 at *DOG1*

Reduced expression of *HD2A* and *HD2B* resulted in decrease of total HDA activity **(Figure 6A)**, which in turn, led to increased global acetylation of histone H4 and H4K5 **(Figure 6B, C)**. However, plant-specific HDAs itself do most like not possess HDA activity, but are rather required for the activity of RPD3-like HDAs. Previous studies (Luo *et al.*, 2012b; Chen *et al.*, 2018) provided evidence that HDAs are acting in multiple protein complexes. HD2A and HD2B seem to be two key subunits of such a HDA complex. Therefore, loss of HD2A and HD2B function indirectly reduced HDA activity via disturbing the HDA protein complex. *DOG1* expression negatively correlated with the expression of *HD2A* and *HD2B* **(Figure 5A)**. Moreover, the *hd2ahd2b* line has a significantly higher *DOG1* expression level and a stronger seed dormancy phenotype than the corresponding single mutants pointing to an overlapping function of HD2A and HD2B **(Figure 1C, D, Figure 5A, B and Supplemental Figure 1)**. Loss of *DOG1* function in *hd2ahd2b* genetic background rescued the seed dormancy phenotype **(Figure 5C)**, indicating that the upregulation of *DOG1* is the reason for the seed dormancy phenotype in *hd2ahd2b*. HD2B binds to the first exon of *DOG1* and deacetylates H4K5 and probably other acetylation mark of H4, too **(Figure 6B, C and Figure 7B, C)**. The distance of around 200 - 500 bp downstream of the transcription start (TSS) are typically regulatory regions, where HDAs act. E. g. in S-nitrosoglutathione-treated Arabidopsis seedlings hyperacetylation of H3K9/14 was observed predominantly around 400 bp downstream of TSS (Mengel et al., 2017). Moreover, in *hda6* Arabidopsis mutant hyperacetylation of DNA was peaked 200 – 300 bp downstream of TSS (Ageeva-Kieferle et al., 2021).

HD2A and HD2B are interacting with each other **(Figure 8A) and** both plant-specific HDAs may function on *DOG1* binding sites. HDAs can be recruited to different DNA binding sites at different developmental stages and in different cell types via different complex partners or DNA binding proteins, such as transcription factors (Liu *et al.*, 2014). Using a BIFC and Co-IP approach, we demonstrated that HD2A and HD2B interact with the transcriptional repressors HSI2 and HSL1 **(Figure 8B, C)**, suggesting that during seed development and imbibition, HD2A and HD2B are recruited by transcriptional repressors HSI2 and HSL1 to *DOG1*. This results in H4K5 deacetylation of *DOG1* and consequently in a decrease in the accessibility of

560 *DOG1* for the transcription machinery. It was reported that the selectivity of the HDA activity
561 largely depends on additional modifications of the substrate as well as corepressor binding
562 (Riester *et al.*, 2007; Liu *et al.*, 2014). In yeast, the methyltransferase activity of DOT1 can be
563 specifically activated by H4K16ac sites and is further enhanced by H2B ubiquitination
564 (Valencia-Sánchez *et al.*, 2021). Besides interacting with HD2A and HD2B, HSI2 and HSL1
565 also recruit CLF and LHP1 for consequent deposition of H3K27me3 marks at DOG1 to inhibit
566 *DOG1* expression (Chen *et al.*, 2020). In sum, repression of *DOG1* by HSI2 and HSL1 includes
567 a combinatorial regulation via histone acetylation and methylation.
568
569 **Regulatory function of HD2A and HD2B in seed development, seed dormancy,**
570 **germination, and seedling development**
571 Generally, a fully developed embryo, proper seed storage regents, and well seed coat
572 characteristics (impermeable to water and/or oxygen and low mechanical resistance) are
573 essential for seed dormancy and germination (Focks & Benning, 1998; Wang *et al.*, 2016;
574 Debeaujon *et al.*, 2018). DOG1 plays a central role in regulating seed germination and is also
575 involved in multiple aspects of seed maturation by interfering with ABA signaling components
576 ABI3 and ABI5 (Dekkers *et al.*, 2016).
577 We demonstrated that HD2A- and HD2B-mediated repression of *DOG1* is essential during seed
578 development, maturation, and storage **(Figure 5B)**, and loss of HD2A and HD2B function
579 caused multiple defects in seeds **(Figure 9A-H)**. Our transcriptomic data provided a general
580 insight into the functions of HD2A and HD2B. GO enrichment analysis demonstrated that these
581 genes are involved in seed maturation and seed dormancy process. It was reported, that DOG1
582 mediates a conserved coat dormancy mechanism that controls seed germination through the
583 regulation of GA metabolism (Graeber *et al.*, 2014). In this context, it is important to note that
584 the expression of genes involved in "GA biosynthetic processes" and "response to GA" is
585 disturbed in *hd2ahd2b* **(Figure 10C)**. Interestingly, HD2A and HD2B function is also required
586 to control the expression of genes responding to different types of stimuli **(Figure 10C)**. The
587 coordinated responses to external or environmental stimuli are important to coping with
588 environmental changes.
589 In sum, we showed, that the Arabidopsis plant-specific histone deacetylases HD2A and HD2B
590 have a redundant function and are involved in controlling seed development and germination
591 by coordinating *DOG1* expression. Based on our results, we propose a model for the regulatory
592 function of HD2A and HD2B in seed development/germination processes **(Figure 11)**.

Acetylation of H4K5 at the 5′-end of the coding region of *DOG1* enables its transcription and establishes seed dormancy. During seed maturation and imbibition, HSI2 and HSL1 recruit HD2A and HD2B to the 5′-end of the coding region of *DOG1*. Consequently, this region is deacetylated at H4K5 resulting in repression of *DOG1*.

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Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

Table 1. Summary of mean measured seed traits of WT and *hd2ahd2b*. SD = standard deviation. Effect sizes of differences in means between genotypes were estimated by Cohen's d value (d < 0.2 means no or very small effects).

		Mass (mg)	Volume (mm^3)	Density (mg/mm^3)	Length (mm)	Width (mm)	Size (mm^2)
WT	n	99	96	96	101	101	101
	Mean	0.0154	0.0149	1.044	0.499	0.326	0.126
	SD	0.0026	0.0024	0.034	0.036	0.024	0.014
hd2ahd2b	n	147	143	141	148	148	148
	Mean	0.0160	0.0152	1.062	0.524	0.319	0.128
	SD	0.0043	0.0041	0.062	0.055	0.032	0.019
	Cohen's d	0.012	0.004	0.080	0.117	0.044	0.014

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Figure legends

Figure 1. *hd2ahd2b* double KO line shows enhanced seed dormancy.

A) Gene structure of HD2A, HD2B, HD2C and HD2D and T-DNA insertion sites are shown. Exons, introns and T-DNA insertions are represented by black boxes, lines and triangles, respectively. **B)** RT-qPCR analysis of *HD2A*, *HD2B*, *HD2C and HD2D* expression levels in WT and *HD2s* single mutant lines of 24h-imbibed seeds. RT-qPCR signals were normalised to *UBQ5* expression levels. **C)** Germination percentage of fresh harvested wild-type and HD2s mutant seeds. The seeds were sown on water-saturated filter paper. After 3 days of incubation, the germination rates were analysed. **D)** Germination percentage of non-stratified wild-type, *hd2a*, *hd2b* and *hd2ahd2b* seeds after different periods of dry storage. The seeds were sown on water-saturated filter paper. After 3 days of incubation, the germination percentages were analysed. Data represent are averages ±SE of three independent experiments. Asterisks in (**B**) indicate a significant difference between the mutant and wild type (**$P < 0.01$). Lowercase letters indicate significant differences compared with the wild type in **(C)** ($P < 0.01$) and significant differences ($P < 0.01$) between different samples in **(D)**, One-Way ANOVA (Tukey-Kramer test) analysis was performed.

Figure 2. *HD2A* and *HD2B* expression pattern during maturation and imbibition of wild type seeds.

The expression of *HD2A* and *HD2B* at different seed developmental and imbibition stages was analysed by RT-qPCR. Expression of *UBQ5* was used for normalisation. The expression was analysed 12 days after pollination (DAP), 15 DAP, 18 DAP and in freshly harvested dry seeds (ds). Moreover, mature seeds were analysed 6 h, 12 h and 24 h of imbibition at 20°C under light. Three biological replicates were performed. The average (±SD) values are shown. Lowercase letters indicate significant differences ($P < 0.05$) between the different values. One-Way ANOVA (Tukey-Kramer test) analysis was performed.

Figure 3. Endogenous ABA and GA3 levels and expression of genes involved in ABA and GA metabolism and signal transduction-pathways in WT and *hd2ahd2b.*

ABA **(A)** and GA3 **(B)** content in dry seeds and seeds imbibed for 24h. The phytohormone content of the seeds was determined by LC-MS. Changes in transcript levels of genes involved in ABA **(C)** and GA **(D)** biosynthesis, catabolism and signal transduction were analysed in 24 h imbibed seeds analysed by RT-qPCR. Expression of *UBQ5* was used for normalisation. Error

bar represent the ±SD of 3 biological replicates. Asterisks in **(C)** and **(D)** indicate a significant difference between *hd2ahd2b* and WT based on One-Way ANOVA (Tukey-Kramer test) (**P < 0.01).

Figure 4. Germination analysis of different *hd2* lines.

A) Germination of WT, *hd2a*, *hd2b* and *hd2ahd2b* seeds in presence of different concentrations of ABA. Sixteen weeks after-ripened seeds were imbibed on ½ MS plate in the presence of 0, 0.1, 0.2 and 0.5 µM of ABA. The germination percentage was scored after 3 days. **B)** Germination of fresh and 16 weeks old *hd2ahd2b* seeds after stratification and GA3 treatments. For stratification, seeds were placed on water-saturated filter paper and stratified for 3 days at 4°C before transferring to the growth chamber. For GA3 treatment, non-stratified seeds were sown on filter paper saturated with 100 µM of GA3. The germination percentages were scored 3 days after incubation. Statistics: Error bar represent the ±SD of at least 3 biological replicates. Lowercase letters indicate significant differences (P < 0.05) between the different values. One-Way ANOVA (Tukey-Kramer test) analysis was performed.

Figure 5. *HD2B* negatively regulates *DOG1* expression and promotes seed germination.

A) RT-qPCR analyses of *HD2B* and *DOG1* expression in 24h imbibed seeds of WT, *hd2a*, *hd2b*, *hd2ahd2b, HD2B-OX and* complementation lines *pHD2A:HD2A-GFP* and *pHD2B:HD2B-GFP*. Seeds were stored at room temperature for 16 weeks before imbibition. **B)** *DOG1* expression pattern during seed maturation and imbibition in WT and *hd2ahd2b*. "ns" indicate freshly harvested seeds. Expression of *UBQ5* was used for normalisation. Error bar represent the ±SD of 3 biological replicates. Asterisks in **(A)** indicate a significant difference of the different lines compared with WT. Asterisks in **(B)** indicate significant differences between the different samples. One-Way ANOVA (Tukey-Kramer test) analysis was performed, (*P < 0.05, **P < 0.01). **C)** Germination percentage of the lines with different *DOG1* expression level. Fully after-ripened seeds were stratified for 3 days at 4 °C before incubation in the growth chamber. Error bar represent the ±SD of 3 biological replicates.

Figure 6. Loss of HD2A and HD2B function caused global hyperacetylation on H4 and H4K5 in Arabidopsis.

A) Relative HDA activity in protein extracts of WT and *hd2ahd2b* plants. Total soluble protein of 10 days old seedlings was extracted and the HDA activity was measured using a modified

fluorometric assay. **B)** Detection of histone acetylation levels in WT and *hd2ahd2b* mutant seeds by immunoblotting. Four independent replicates were performed with similar results. **C)** The intensities of the signals of the immunoblot were quantified using the ImageJ software. Four biological replicates (mean ±SD) were normalized to the signals of H4 (H4ac and H4K5ac) and H3 (H3K9ac). The WT signals were set to 1 for each analysed histone modification. Error bars represent the ±SD of 4 biological replicates. Asterisks indicate a significant difference between WT and *hd2ahd2b.* One-Way ANOVA (Tukey-Kramer test) analysis was performed, (**P < 0.01).

Figure 7. Enhanced H4 and H4K5 acetylation levels in *hd2ahd2b* seeds at *DOG1*.

A) Schematic illustration of the *DOG1* genomic region examined by ChIP-qPCR. Promoter, exons and introns are represented by dashed line, black boxes and continuous lines, respectively. The regions analysed in the ChIP-qPCR are indicated above the gene structure as P1 and P2. **B)** ChIP-qPCR analysis of H4, H4K5 and H3K9 acetylation of *DOG1*. Immunoprecipitated DNA was obtain from 24 h imbibed WT and *hd2ahd2b* seeds using the indicated specific antibodies against the analysed histone marks. Specific primers for P1 and P2 were used. **C)** ChIP-qPCR analysis of *35Spro:HD2B-GFP* complemention line with GFP antibody. Immunoprecipitated DNA was obtain from 10 days seedlings of WT and *35Spro:HD2B-GFP* with anti-GFP antibody. The relative amount of PCR products using P1 and P2 specific primers were quantified and normalized to an internal control (*s16*). The values shown are means ± SD. Error bars represent the SD of 3 biological replicates for each ChIP-qPCR experiment.

Figure 8. HD2A and HD2B interact with HSI2 and HSL1 in vivo.

A) Bimolecular fluorescence complementation (BiFC) showing protein– protein interactions between HD2A and HD2B. HD2A was fused to N-terminus of YFP (nYFP) and HD2B was fused to C-terminus of YFP (cYFP). Both constructs were co-transfected into Arabidopsis protoplast and visualized using confocal microscope after cultivating for 24 hours at 25℃. **B)** BiFC showing protein-protein interactions between HD2A, HD2B, HSL1 and HSI2 in Arabidopsis mesophyll protoplasts. HD2A and HD2B were fused to N-terminus of YFP (nYFP) and HSL1 and HSI2 were fused to C-terminus of YFP (cYFP). The constructs were co-transfected into Arabidopsis mesophyll protoplasts as indicated and visualized using confocal microscope after cultivating for 24 hours at 25℃. As negative control, empty plasmids containing cYFP and HD2A or HD2B fused with nYFP were co-transfected into Arabidopsis

mesophyll protoplasts. Bar, 20µm. **C)** Co-immunoprecipitation assays demonstrating interactions between HD2A, HD2B, HSL1 and HSI2 in vivo. Myc-tagged HSL1 and HSI2 were transfected into Arabidopsis mesophyll protoplast of HD2A-GFP and HD2B-GFP overexpression line. Total protein was extracted, HD2A-GFP and HD2B-GFP were immunoprecipitated with anti-GFP antibody and the immunoblot was detected with anti-GFP anti-Myc antibody.

Figure 9. Seed and seedling phenotypes of WT and *hd2ahd2b* mutants. Example pictures of single seeds at the nozzle of the *pheno*Seeder. **(A-B)** small and large seed of Col-0 WT, respectively, **(C-D)** small and large seed of *hd2ahd2b*, respectively. Scale bar 1 mm. Seed traits of Arabidopsis genotypes Col-0 and *hd2ahd2b*. Frequency histograms of volume **(E)** and seed mass **(F)** with corresponding normal distribution fits. **(G)** Microscope images of WT, *hd2ahd2b* and *hd2ahd2bdog1-4* mature dry seeds. **(H)** Color phenotype of WT and *hd2ahd2b* seeds. **(I)** Phenotype of WT, *hd2ahd2b* and *hd2ahd2bdog1-4* seedlings 14d after germination. Numbers below the pictures indicate the observed frequency of each phenotype. Red arrows indicate the cotyledons.

Figure 10. HD2A and HD2B function is required for downregulation of genes involved in stress response and seed development in 10 days old seedlings. A) RNA-seq analysis of 10 days old wt and *hd2ahd2b* seedlings. Volcano plots showing differentially expressed genes in *hd2ahd2b* seedlings in comparison to wt. Genes with an adjusted *P* value of < 0.05 and a $\log_2$ fold-change ≥2 or $\log_2$ fold-change ≤ -2 are highlighted in red and green. B) Multi-dimensional scaling analysis of significantly enriched GO terms (adjusted p-value < 0.05) among the significantly up-regulated or down-regulated genes (adjusted p-value < 0.05) changed for *hd2ahd2b* vs. wt. Only GO terms from the biological process ontology are shown in the plot. Each circle corresponds to an enriched GO term. Its size is proportional to the number of differentially regulated genes assigned to the GO term. The enriched GO terms are arranged in two dimensions such that their distance approximately reflects how distinct the corresponding sets of differential genes are from each other, i.e. neighboring circles share a large fraction of genes. Each enriched GO term is colored by its membership in the top level categories, which are grouped into five themes. If a GO term belongs to multiple top level terms, a pie chart within the circle indicates the relative fraction of each theme. The total distribution of themes across all enriched GO terms is depicted in bar plots below. C) Significantly up- (red)

and down-regulated (blue) enriched GO terms. Grey scale indicates the number of up- and down-regulated genes in the corresponding enriched GO term. GO terms related to seed dormancy and germination are highlighted.

Figure 11. Proposed model for the regulation of seed dormancy and germination mediated by HD2A and HD2B. H4ac and H4K5ac at *DOG1* results in open chromatin structure and enables *DOG1* transcription. The hetero-oligomer of HD2A and HD2B is recruited by HSI2 and HSL1 and directly binds to the coding region of *DOG1*, causing a decreased of H4ac and H4K5ac levels. Consequently, *DOG1* expression level is reduced during seed maturation and imbibition. The gradually increased expression of *HD2A* and *HD2B* guarantees normal seed development during seed maturation and release of the seed dormancy during seed imbibition. AC, acetyl groups.

Supporting Information

Supplemental Information: Methods

Supplemental Figure 1: Germination analysis of different *hd2* lines.

Supplemental Table S1: RNA-seq data set_control_ab vs wt

Supplemental Table S2: List of oligonucleotides

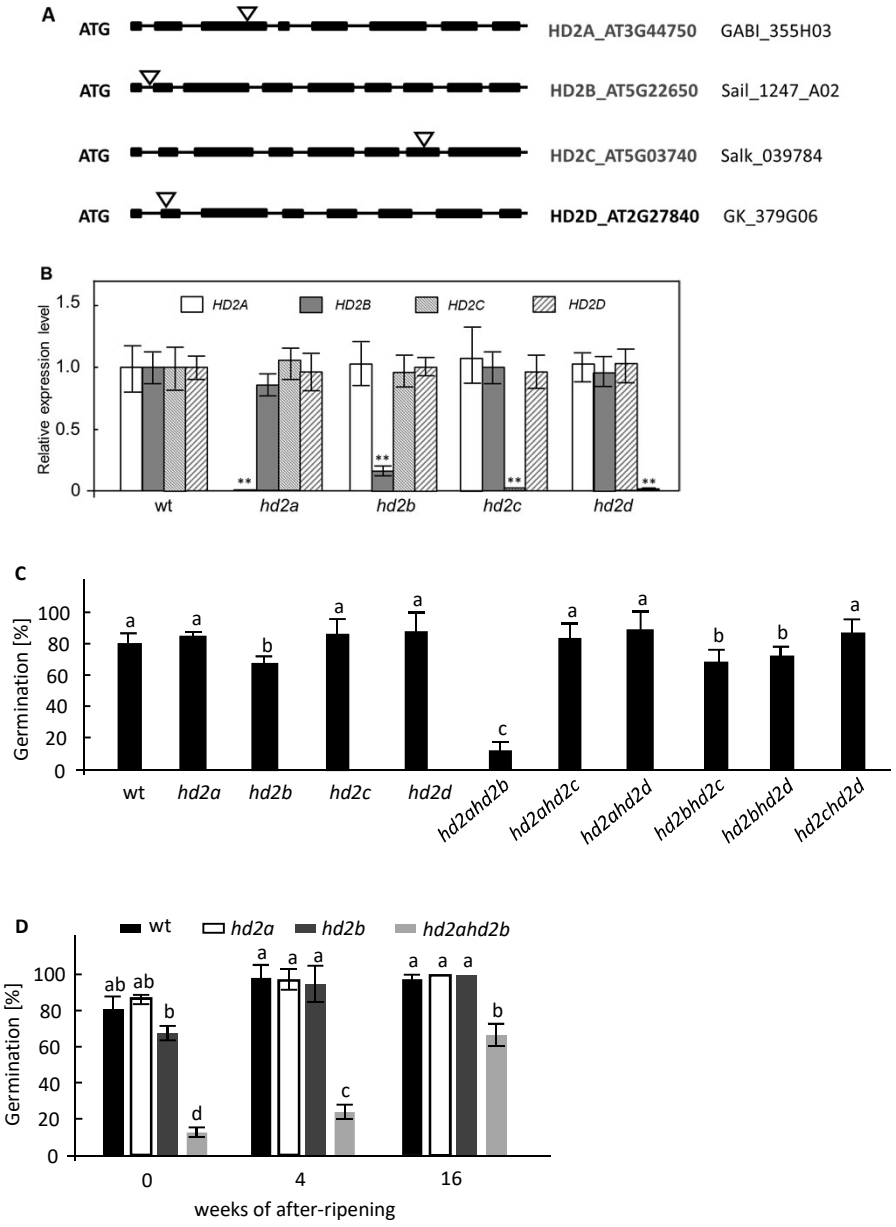

A
ATG
HD2A_AT3G44750
GABI_355H03
ATG
HD2B_AT5G22650
Sail_1247_A02
ATG
HD2C_AT5G03740
Salk_039784
ATG
HD2D_AT2G27840
GK_379G06
B
HD2A
HD2B
HD2C
HD2D
Relative expression level
1.5
1.0
0.5
0
wt
hd2a
hd2b
hd2c
hd2d
C
Germination [%]
100
80
60
40
20
0
wt
hd2a
hd2b
hd2c
hd2d
hd2ahd2b
hd2ahd2c
hd2ahd2d
hd2bhd2c
hd2bhd2d
hd2chd2d
D
wt
hd2a
hd2b
hd2ahd2b
Germination [%]
0
4
16
weeks of after-ripening

Figure 1

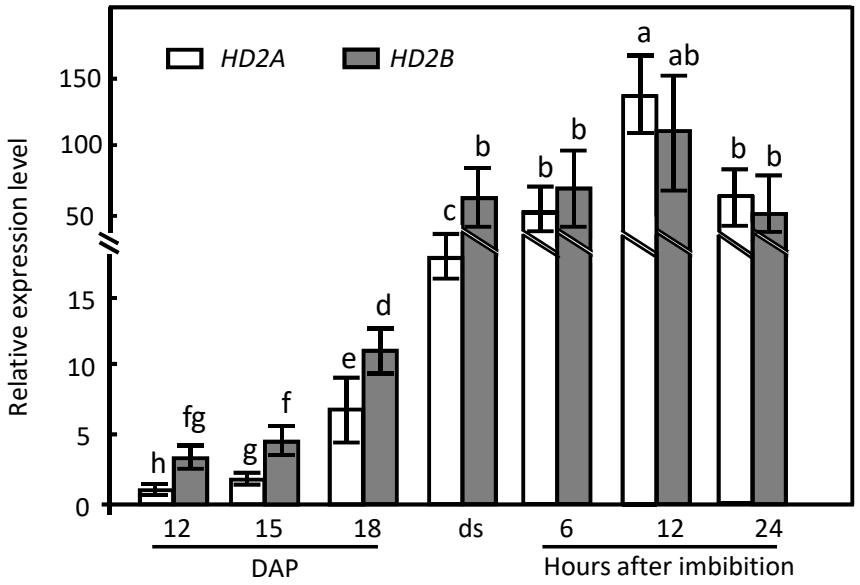

HD2A
HD2B
Relative expression level
150
100
50
15
10
5
0
h
fg
g
f
e
d
c
b
b
b
a
ab
b
b
12
15
18
ds
6
12
24
DAP
Hours after imbibition

Figure 2

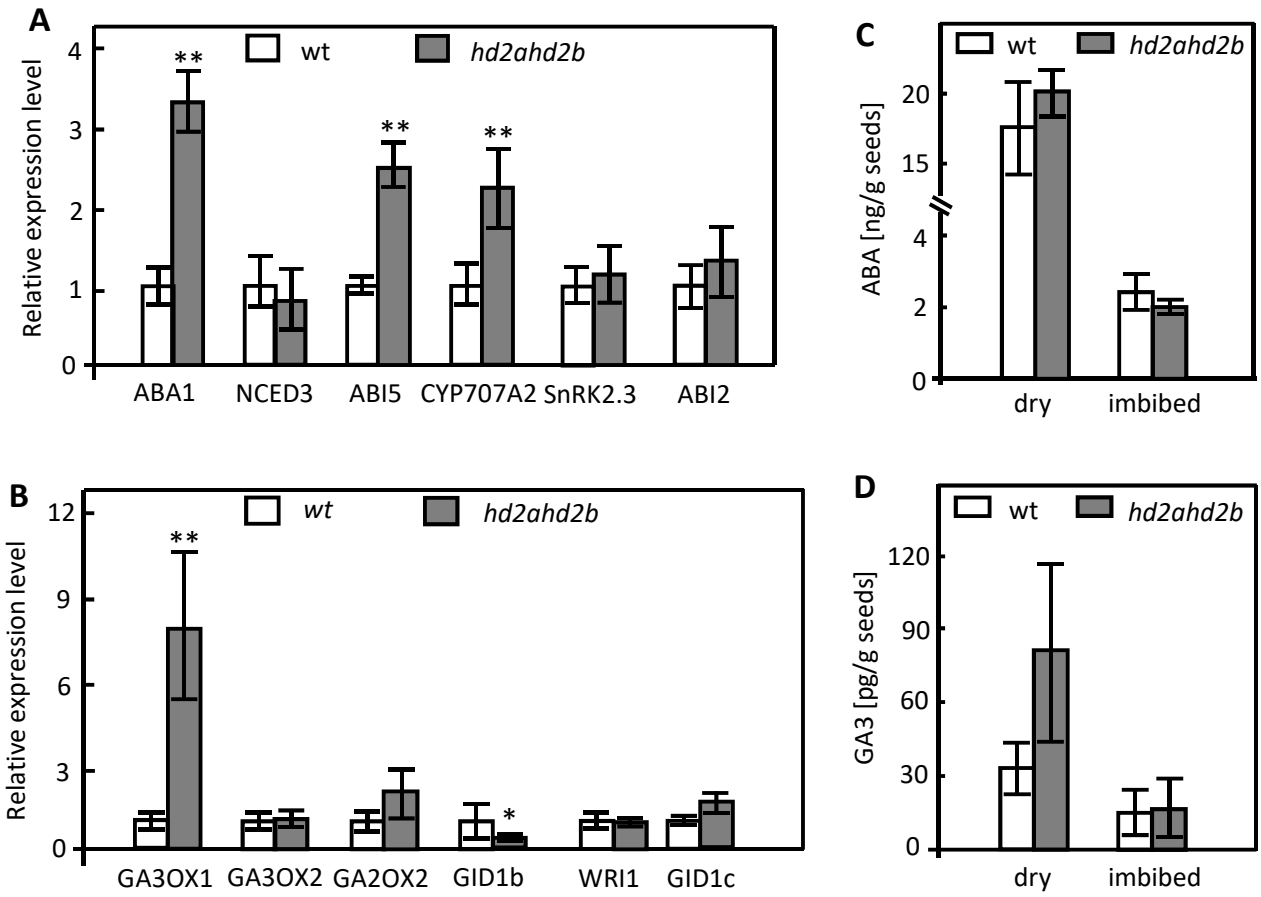

A
Relative expression level
wt
hd2ahd2b
ABA1
NCED3
ABI5
CYP707A2
SnRK2.3
ABI2
B
Relative expression level
wt
hd2ahd2b
GA3OX1
GA3OX2
GA2OX2
GID1b
WRI1
GID1c
C
ABA [ng/g seeds]
wt
hd2ahd2b
dry
imbibed
D
GA3 [pg/g seeds]
wt
hd2ahd2b
dry
imbibed

Figure 3

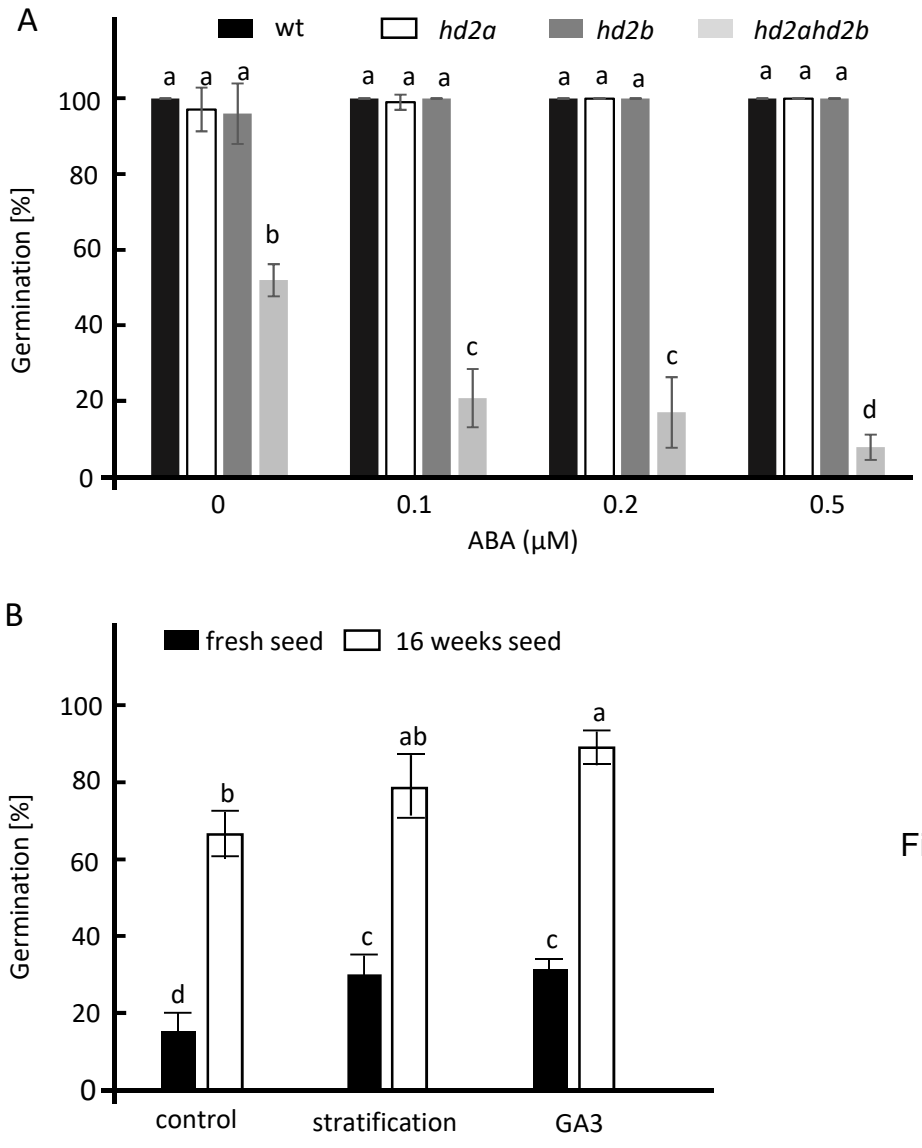

A
wt
hd2a
hd2b
hd2ahd2b
Germination [%]
ABA (µM)
B
fresh seed
16 weeks seed
Germination [%]
control
stratification
GA3

Figure 4

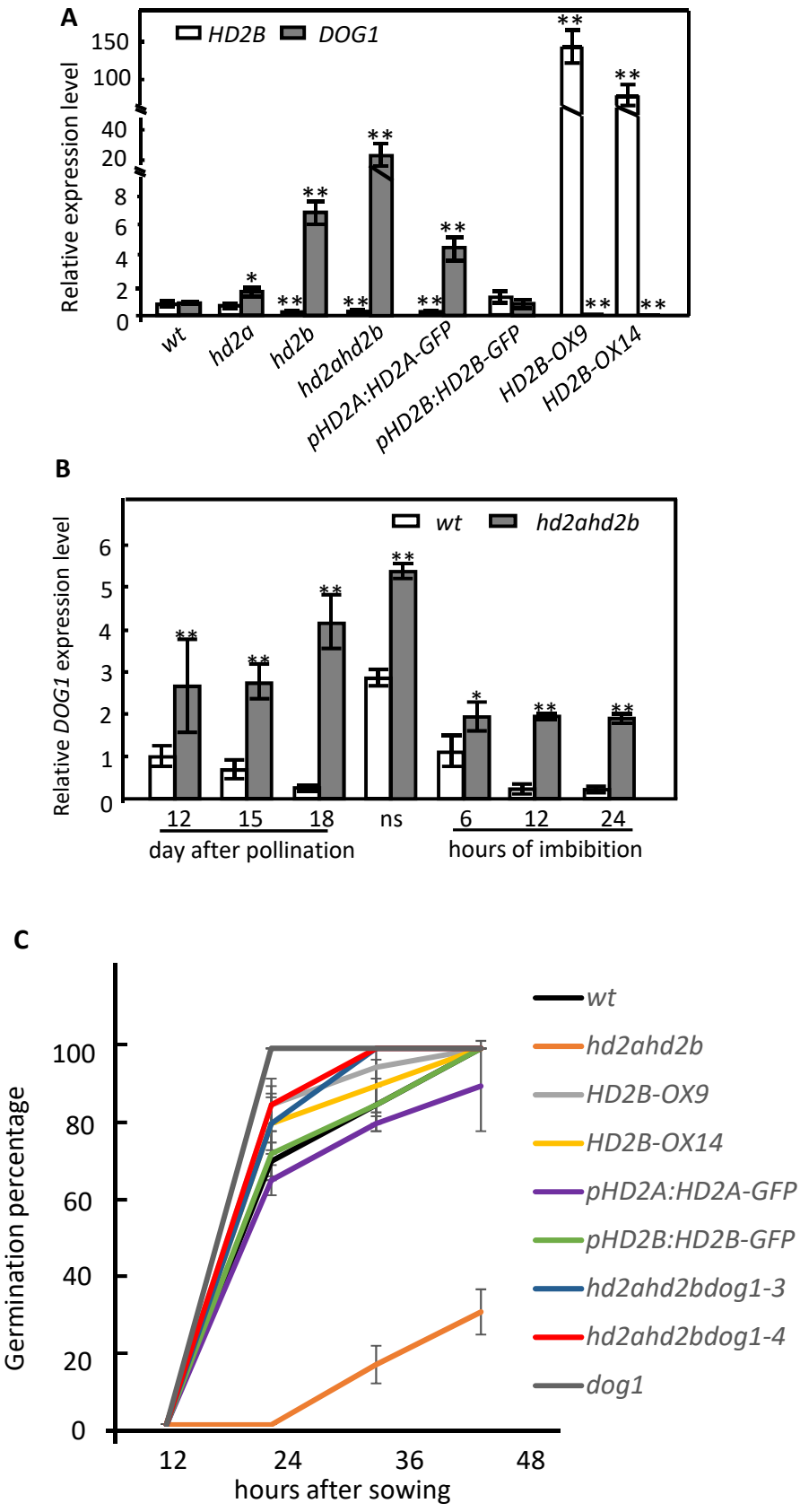

A
HD2B
DOG1
Relative expression level
150
100
40
20
8
6
4
2
0
wt
hd2a
hd2b
hd2ahd2b
pHD2A:HD2A-GFP
pHD2B:HD2B-GFP
HD2B-OX9
HD2B-OX14
B
wt
hd2ahd2b
Relative DOG1 expression level
6
5
4
3
2
1
0
12
15
18
ns
6
12
24
day after pollination
hours of imbibition
C
Germination percentage
100
80
60
40
20
0
12
24
36
48
hours after sowing
wt
hd2ahd2b
HD2B-OX9
HD2B-OX14
pHD2A:HD2A-GFP
pHD2B:HD2B-GFP
hd2ahd2bdog1-3
hd2ahd2bdog1-4
dog1

Figure 5

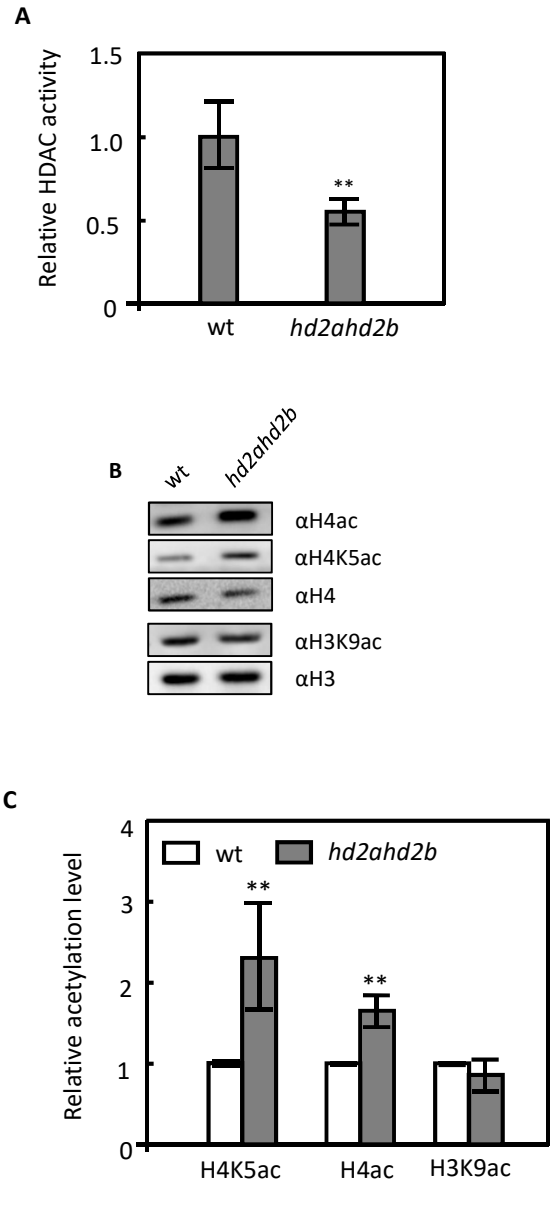

A
Relative HDAC activity
1.5
1.0
0.5
0
**
wt
hd2ahd2b
B
wt
hd2ahd2b
αH4ac
αH4K5ac
αH4
αH3K9ac
αH3
C
Relative acetylation level
4
3
2
1
0
wt
hd2ahd2b
**
**
H4K5ac
H4ac
H3K9ac

Figure 6

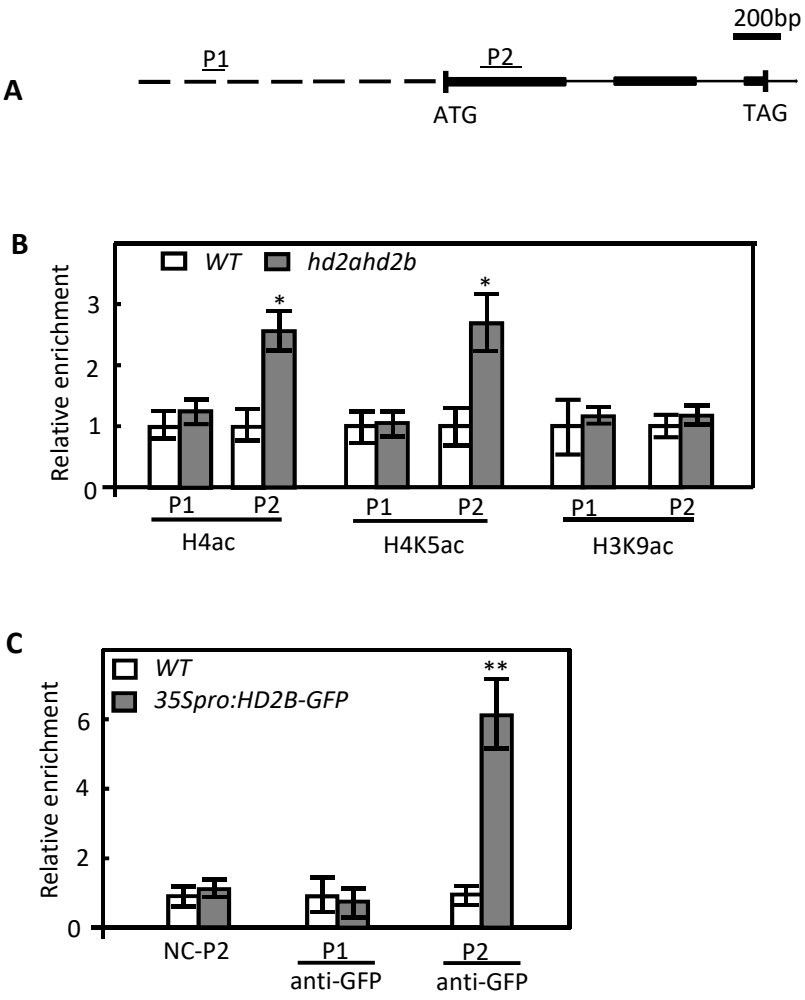

A
200bp
P1
P2
ATG
TAG
B
WT
hd2ahd2b
Relative enrichment
0
1
2
3
*
*
P1
P2
P1
P2
P1
P2
H4ac
H4K5ac
H3K9ac
C
WT
35Spro:HD2B-GFP
Relative enrichment
0
2
4
6
**
NC-P2
P1
anti-GFP
P2
anti-GFP

Figure 7

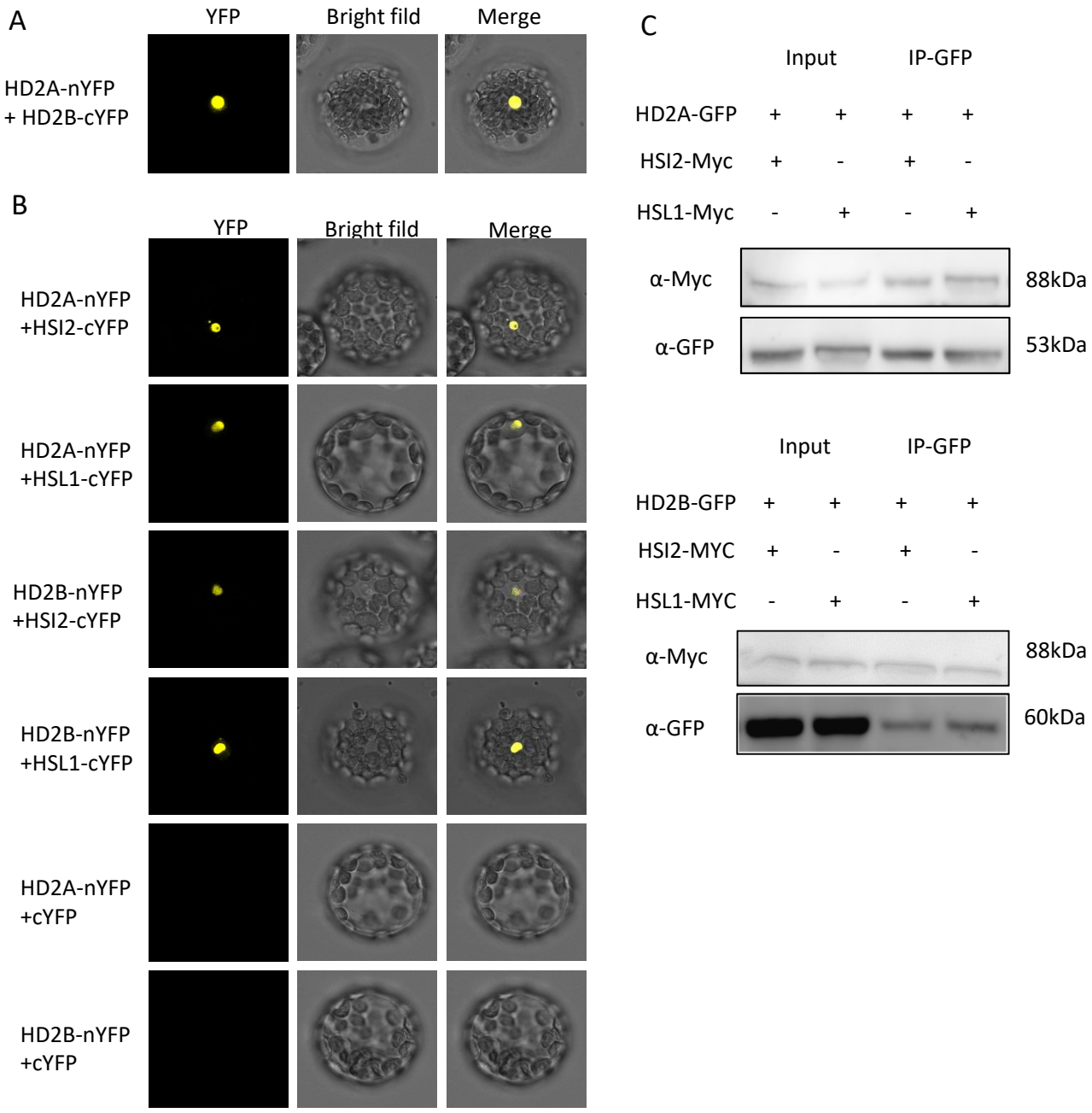

A
YFP
Bright fild
Merge
HD2A-nYFP
+ HD2B-cYFP
B
YFP
Bright fild
Merge
HD2A-nYFP
+HSI2-cYFP
HD2A-nYFP
+HSL1-cYFP
HD2B-nYFP
+HSI2-cYFP
HD2B-nYFP
+HSL1-cYFP
HD2A-nYFP
+cYFP
HD2B-nYFP
+cYFP
C
Input
IP-GFP
HD2A-GFP + + + +
HSI2-Myc + - + -
HSL1-Myc - + - +
α-Myc
88kDa
α-GFP
53kDa
Input
IP-GFP
HD2B-GFP + + + +
HSI2-MYC + - + -
HSL1-MYC - + - +
α-Myc
88kDa
α-GFP
60kDa

Figure 8

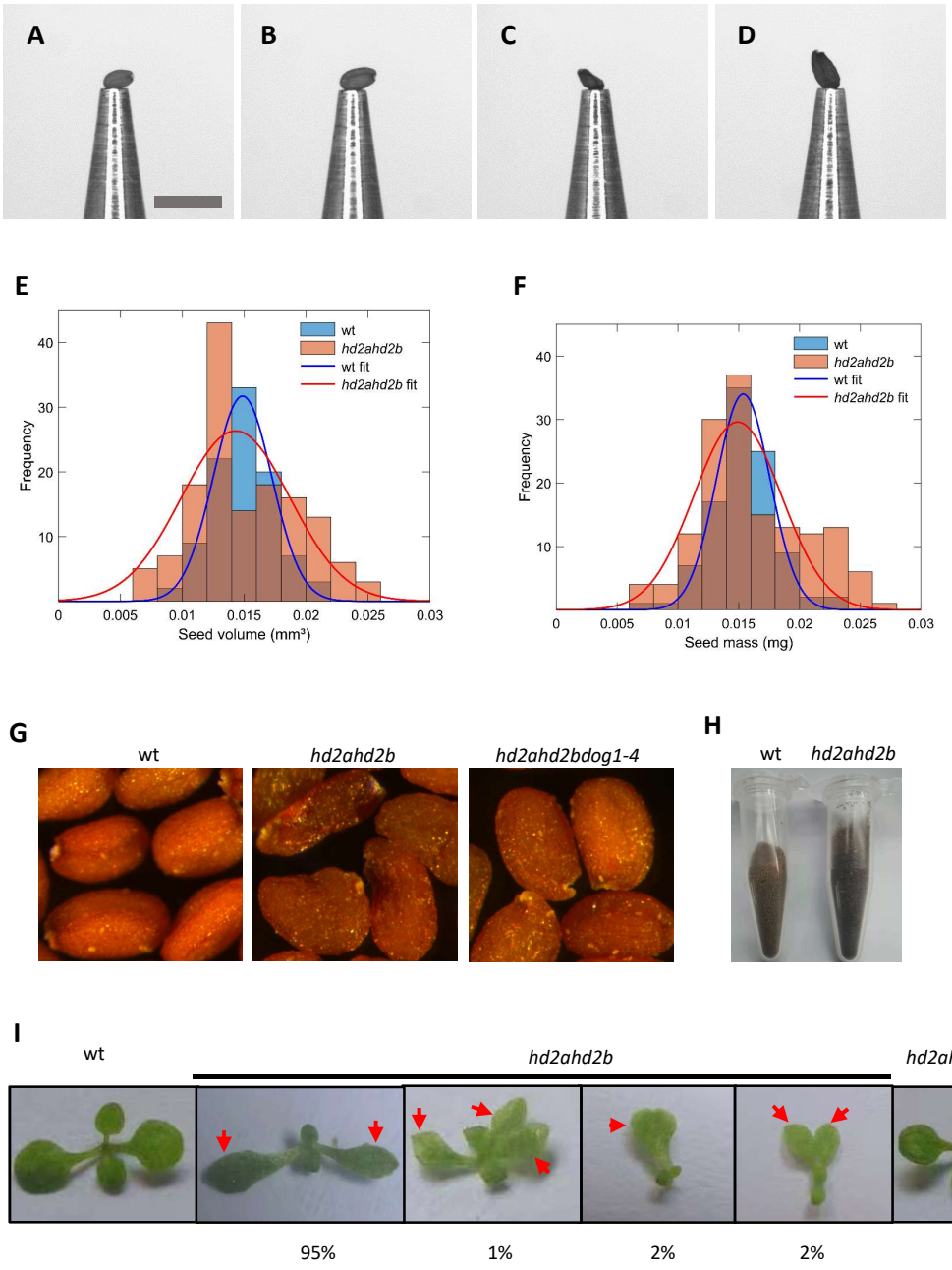

A
B
C
D
E
wt
hd2ahd2b
wt fit
hd2ahd2b fit
Frequency
Seed volume (mm³)
F
Seed mass (mg)
G
wt
hd2ahd2b
hd2ahd2bdog1-4
H
I
95%
1%
2%
2%

Figure 9

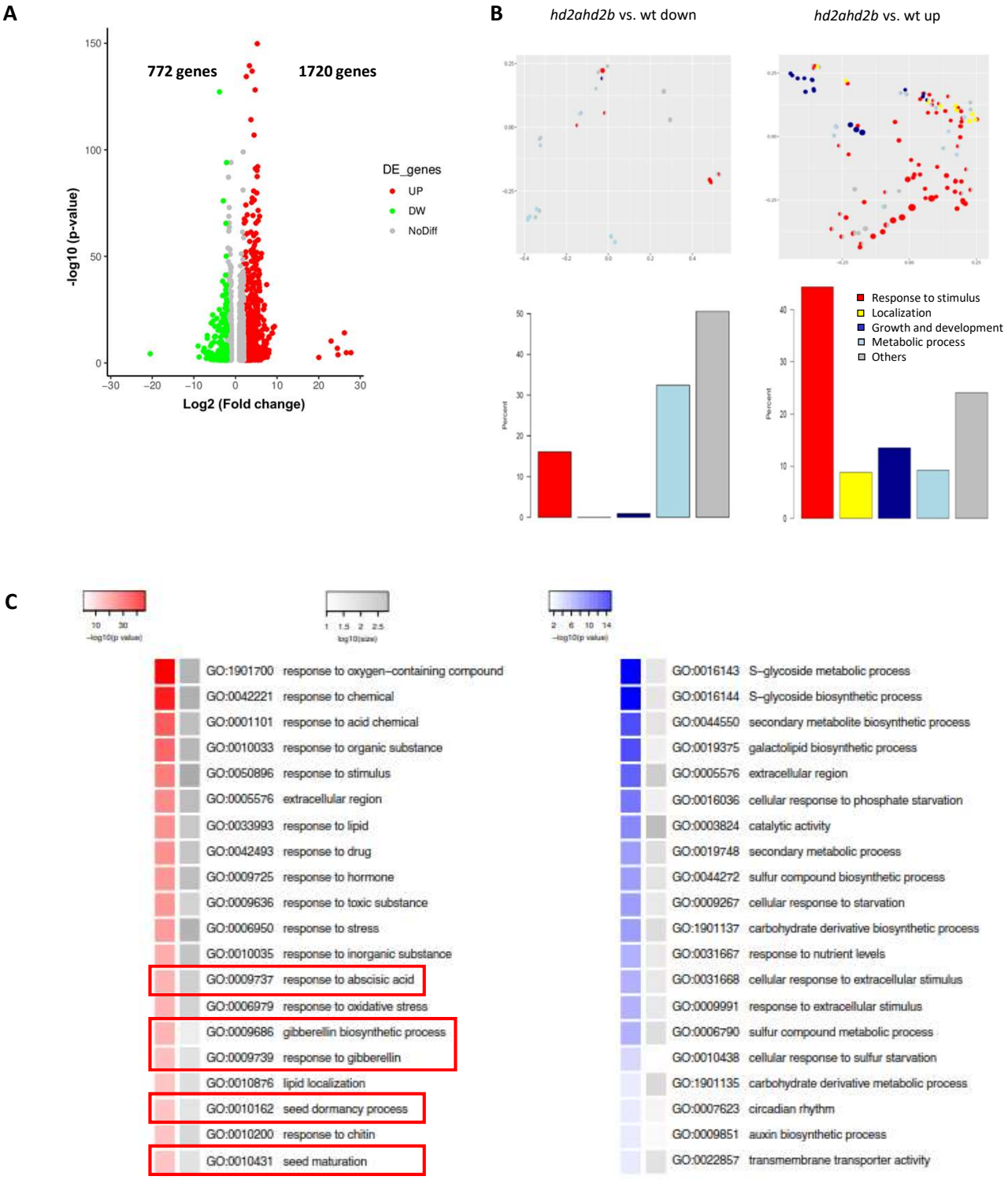

A
772 genes
1720 genes
DE_genes
UP
DW
NoDiff
-log10 (p-value)
Log2 (Fold change)
B
hd2ahd2b vs. wt down
hd2ahd2b vs. wt up
Response to stimulus
Localization
Growth and development
Metabolic process
Others
Percent
C
GO:1901700 response to oxygen−containing compound
GO:0042221 response to chemical
GO:0001101 response to acid chemical
GO:0010033 response to organic substance
GO:0050896 response to stimulus
GO:0005576 extracellular region
GO:0033993 response to lipid
GO:0042493 response to drug
GO:0009725 response to hormone
GO:0009636 response to toxic substance
GO:0006950 response to stress
GO:0010035 response to inorganic substance
GO:0009737 response to abscisic acid
GO:0006979 response to oxidative stress
GO:0009686 gibberellin biosynthetic process
GO:0009739 response to gibberellin
GO:0010876 lipid localization
GO:0010162 seed dormancy process
GO:0010200 response to chitin
GO:0010431 seed maturation
GO:0016143 S−glycoside metabolic process
GO:0016144 S−glycoside biosynthetic process
GO:0044550 secondary metabolite biosynthetic process
GO:0019375 galactolipid biosynthetic process
GO:0005576 extracellular region
GO:0016036 cellular response to phosphate starvation
GO:0003824 catalytic activity
GO:0019748 secondary metabolic process
GO:0044272 sulfur compound biosynthetic process
GO:0009267 cellular response to starvation
GO:1901137 carbohydrate derivative biosynthetic process
GO:0031667 response to nutrient levels
GO:0031668 cellular response to extracellular stimulus
GO:0009991 response to extracellular stimulus
GO:0006790 sulfur compound metabolic process
GO:0010438 cellular response to sulfur starvation
GO:1901135 carbohydrate derivative metabolic process
GO:0007623 circadian rhythm
GO:0009851 auxin biosynthetic process
GO:0022857 transmembrane transporter activity

Figure 10

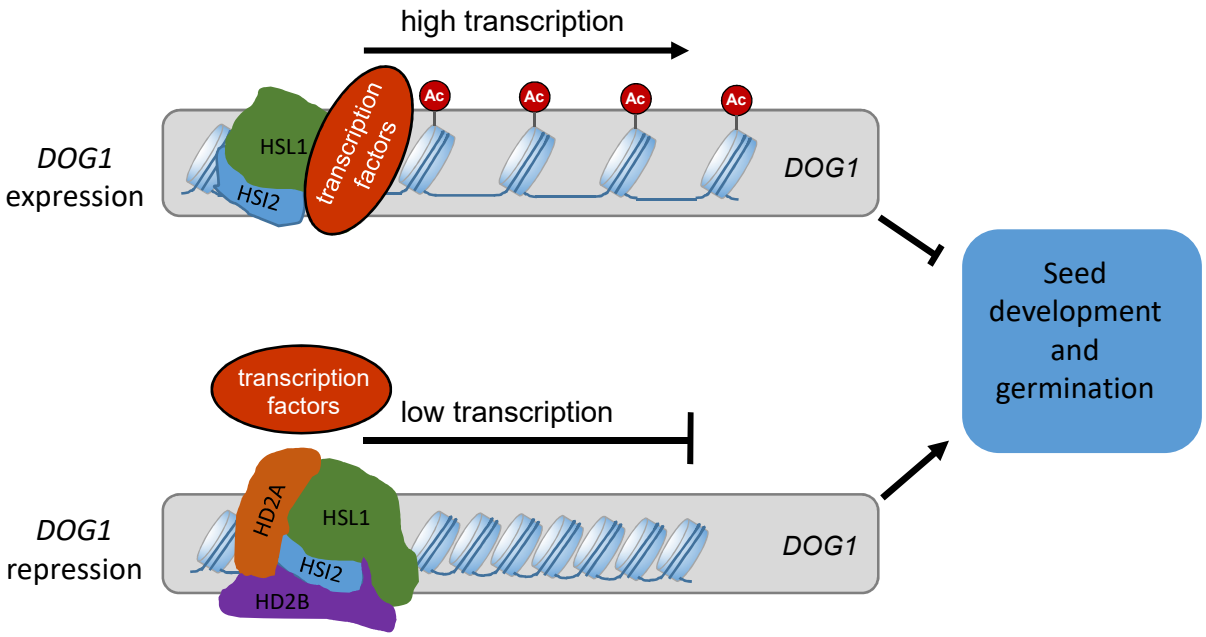
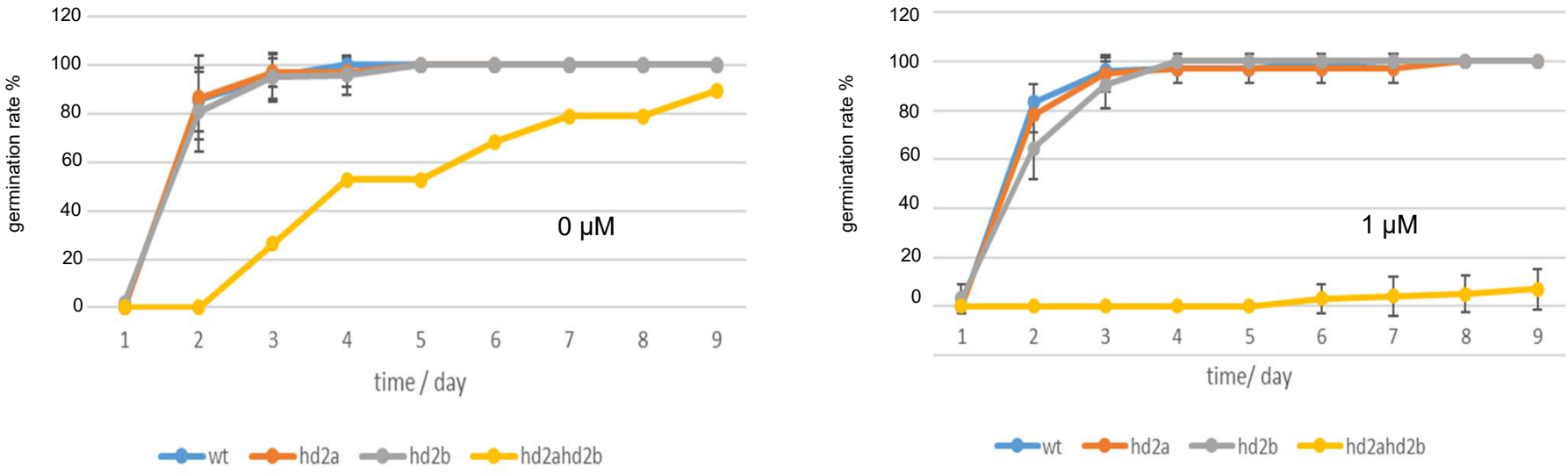

high transcription
DOG1
expression
HSL1
HSI2
transcription
factors
Ac
Ac
Ac
Ac
DOG1
Seed
development
and
germination
transcription
factors
low transcription
DOG1
repression
HD2A
HSL1
HSI2
HD2B
DOG1

Figure 11



Supplemental Figure 1. Germination analysis of different *hd2* lines.
Germination of WT, *hd2a*, *hd2b* and *hd2ahd2b* seeds in absence or presence of 1 µM ABA. Sixteen weeks after-ripened seeds were imbibed on ½ MS plate in absence (left) or presence (right) of 1 µM ABA. The germination percentage was monitored daily for nine days. Statistics: Error bar represent the ±SD of at least 3 biological replicates. One-Way ANOVA (Tukey-Kramer test) analysis was performed.