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Expression and function of HD2-type histone deacetylases in *Arabidopsis* development

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Summary

HD2 (histone deacetylase) proteins are plant-specific histone deacetylases (HDACs). The *Arabidopsis* genome contains four *HD2* genes, namely *HD2A*, *HD2B*, *HD2C*, and *HD2D*. We have previously demonstrated that HD2A, HD2B, and HD2C can repress transcription directly by targeting to promoters *in planta*. Here, we show that the N-terminal conserved motif (EFWG) and histidine 25 (H25), a potential catalytic residue, were important for the gene repression activity of HD2A. *In situ* hybridization indicated that *HD2A*, *HD2B*, and *HD2C* were expressed in ovules, embryos, shoot apical meristems, and primary leaves. Furthermore, all three genes were strongly induced during the process of somatic embryogenesis. *HD2D* mRNA was only detected in the stems and flowers with young siliques and may have adopted different functions. Using green fluorescent protein (GFP) fusions, we demonstrated that HD2A, HD2B, and HD2C accumulated in the nuclei of *Arabidopsis* cells. Overexpression of *35S::GFP–HD2A* in transgenic *Arabidopsis* plants generated pleiotropic developmental abnormalities, including abnormal leaves, delayed flowering, and aborted seed development. The data showed that normal pattern of *HD2* expression was essential for normal plant development and that HD2A, HD2B, and HD2C may be needed for embryogenesis and embryo development. Reverse transcriptase (RT)-PCR analysis revealed that a number of genes involved in seed development and maturation were repressed in the *35S::GFP–HD2A* plants, supporting a role of HD2A in the regulation of gene expression during seed development.

Keywords: histone deacetylase, HD2, gene repression, development, *Arabidopsis*.

Introduction

In eukaryotic chromatin, the strong interaction of DNA with histones H2A, H2B, H3, and H4 restricts access of regulatory protein factors involved in DNA replication and transcription to their target sequences. The core histones can be reversibly modified through post-translational modifications. Acetylation of histones is generally correlated with transcriptionally active euchromatin, whereas deacetylation is correlated with transcriptionally silent heterochromatin. The level of acetylation depends on the competing activities of the enzymes histone acetyltransferases (HATs) and histone deacetylases (HDACs). The finding that many transcription complexes possess HAT or HDAC activities in yeast and animals indicates that histone acetylation has been conserved as a fundamental and central mechanism used to regulate transcription (Khochbin *et al.*, 2001).

Different families of HDACs have been identified in plants, yeast, and animals (Pandey *et al.*, 2002; Yang and Seto, 2003): the reduced potassium dependency (RPD)3/histone deacetylase (HDA)1 family and SIR2 family. SIR2 differs from the others in that it depends on nicotinamide adenine dinucleotide for enzymatic activity. It has been shown that RPD3/HDA1-type HDACs in maize efficiently repress transcription (Pipal *et al.*, 2003; Rossi *et al.*, 2003). In plants, specific functions have not been assigned to these specific enzymes; however, it is possible that they play diverse roles because pleiotropic effects were observed when the *Arabidopsis RPD3A* gene was silenced by an antisense construct (Tian and Chen, 2001).

Plants differ from other eukaryotes in that they possess an additional HDAC family, the HD2 (histone deacetylase)

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family. Four HD2 proteins, HD2A (HDT1), HD2B (HDT2), HD2C (HDT3), and HD2D (HDT4), were identified in *Arabidopsis* (Dangl *et al.*, 2001; Pandey *et al.*, 2002; Wu *et al.*, 2003). The evolution of a new family is interesting because it raises the possibility of functional specialization. The first member of the HD2 family to be identified was purified from maize chromatin as a high-molecular weight complex composed of three almost identical acidic polypeptides (Lusser *et al.*, 1997). The active enzyme is a phosphoprotein, and therefore it may be subject to post-translational control through phosphorylation pathways. The maize HD2 is tightly bound to chromatin, located in the nucleolus, and shares homology with other nucleolar proteins (Lusser *et al.*, 1997). The nucleolar location of the maize HD2 led to speculation that it could be involved in rDNA chromatin organization and expression. However, silencing of *Arabidopsis HD2A* resulted in aborted seeds without general pleiotropic effects, indicating a role in seed development (Wu *et al.*, 2000a). Although little is known about the mechanism of action, composition of multiprotein complexes, and downstream targets of HD2 proteins in plants, we have demonstrated that *Arabidopsis* HD2A, HD2B, and HD2C proteins can repress transcription (Wu *et al.*, 2000a, 2003).

Here, we extend the functional analysis of the HD2 family in *Arabidopsis*. We used site-directed mutagenesis to identify amino acid residues essential for the gene repression activity of HD2A. Studies on the spatial expression patterns of *HD2A*, *HD2B*, and *HD2C* genes in *Arabidopsis* revealed selective expression in meristematic and embryonic cells. Somatic embryogenesis was induced using the transcription factor BABYBOOM (BBM; Boutilier *et al.*, 2002) to confirm the induction of the *HD2* genes during embryogenesis as well as the subsequent stages of embryo development. The green fluorescent protein (GFP)–HD2 fusion proteins were expressed in transgenic *Arabidopsis* plants to monitor the nuclear location of ectopically expressed HD2 proteins. The *35S::HD2A* transgenic plants were found to display pleiotropic developmental abnormalities, including abnormal leaves, delayed flowering, and aborted seed development, indicating that ectopically expressed HD2A can interrupt critical developmental processes. Our studies provide evidence that the HD2 family may play a role in the regulation of gene expression essential for development, including embryogenesis and embryo development.

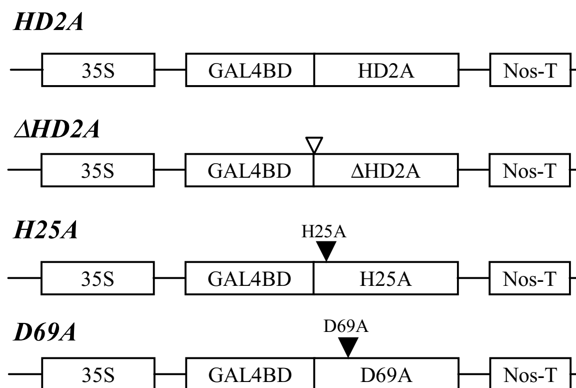
Results

Identification of amino acid residues essential for gene repression activity

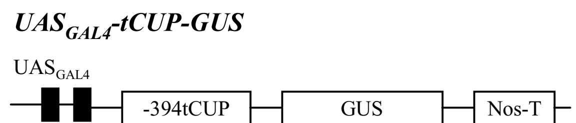
We have previously demonstrated that HD2A, HD2B, and HD2C repressed transcription as GAL4 fusion proteins when directed to a promoter containing GAL4-binding sites (Wu *et al.*, 2003). Here, we used site-directed mutagenesis to identify residues in HD2A that are required for gene repression activity. Sequence alignments of all known and putative HD2 proteins revealed that the N-terminal region of HD2 proteins contains an EFWG motif as well as 18 conserved amino acid residues (Dangl *et al.*, 2001; Wu *et al.*, 2003). As shown in Figure 1, deletion of the N-terminal EFWG motif resulted in loss of gene repression activity.

(a)

Effector Plasmids



Reporter Plasmid



(b)

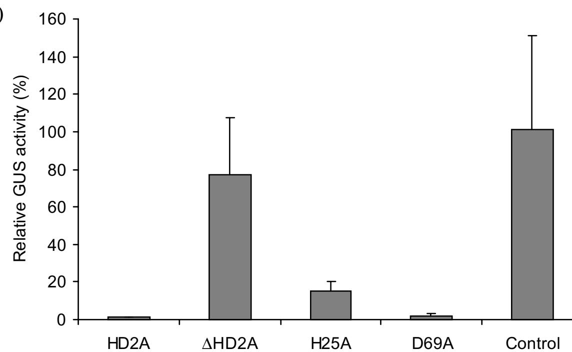


Figure 1. Repression of the *UAS_{GAL4}-tCUP–GUS* fusion gene by *HD2A* and *HD2A* mutants in transient expression assays.
(a) Schematic diagram of the effector and reporter constructs used in co-bombardment experiments. The reporter construct contains *UAS_{GAL4}* repeated twice and fused to the *−394tCUP* promoter–*GUS* construct. The effector constructs contain the *GAL4* DNA-binding domain (*GAL4BD*) fused to the full-length HD2A, the N-terminal EFWG deletion *ΔHD2A*, *H25A*, and *D69A* mutants.
(b) Repression of the *UAS_{GAL4}-tCUP–GUS* fusion gene by *HD2A*, *ΔHD2A*, *H25A*, and *D69A* mutants. The reporter plasmid was co-bombarded with each effector plasmid or the control plasmid *pUC19*. Bars indicate the SE of three replicates.

Among the conserved amino acid residues, it was suggested that histidine 25 (H25) and aspartic acid 69 (D69) have potential catalytic properties (Aravind and Koonin, 1998). We therefore mutated these residues to alanine (A) by site-directed mutagenesis to test if they are required for gene repression activity. As shown in Figure 1, the H25A mutant yielded decreased gene repression activity compared with wild-type protein. The D69A mutant, however, showed little change in gene repression activity compared with wild-type protein. The experiment was repeated thrice with similar results. Our data suggest that the N-terminal EFWG motif is essential for gene repression activity, and that amino acid residue H25 may be important but to a lesser extent.

Spatial patterns of HD2A*,* HD2B*,* HD2C*, and* HD2D *expression*

The mRNA accumulation patterns were examined for all of the *Arabidopsis HD2* genes by reverse transcriptase (RT)-PCR. As shown in Figure 2, *HD2A*, *HD2B*, and *HD2C* mRNAs were detected in leaves, roots, stems, young plantlets, and a sample containing flowers and siliques; however, the levels of accumulation differed among the organs. Our RT-PCR analysis data are consistent with previously published data of northern analysis (Wu *et al.*, 2000a; plant chromatin database: http://www.chromdb.org). In contrast, the *HD2D* mRNA was only detected in the stems and flowers with young siliques, suggesting that certain *HD2* genes may be differentially expressed in *Arabidopsis*.

The spatial patterns of *HD2A*, *HD2B*, and *HD2C* mRNA accumulation were examined by *in situ* hybridization. The gene-specific probes corresponding to the less-conserved C-terminal regions of the HD2 proteins (Wu *et al.*, 2003) were generated by PCR. Low levels of expression were detected in all of the organs examined, but the highest levels of accumulation were found in the ovules (Figure 3a), embryos (Figure 3e,i), shoot apical meristem (Figure 3c), and first leaves (Figure 3c). Figure 3 shows the spatial expression pattern for *HD2B* mRNA accumulation and the data were identical for *HD2A* and *HD2C* (data not shown).

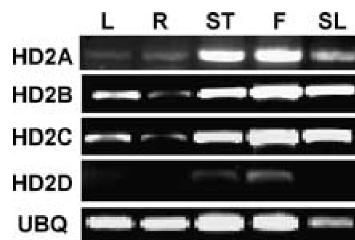


Figure 2. RT-PCR analysis of expression of *HD2* genes in *Arabidopsis*. Total RNA for RT-PCR analysis was isolated from leaves (L), roots (R), stems (ST), flowers and young siliques (F), and seedlings (SL) of *A. thaliana. UBQ* was shown as an internal control.

Induction of HD2A*,* HD2B*, and* HD2C *in somatic embryos*

The selective expression of the *HD2* genes in the embryos of seeds indicated a potential functional role in embryogenesis and embryo development that may be addressed experimentally through somatic embryogenesis. Although somatic embryogenesis is difficult to achieve in wild-type *Arabidopsis* (Mordhorst *et al.*, 2002) the ectopic expression of certain transcription factors, such as BBM from *Brassica napus*, can induce the proliferation of somatic embryos on transgenic plant tissues (Boutilier *et al.*, 2002). Figure 4(b) illustrates the differentiation of somatic embryos on the cotyledons of transgenic *Arabidopsis* seedlings expressing a *35S::BBM-nos* gene. Compared with wild-type plantlets (Figure 4a), the transgenic plantlets have shortened roots, swollen dedifferentiating hypocotyls, and abnormal cotyledons with somatic embryos differentiating mostly at the tips (Figure 4b). *In situ* hybridization with an *HD2B*-specific probe shows that the *HD2B* mRNA is accumulating throughout the transgenic cotyledonary tissues but particularly in the somatic embryos and underlying tissues (Figure 4c). In contrast, *HD2B* mRNA did not accumulate in the cotyledons of wild-type plants (Figure 3c). The same results were found with an *HD2A*-specific probe (Figure 4d) and with an *HD2C*-specific probe (data not shown). The induction of the HD2 family of genes therefore appears to be correlated with the competence of tissues to undergo somatic embryogenesis and the early stages of somatic embryo development. This pattern of expression is similar to that seen with seed-derived embryos (Figure 3e,i). It is unclear from this experiment whether the increased levels of *HD2* gene expression was caused by the induction of somatic embryos or overexpression of the BBM transcription factor.

Targeting of AtHD2A*,* AtHD2B *and* AtHD2C *into the nucleus*

To investigate the cellular distribution of HD2 proteins, we performed an *in vivo* targeting experiment using a GFP. *GFP–HD2A*, *GFP–HD2B*, and *GFP–HD2C* gene fusions were created and introduced into *Arabidopsis* under the 35S promoter to achieve high levels of constitutive expression. To confirm that the fusion proteins entered the nucleus and associated with chromatin, we monitored the fluorescence of GFP at the cellular level. Protoplasts were isolated from seedlings of transgenic *Arabidopsis*, and localization of the fusion protein was determined by fluorescence microscope. As shown in Figure 5, the GFP–HD2B fusion protein was localized in the nucleus of the *Arabidopsis* cells. The maize HD2 was previously localized in the nucleolus (Lusser

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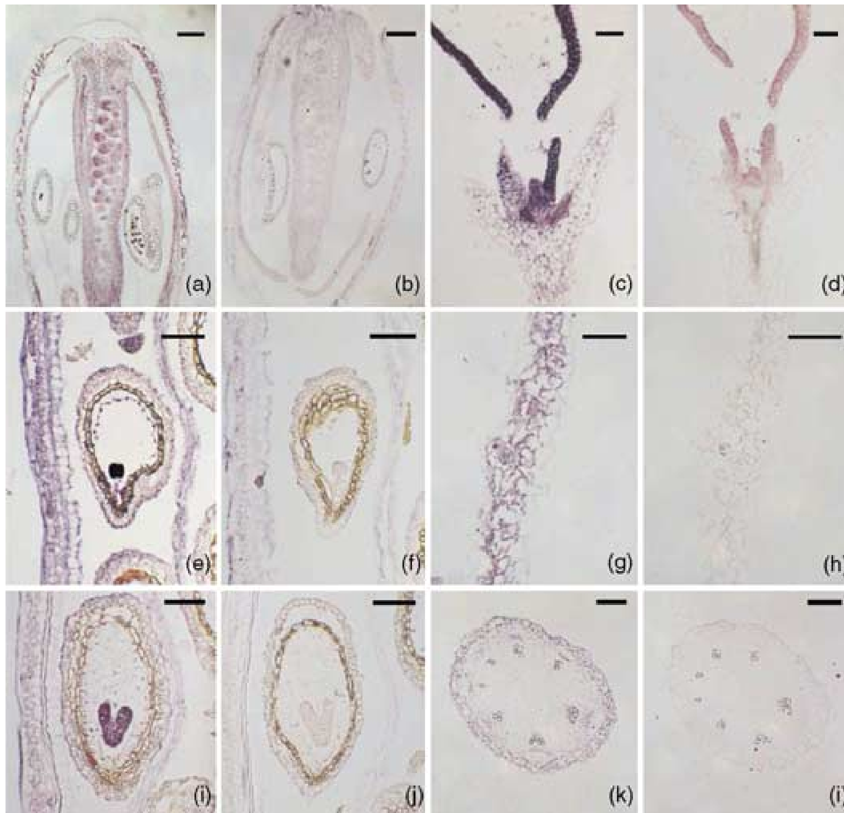
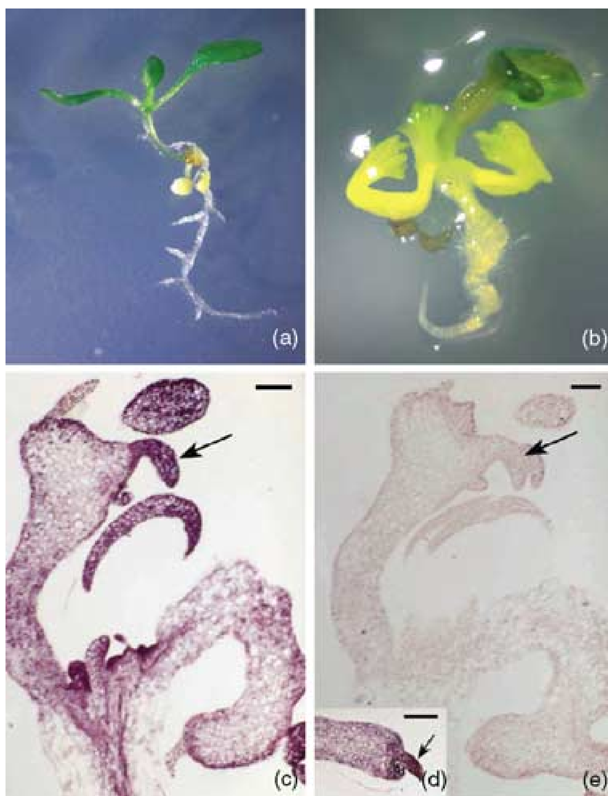


Figure 3. *In situ* hybridization of wild-type *Arabidopsis* organs using an antisense *HD2B*-specific RNA probe (a,c,e,g,i,k) and the sense control probe (b,d,f,h,j,l).
Floral buds (a,b), developing siliques (e,f,i,j), leaf (g,h), and stem (k,l). The bar represents 100 μm.



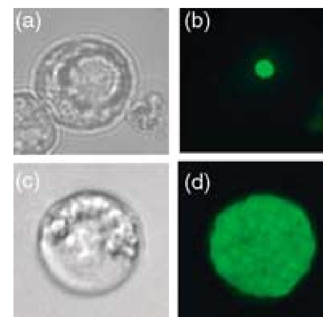


Figure 5. Subcellular localization of the HD2B.
Protoplasts were isolated from the leaves of *35S::GFP–HD2B* (a,b) and *35S::GFP* (c,d) transgenic *Arabidopsis* plants. GFP fluorescence was examined after 12 h by fluorescence microscopy under white light (a,c) and UV light (b,d).

Figure 4. Induction of HD2A and HD2B in somatic embryo.
In situ hybridization of transgenic *Arabidopsis* plantlets expressing the *B. napus* transcription factor BBM using the 35S promoter 10 days after germination (b–e). The *HD2B*-specific antisense probe (c), the *HD2B*-specific sense probe (e), and the *HD2A*-specific antisense probe (d) were used. The morphology of the wild-type (a) and transformed (b) *Arabidopsis* plantlets is shown 10 days after germination. The arrows indicate somatic embryos. The bar represents 100 μm.

et al., 1997). The GFP–HD2B fusion protein did not localize uniformly to the entire nucleus, but rather to a much smaller area with sharp boundaries, suggesting possible nucleolus localization. Similar results were observed with the GFP–HD2A and GFP–HD2C. The GFP alone, however, was uniformly distributed throughout the cell (Figure 5d).

Ectopic expression of GFP–HD2A *resulted in pleiotropic developmental defects*

We analyzed the phenotypes of the transgenic *Arabidopsis* plants that expressed the *GFP–HD2A* gene. The expression of the *GFP–HD2A* transcript was determined by using a ^{32}P-labeled *HD2A* cDNA probe. Four independent transgenic lines, 21N-1, 21N-2, 21N-3, and 21N-4, with high levels of *GFP–HD2A* expression were identified (Figure 6). Low level of endogenous *HD2A* expression was detected in the leaf tissues by northern analysis, which is consistent with the RT-PCR data in Figure 3. Transgenic *Arabidopsis* plants displayed pleiotropic developmental abnormalities at very high frequencies compared with wild-type and *35S::GFP* plants (Table 1). These included abnormal leaf (Figure 7b–d) and flower morphologies (Figure 7h,i,k,l), delayed flowering (Figure 7e), and aborted seed development (Figure 7n,o). Among four transgenic lines analyzed, about 20–70% of the transgenic plants were sterile (Table 1). In sterile plants, the sizes of the filaments were significantly shorter than those in normal plants (Figure 7k,l) but the pollen was fertile when crossed to wild-type plants. The data showed that ectopic expression of *HD2A* may interfere with several normal developmental processes, indicating that the spatial regulation of the *HD2A* gene observed earlier may be essential for normal plant development. Developmental abnormalities were observed in the plants from four independent transgenic lines, but not in wild-type control plants or in plants transformed with the *GFP* alone. The developmental abnormalities persisted over five generations of selfing. Our data suggest that the abnormal phenotypes of the transgenic plants were co-inherited with the *GFP–HD2A* transgene.

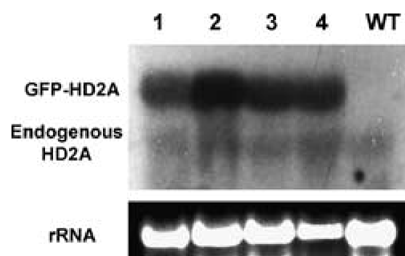


Figure 6. Northern analysis of *HD2A* expression in transgenic plants.
Analysis of endogenous *HD2A* and *GFP–HD2A* transgene expression in wild-type (WT) line and four transgenic lines, 21N-1 (lane 1), 21N-2 (lane 2), 21N-3 (lane 3), and 21N-4 (lane 4). Twenty micrograms of total RNA isolated from leaves were probed with an *HD2A* cDNA probe. Ethidium bromide-stained 26S rRNA is shown to illustrate the gel loading.

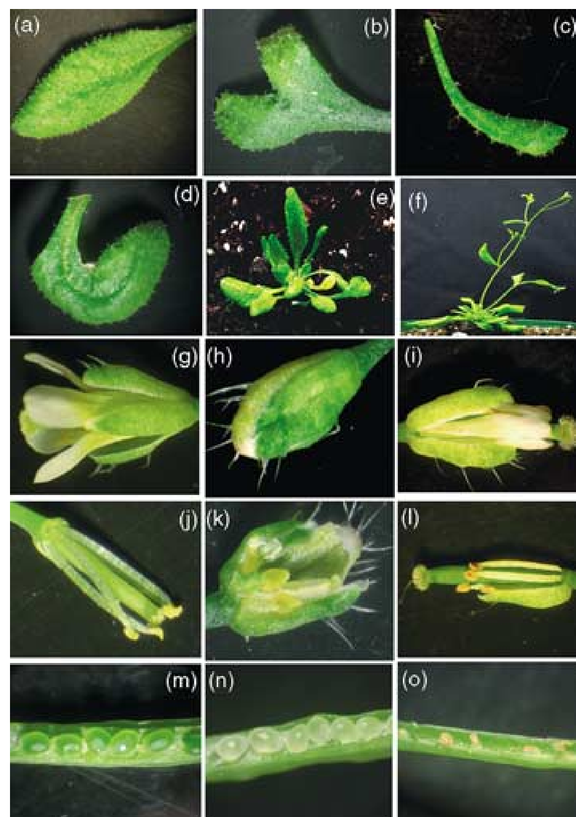


Figure 7. Phenotype of *35S::GFP–HD2A* transgenic plants.
(a–d) The 21N-2 transgenic plants from the T_2 line with branching-leaf (b), narrow-leaf (c), and curve-leaf phenotypes (d), compared with a normal leaf (a).
(e, f) A 6-week-old 21N-2 plant (e) from the T_2 line showed delayed flowering when compared with a 6-week-old wild-type plant (f).
(g–l) The 21N-2 transgenic plants from the T_2 line showed aberrant flowers with shorter filaments (h,i,k,l) compared with the wild-type (g,j).
(m–o) The 21N-2 transgenic plants from the T_2 line showed aborted seed development (n,o) compared with the wild-type (m).

GFP–HD2A *overexpression affected gene expression involved in seed development*

To investigate the transcriptional regulatory roles of *HD2A* in plants, the expression of several genes involved in seed development in *35S::GFP–HD2A* plants was determined by RT-PCR. As shown in Figure 8, the transcript levels of a number of seed development-related genes were repressed in two *35S::GFP–HD2A* transgenic lines, 21N-1 and 21N-2. These include late embryogenesis-abundant protein (Fujiwara *et al*., 2002), cruciferin, lectin, oleosin, seed maturation protein, albumin, legumin, peroxiredoxin (Haslekas *et al*., 2003), and abscisic acid-responsive elements-binding factor 3 (ABF3; Brocard *et al*., 2002; Kang *et al*., 2002). The expression of these genes was more significantly repressed in 21N-2 plants compared with 21N-1 plants. 21N-2 plants had a higher expression level of *GFP–HD2A* compared with 21N-1 plants (Figure 6),

Table 1 Frequency of abnormal phenotypes in *35S::GFP–HD2A* transgenic lines

Arabidopsis lines[a]	Abnormal leaves[b] (%)	Delayed flowering[c] (%)	Sterile[d] (%)
Wild-type (29)	0	0	0
35S::GFP (23)	0	0	0
35S::GFP–HD2A			
21N-1 (26)	30.8	28.7	20.5
21N-2 (26)	46.2	19.4	69.2
21N-3 (21)	38.1	47.6	33.3
21N-4 (21)	28.6	38.1	19.0

[a]The numbers in the parentheses are the total plants analyzed. The frequencies are for the transgenic progenies only, the non-transgenic segregants are not included.
[b]Abnormal leaves include branching-leaf, narrow-leaf, and curled-leaf phenotypes.
[c]Wild-type *Arabidopsis* plants flowered at 26–33 days, whereas the later flowering plants flowered at 35–43 days.
[d]Sterile plants did not produce seeds.

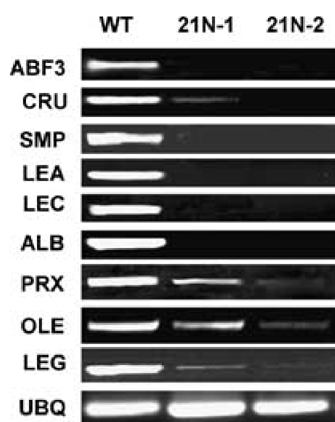


Figure 8. RT-PCR analysis of gene expression in *35S::GFP–HD2A* transgenic plants.
Total RNA was isolated from the leaf tissues of the wild-type (WT) plants, and two independent *35S::GFP–HD2A* transgenic lines, 21N-1 and 21N-2. The expression of ABF3, cruciferin (CRU), seed maturation protein (SMP), late embryogenesis abundant protein (LEA), lectin (LEC), albumin (ALB), peroxiredoxin (PRX), oleosin (OLE), and legumin (LEG) was analyzed by RT-PCR. *UBQ* was shown as an internal control.

suggesting a correlation between *HD2A* expression and gene repression.

Discussion

Three distinct domains have been identified in the HD2 family of proteins: the predicted N-terminal deacetylase catalytic domain, the middle region consisting of the extended acidic domain, and the C-terminal domain (Aravind and Koonin, 1998; Dangl *et al.*, 2001; Lusser *et al.*, 1997; Wu *et al.*, 2000a). Both HD2A and HD2C have a putative zinc-finger domain in their C-terminal, whereas HD2B and HD2D do not. Deletion of the C-terminal domain from HD2A did not affect gene repression activity, indicating that this domain is not required for gene repression (Wu *et al.*, 2000a). Deletion of the extended acidic domain and the domain containing predicted catalytic residues of HD2A resulted in the loss of gene repression activity, suggesting that both domains may be essential for HD2A function. Our studies indicate that the N-terminal EFWG motif is essential, and H25 is important for gene repression activity of HD2A. H25 has been suggested to have potential catalytic properties in HD2-type HDACs (Aravind and Koonin, 1998).

The control of intracellular location is an important regulatory mechanism for HDAC proteins in yeast and mammalian cells (Bjerling *et al.*, 2002; Hirschler-Laszkiewicz *et al.*, 2001). It was demonstrated that mammalian HDA1-type HDACs were mobilized from the cytoplasm to the nucleus by phosphorylation. Using GFP fusions, we demonstrated that HD2A, HD2B, and HD2C accumulated in the nuclei of *Arabidopsis* cells. Recently, it was found that *Arabidopsis* HD2A is present in the nuclear matrix (Calikowsk *et al.*, 2003). In addition, maize HD2 was also localized in the nucleolus (Lusser *et al.*, 1997). Our studies indicate that *Arabidopsis* HD2 proteins may also be localized into nucleolus. The nucleolar localization of other types of HDACs has also been demonstrated in yeast and mammalian cells (Bjerling *et al.*, 2002; Hirschler-Laszkiewicz *et al.*, 2001). The nucleolus is recognized as the site of rRNA transcription, rRNA processing, and ribosome assembly; however, recent studies suggest that the nucleolus functions more broadly in gene expression (Pederson, 1998; Scherl *et al.*, 2003) and may play a crucial role in cellular processes such as the control of cell cycle, aging, and mRNA export.

Our study indicated the potential for differential expression among *HD2* genes. *HD2A*, *HD2B*, and *HD2C* appeared to have identical spatial expression patterns. The mRNAs could be detected in all the tissues at low levels but selectively accumulated in ovules, embryos, shoot apical meristems, and primary leaves. Similar spatial patterns of expression have been observed for genes that control embryogenesis, such as *WUSCHEL* and somatic embryogenesis receptor kinase (*SERK*) (Hecht *et al.*, 2001; Mayer *et al.*, 1998) where they are involved in the maintenance of both shoot apical meristems and embryonic stem cells (Zuo *et al.*, 2002). As the *in situ* hybridization data showed the strongest expression of the *HD2A*, *HD2B*, and *HD2C* genes in embryos, we considered the possibility that the aborted seed phenotype that we observed in the *HD2A* antisense plants (Wu *et al.*, 2000a) might result from a defect in embryogenesis, resulting from the silencing of *HD2A*. Somatic embryogenesis can be a valuable model system for testing this hypothesis and characterizing the processes. Although difficult to achieve in *Arabidopsis* (Mordhorst *et al.*, 2002), the *BBM* gene that codes for an AP (apetula)2-domain transcription factor will generate

somatic embryos on transgenic *Arabidopsis* cotyledons when ectopically expressed at high levels (Boutilier *et al.*, 2002). Ectopic expression of *BBM* induced the expression of *HD2A*, *HD2B*, and *HD2C*, particularly in pre-embryonic tissues and somatic embryos. The data indicate that the expression of these genes is tightly correlated with both somatic and zygotic embryogenesis and is likely to be essential for embryo development. The somatic embryo system may generate enough material for the isolation and characterization of the HDAC complexes, which is needed to characterize the repression mechanisms participating in the control of embryo development.

Different HDACs, even within single families, may play different roles in plant growth and development. High levels of HD2 expression were found in the shoot apical meristem and primary leaves as well as in embryonic cells. Yet, developmental defects were only observed in seeds of plants in which *HD2A* was silenced (Wu *et al.*, 2000a). Evidence for functional specialization of plant HDACs exists in the literature. The silencing of an *RPD3*-type *HDAC* gene, *RPD3A* (*HD1*), resulted in general pleiotropic effects, indicating that this gene plays diverse functional roles as one of the five members of the RPD3 gene family in *Arabidopsis* (Tian and Chen, 2001; Wu *et al.*, 2000b). The *Arabidopsis axe1* (auxin gene expression 1) mutant that is defined by a mutation in the *RPD3B* (*HDA6*) gene had a distinctive phenotype with a higher number of root hairs compared with wild-type plants (Devoto *et al.*, 2002; Murfett *et al.*, 2001).

The similarity of *HD2A*, *HD2B*, and *HD2C* expression patterns raised the possibility of functional redundancy. Indeed, no obvious phenotype was observed in the T-DNA insertion lines and RNAi lines in which these genes were silenced (Zhou *et al.*, unpublished data; plant chromatin database: http://chromdb.org/). Suppression of *HD2A* by an antisense construct that was likely to have partially suppressed the expression of other *HD2* genes caused aborted seed development (Wu *et al.*, 2000a). Ectopic expression of *HD2A* as a fusion protein disrupted normal development and generated many pleiotropic effects in a variety of somatic and reproductive tissues. Similar results were obtained by ectopic expression of *HD2A* alone (data not shown). This observation indicated that the components needed for specificity may be absent in non-target tissues and/or that ectopically expressed *GFP–HD2A* may be acting on atypical non-specific target sites. However, the data from the transgenic plants overexpressing *HD2A* need to be interpreted carefully. Overexpression of *HD2A* may induce direct or secondary effects on gene expression. Moreover, overexpressing *HD2A* may induce silencing of endogenous *HD2* genes. Our Northern analysis data suggest that the expression of endogenous *HD2A* gene was not affected in the *GFP–HD2A* transgenic plants.

RT-PCR analysis revealed that a number of genes involved in seed development and maturation were repressed in transgenic *Arabidopsis* overexpressing *HD2A.* HD2A may therefore play a role in the regulation of gene expression during *Arabidopsis* seed development. In particular, the expression of the transcriptional factor ABF3 was downregulated in *35S::GFP–HD2A* transgenic plants. ABF3 is a basic leucine zipper (bZIP) protein similar to ABA insensitive (ABI)5 that regulates key aspects of plant seed development and abscisic acid signaling (Brocard *et al.*, 2002; Kang *et al.*, 2002). Downregulation of ABF3 may affect the expression of downstream genes, resulting in aborted seed development. The pleiotropic developmental defects of the *35S::GFP–HD2A* transgenic plants, however, indicated that the importance of the spatial control of *HD2A* expression extended beyond seed development and it was essential for overall plant development.

The circumstantial evidence for the direct participation of HDACs in embryogenesis is very strong. In *Drosophila* early embryogenesis, it has been shown that the chromatin-remodeling protein Mi2 interacts with polycomb group (PcG) proteins in the repression of homeotic genes (Kehle *et al.*, 1998) and with HDACs in nucleosome remodeling and histone deacetylation (NuRD) complexes. In *Arabidopsis* seeds, PICKLE represents a homolog of Mi2 and represses the embryonic phenotype in germinated seedlings. Mutations in *PICKLE* result in the maintenance of the embryonic phenotype in roots where somatic embryos will form (Ogas *et al.*, 1999). Another member of this gene family, *GYMNOS*, is required for carpel development (Eshed *et al.*, 1999). It is believed that most developmental decisions in eukaryotes involving transcriptional repressors will involve HDAC multiprotein complexes that have been adapted for specific roles in development.

Our study demonstrated that *HD2A*, *HD2B*, and *HD2C* were expressed strongly in embryonic tissues and that ectopic expression of *HD2A* disrupted the normal patterns of gene expression and development. We provide evidence that functional specialization of the HD2 members may exist and that regulation of *HD2* expression is essential for normal development in *Arabidopsis*. It seems likely that the target genes undergoing repression by HD2 are mediated through protein complexes by specific transcription factors. Further identification of the target genes and proteins interacting with HD2 are essential to elucidate their functions and mechanisms of action in plants. The somatic embryogenesis system described here will be needed for these experiments.

Experimental procedures

Plasmid construction

The upstream activating sequence of *GAL4* tandem (UAS_{GAL4})-*tCUP–GUS* reporter and *35S::GAL4–HD2A* effector constructs were generated as described by Wu *et al.* (2000a). The N-terminal EFWG deletion (ΔHD2A) of HD2A was generated by PCR using the primer

pair PRM1s and PR4. The *H25A* and *D69A* mutants of HD2A were generated by PCR-based overlap extension mutagenesis (Sambrook and Russell, 2001). Two PCRs were set up simultaneously using the HD2A cDNA as a template. The primer pairs for generating H25A are: FM1a and PR1 for the first PCR, and RM1s and PR4 for the second PCR. The primer pairs for generating D69A are: FM2a and PR1 for the first PCR, and RM2s and PR4 for the second PCR. To construct *35S::GFP–HD2A* plasmid, we replaced the *GAL4* cDNA in the *35S::GAL4–HD2A* (Wu *et al.*, 2000a) with the *GFP* cDNA. Pfu Polymerase (Stratagene, La Jolla, CA, USA) was used in PCR to minimize undesired mutations in the sequences. The sequences of the primers used for the mutagenesis are listed: PRM1s, 5′-aattcccgggaattgaagttaaatcaggaaagc-3′; PR4, 5′-acgtgagctcagaaaccacttcacttggc-3′; FM1a, 5′-ctgagaaacggcgataagaatgccttcttcag-3′; RM1s, 5′-cattcttatcgccgtttctcaggcatcgcttg-3′; FM2a, 5′-tccccaagtggcagaaagctcaaactccttg-3′; and RM2s, 5′-gagctttctgccacttggggaaaaggaagtg-3′.

DNA and protein sequence analysis

Dye primer sequencing of plasmids was performed using an automated sequencing system (Applied Biosystems, Foster City, CA, USA). DNA and protein sequence analysis was carried out using BLAST searches (Altschul *et al.*, 1990) and the VECTOR NTI suite program (InforMax Inc., Bethesda, MD, USA).

Southern and Northern blot analysis

Total genomic DNA from *Arabidopsis* was extracted as described by Dellaporta *et al.* (1983). For Southern blots, *Arabidopsis* genomic DNA was digested with restriction enzymes, separated by agarose gel electrophoresis, and transferred to nylon membranes (Sambrook and Russell, 2001). For Northern analysis, total RNA was isolated from 100 to 200 mg of *Arabidopsis* leaf tissues using Trizol reagent as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). Northern blots were prepared by electrophoresis of 5–10-μg samples of total RNA through agarose gels in the presence of formaldehyde, followed by transfer to nylon membranes.

Southern and Northern blots were probed with ^{32}P-labeled probes. Pre-hybridization and hybridization were performed at 65°C in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, and 1 mM EDTA (Strommer *et al.*, 1993). Filters were washed once for 15 min in 2× SSC with 0.1% SDS at room temperature, and then twice for 20 min in 0.1× SSC, 0.1% SDS at 65°C. The damp filters were autoradiographed at −80°C using two intensifying screens. Filters were stripped in 5 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.05% SDS at 100°C for 2 min when re-probing was required.

RT-PCR analysis

One microgram of total RNA was used for the first-strand cDNA synthesis after incubation at 65°C for 10 min. cDNA was synthesized in a volume of 20 μl that contained MoMLV RT buffer (Promega, Madison, WI, USA), 10 mM dithiothreitol, 1.5 μM poly(dT) primer, 0.5 mM dNTPs, 2 U of MoMLV RT at 37°C for 1 h. All PCR reactions were performed with 0.5 U of Taq polymerase (PGC Scientific, Gaithersburg, MD, USA), the buffer provided by the supplier, 0.2 mM dNTPs, and a pair of primers (0.1 μM each) in a final volume of 20 μl. PCR parameters differed for each gene: thermocycling conditions were 94°C for 2 min followed by 25–40 cycles of 94°C for 1 min, 62–65°C for 1 min, and 72°C for 2 min, with a final polymerization step at 72°C for 10 min. The gene-specific primer pairs used for the RT-PCR are listed: *HD2A*, 5′-atggagttctggggaatttg-3′ and 5′-cgtgcttggccttgttgtgag-3′; *HD2B*, 5′-atggagttctggggtgttgaag-3′ and 5′-tcaagcagctgcactgtgtttg-3′; *HD2C*, 5′-atggagttctggggtgttgaag-3′ and 5′-tcaagcagctgcactgtgtttg-3′; *HD2D*, 5′-atggagttttggggtatcg-3′ and 5′-ctactttttgcaagagggac-3′; and *UBIQUITIN* (*UBQ*), 5′-gatctttgccggaaaacaattggaggatggt-3′ and 5′-cgacttgtcattagaaagaaagagataacagg-3′. Primers used for checking expression of the genes involved in seed development are listed in the Table 2.

Plant growth and transformation

Arabidopsis thaliana (ecotype Columbia) was grown in a growth chamber (16 h of light and 8 h of darkness at 23°C) after a 2–4-day vernalization period. For growth under sterile conditions, seeds were surface sterilized (15 min incubation in 5% (v/v) sodium hypochlorite, and rinsed thrice with sterile distilled water) and sown on half-strength Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) supplemented with 1% sucrose, pH 5.7, and 0.8% (w/v) agar in Petri dishes.

Plant transformation plasmids were electroporated into *Agrobacterium tumefaciens* GV3101 (Van Larebeke *et al.*, 1974) as described by Shaw (1995). The *Agrobacterium*-mediated transformation of *A. thaliana* was performed as described by Clough and Bent (1998). T_1 seeds were harvested, dried at 25°C, and germinated on sterile medium containing 40 μg ml^{-1} kanamycin to select the transformants. Surviving T_1 plantlets were transferred to soil to set seeds (T_2).

GFP localization

Protoplasts were isolated from *Arabidopsis* seedlings as described by Weigel and Glazebrook (2002). The fluorescence

Table 2 Primers for RT-PCR analysis

Gene symbols[a]	AGI #	Primer sequences
CRU	AT4G28520	5′-acaacatgaacgctaacgaga-3′, 5′-ctcctcgatcaactgttgtt-3′
LEC	AT3G16420	5′-ctgataatgcccttctgagc-3′, 5′-tctttaagaggcaagaccc-3′
OLE	AT4G25140	5′-atggcggatacagctagagg-3′, 5′-ttaagtagtgtgctggccac-3′
LEA	AT5G06760	5′-atgcagtcgatgaaagaaac-3′, 5′-gaccagttccagtgttcctt-3′
SMP	AT2G21820	5′-atgtcgggagcacaaggag-3′, 5′-accaagatcctgcttcttg-3′
ALB	AT4G27170	5′-tgggtgatgaacgagtttcg-3′, 5′-gtgtaagattctggagccat-3′
LEG	AT5G44120	5′-cactctcaacagttacgatc-3′, 5′-gtgagtcaaagtggtctcga-3′
PRX	AT1G65970	5′-cactcccacatgcagtatga-3′, 5′-atgccaagcaagtcaaacac-3′
ABF3	AT4G34000	5′-gaagccagatgtcacctgat-3′, 5′-caatgtccttcgcaagcatt-3′

CRU, cruciferin; LEC, lectin; OLE, oleosin; LEA, late embryogenesis abundant protein; SMP, seed maturation protein; ALB, albumin; LEG, legumin; PRX, peroxiredoxin; and ABF3, abscisic acid responsive elements-binding factor 3.

photographs of protoplasts were taken using an Olympus florescent microscope fitted with fluorescein isothiocyanate filters (excitation filter, 450–490 nm; emission filter, 520 nm; and dichroic mirror, 510 nm).

Particle gun delivery assays

Tobacco (SR1) plants were grown *in vitro* in half-strength MS medium (Murashige and Skoog, 1962) in Magenta boxes (Magenta Corp., Chicago, USA) and kept in a growth chamber at 25°C. After transfer to fresh medium for 2–3 weeks, uniform-sized leaves (about 3 cm in width) were cut off from the plants and placed on a medium consisting of MS salts, B5 vitamins (Gamborg *et al*., 1968), 1 mg l^{-1} 6-benzyladenine, 0.1 mg l^{-1} naphthalene acetic acid, 3% sucrose, and 0.25% Gelrite in a 20 mm × 15 mm Petri dish. The leaves were pre-conditioned on this medium for 1 day prior to gene delivery.

Plasmid DNA was isolated using the Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA, USA). The reporter plasmid was mixed with an effector plasmid at a 1 : 1 ratio (weight). In the control, the reporter plasmid was mixed with an equal amount of the control plasmid pUC19. A modified particle inflow gun (Brown *et al*., 1994) was used for DNA delivery to the tobacco leaves. Twenty-four hours after bombardment, GUS gene expression was determined by recording the number of GUS reporter gene expression events as indicated by the number of blue foci per explant (Jefferson *et al*., 1987).

In situ *hybridization*

The samples were fixed in 3.7% formaldehyde, 50% ethanol, and 5% acetic acid, and then dehydrated, infiltrated in Paraplast, and sectioned as described previously by Gijzen *et al*. (1999). *In situ* hybridization conditions and probe preparation were performed as described by Gijzen *et al*. (1999). The gene-specific probes were generated by PCR using the primer pairs: *HD2A*, 5′-gagaagcttgacggaatggatgaagatg-3′ and 5′-cagctcgagcaagtgcattccctgagttg-3′; *HD2B*, 5′-gtcaattaaccctcactaaagggaagcttccaaaacacccgtctctggaa-3′ and 5′-ctagtaatacgactcactatagggctcgagccctttcccttgcccttgtt-3′; and *HD2C*, 5′-acgtaagcttccgcatgtccatgttgc-3′ and 5′-acgtctcgaggctgcactgtgtttgg-3′.

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