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TECHNICAL ADVANCE

Repression of gene expression by *Arabidopsis* HD2 histone deacetylases

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Summary

The four HD2 proteins of *Arabidopsis thaliana* (AtHD2A–D) belong to a unique class of histone deacetylases that is plant specific. Previously, we have demonstrated that one of the members, AtHD2A, can mediate transcriptional repression when targeted to the promoter of a reporter gene. Here, we report that AtHD2B and AtHD2C can also repress gene expression. AtHD2A and AtHD2C differ from AtHD2B and AtHD2D in the composition of their structural domains. Our data show that both structural types play a role in the repression of gene transcription. We demonstrate that AtHD2A can mediate gene repression through interactions with transcription factors in plants. By fusing AtHD2A with the DNA-binding domain of the plant transcriptional factor Pti4, the expression of a *GCC* box containing reporter gene was repressed. We also demonstrated repression of a GUS gene with *GAL4* enhancers using transgenic plants that expressed a *GAL4/AtHD2A* fusion gene. Furthermore, the expression of the GAL4/AtHD2A protein using the seed-specific napin promoter (*NAP2*) and the constitutive *tCUP* promoter demonstrated that repression of transgenes could be achieved in a tissue-specific or unrestricted manner. Targeting of HD2 proteins to specific promoters using transcription factor DNA-binding domains may therefore provide a new technology for silencing target genes and pathways in plants as well as for assessing the function of unknown transcription factors.

Keywords: histone deacetylases, HD2, gene repression, transgenic plants, *Arabidopsis*.

Introduction

Post-transcriptional modification of histones, in particular acetylation, is an important factor in the regulation of eukaryotic gene expression. Hyperacetylation of histones generally correlates with transcriptionally active chromatin, whereas hypoacetylation correlates with transcriptionally silent chromatin. Histone deacetylases (HDACs) are the enzymes that remove acetyl groups from the core histones. A reduction in the level of histone acetylation results in chromatin condensation, which obstructs access of the transcription machinery to genes resulting in the repression of transcription. In eukaryotes, an increasing number of HDACs are being identified, implying that different classes might have evolved specialized functions (Khochbin *et al.*, 2001).

Three classes of HDACs have been identified in plants, yeast, and animals (Pandey *et al.*, 2002). Class I and class II HDACs are homologous to yeast RPD3 and HDA1, respectively. In plants, functional studies indicate that RPD3 may provide housekeeping HDAC activity (Tian and Chen, 2001; Wu *et al.*, 2000b). Class III HDACs are related to the yeast silencing protein SIR2 and are dependent on nicotinamide adenine dinucleotide (NAD) for enzymatic activity. In addition to these three classes of HDAC (Kolle *et al.*, 1999; Lechner *et al.*, 2000; Murfett *et al.*, 2001; Rossi *et al.*, 1998; Tian and Chen, 2001; Wu *et al.*, 2000b), plants contain a fourth class of HDAC, namely the HD2 class. It appears to be unrelated to the other classes (Aravind and Koonin, 1998; Dangl *et al.*, 2001; Lusser *et al.*, 1997; Wu *et al.*,

2000a) and shares some sequence similarity with the FKBP (FK506 binding protein)-type PPIases (pepetidylprolyl *cis–trans* isomerases) (Aravind and Koonin, 1998; Yang *et al.*, 2001). In germinating maize embryos, the nucleolar location of HD2 led to a speculation that it could be involved in rDNA chromatin organization and expression (Lusser *et al.*, 1997). In *Arabidopsis*, silencing of *AtHD2A* expression resulted in aborted seed development, suggesting that it is important in reproductive development (Wu *et al.*, 2000a). The intracellular location of HD2 has not yet been reported in *Arabidopsis*; therefore, the possibility that HD2 has assumed different functions in different plants has not been investigated.

Many transcriptional activators are modular, consisting of a DNA-binding domain and an activation domain that interact with components of transcriptional machinery assembling at the promoter (Liu *et al.*, 1999). Fusing combinations of these elements together from different kingdoms has created novel hybrid factors with DNA-binding specificity and activity that is specific for target organisms (Guyer *et al.*, 1998; Moore *et al.*, 1998). In contrast, less is known about the molecular mechanisms and potential applications of transcriptional repressors. Recent studies from animal systems indicate that some transcriptional repressors recruit HDACs to the proximity of the basal transcriptional apparatus (Thiel *et al.*, 2001).

Previously, we have demonstrated that *Arabidopsis* AtHD2A can mediate transcriptional repression when targeted to a reporter gene *in vivo* as a fusion protein with a transcription factor (Wu *et al.*, 2000a). Here, we report that HD2 structural variants, such as *Arabidopsis* AtHD2B and AtHD2C, can also repress gene expression when similarly targeted to a promoter used to drive reporter gene expression. To assess the functional significance, we fused AtHD2A with the plant transcriptional factor, Pti4, that can regulate the expression of *GCC* box-containing genes (Wu *et al.*, 2002; Zhou *et al.*, 1997). We demonstrate that the fusion of AtHD2A and the DNA-binding domain of Pti4 (Pti4DB) can inhibit the expression of the *GCC* box-containing reporter gene in transient gene expression, indicating that AtHD2A could mediate gene repression through interactions with plant transcription factors. Finally, to assess the potential for controlling genes and processes in plants, we demonstrated the repression of a target reporter gene using the GAL4/AtHD2A protein expressed constitutively and in specific organs. Targeting of an HD2 protein to specific promoters using transcription factor DNA-binding domains may provide an alternative method for repressing or silencing target genes or blocks of genes that are co-ordinately regulated by transcription factors. This could provide a method for assessing gene function and allow us to exploit transcription factors to control plant developmental processes.

Results

AtHD2B and AtHD2C can repress gene expression

Previously, we characterized two HD2-like genes, *AtHD2A* and *AtHD2B*, from *Arabidopsis* and showed that HD2A was essential for plant reproduction (Wu *et al.*, 2000a). More recently, two additional genes within this HDAC class, *AtHD2C* and *AtHD2D*, were identified in *Arabidopsis* (Dangl *et al.*, 2001). As shown in Figure 1, the predicted HDAC catalytic residues (Aravind and Koonin, 1998) are conserved in the N-terminal domains of AtHD2A, AtHD2B, AtHD2C, and AtHD2D. They all contain an extended acidic domain, with high sequence homology to nucleolar proteins from several organisms (Lusser *et al.*, 1997). A putative zinc finger is present in the C-terminal domain of AtHD2A and AtHD2C, but not AtHD2B and AtHD2D, thus defining two structural variants of HD2.

We have previously demonstrated that AtHD2A repressed transcription when directed to a promoter containing GAL4-binding sites as a GAL4 fusion protein (Wu *et al.*, 2000a). Here, we compare the repression activities of the two structural variants AtHD2B and AtHD2C with that of AtHD2A. As shown in Figure 2, AtHD2A, AtHD2B, and AtHD2C all strongly repressed GUS gene expression compared with expression of the reporter plasmid alone or in combination with a control effector plasmid that expressed the GAL4 protein alone (Figure 2b). Our results suggest that both AtHD2B and AtHD2C may also be functional in *Arabidopsis.*

Pti4DB/AtHD2A *fusion gene inhibits* GCC *box-mediated gene expression*

Pti4 is a tomato transcription factor that belongs to the ethylene-responsive element binding factor (ERF) family of proteins (Zhou *et al.*, 1997). Previously, we have demonstrated that Pti4 can act as a transcriptional activator to regulate expression of *GCC* box-containing genes and enhance *GCC* box-mediated transcription of a reporter gene (Wu *et al.*, 2002). To test whether AtHD2A can mediate repression of *GCC* box-containing genes by interacting with

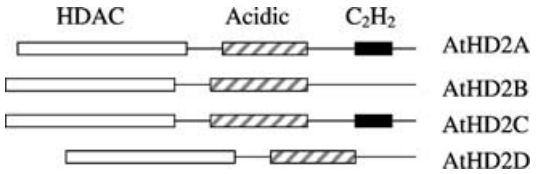

Figure 1. Schematic representation of regions of sequence similarity among *Arabidopsis* HD2-type proteins.
The predicted histone deacetylase catalytic domain (HDAC) is shown as open bars; the acidic region is represented by hatched bars; and the zinc finger (C_2H_2) is denoted by filled bars.

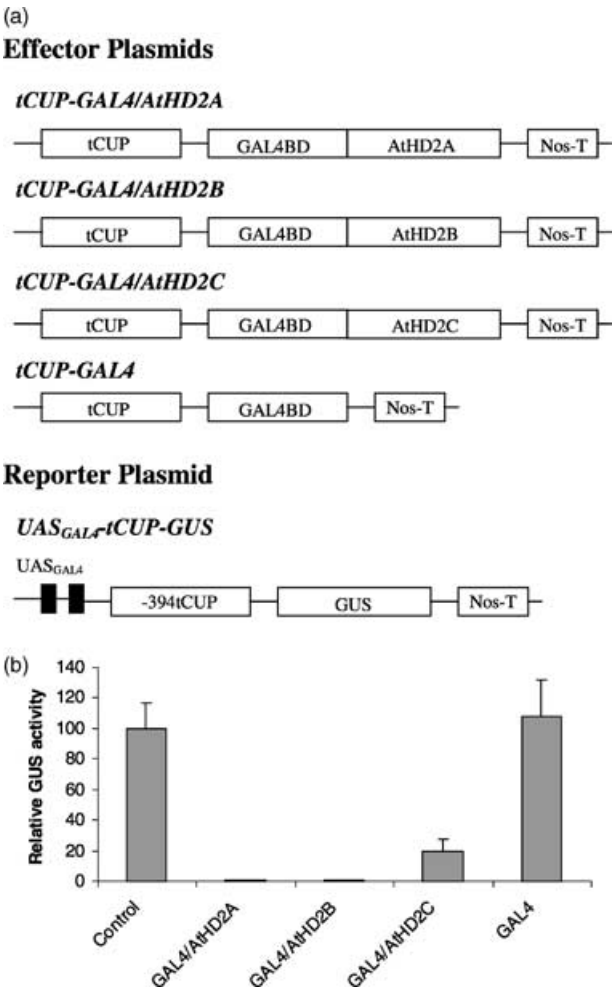

Figure 2. Repression of the UAS_{GAL4}-*tCUP-GUS* fusion gene by *AtHD2A*, *AtHD2B* and *AtHD2C* in transient expression assays.
(a) Schematic diagram of the effector and reporter constructs used in co-bombardment experiments. The reporter construct contains the upstream activating sequence of *GAL4* tandem repeated two times (UAS_{GAL4}) and fused to the *−394tCUP* promoter-*GUS* construct. The effector constructs contain the *GAL4* DNA-binding domain (*GAL4BD*) fused to the full-length *AtHD2A*, *AtHD2B* or *AtHD2C*.
(b) Repression of the UAS_{GAL4}-*tCUP-GUS* fusion gene by AtHD2A, AtHD2B and AtHD2C. The reporter plasmid was co-bombarded with each effector plasmid or the control plasmid *pUC19*. GUS activity was reported as picomoles of 4-methylumbelliferone per milligram of protein per minute. Bars indicate the standard error of three replicates.

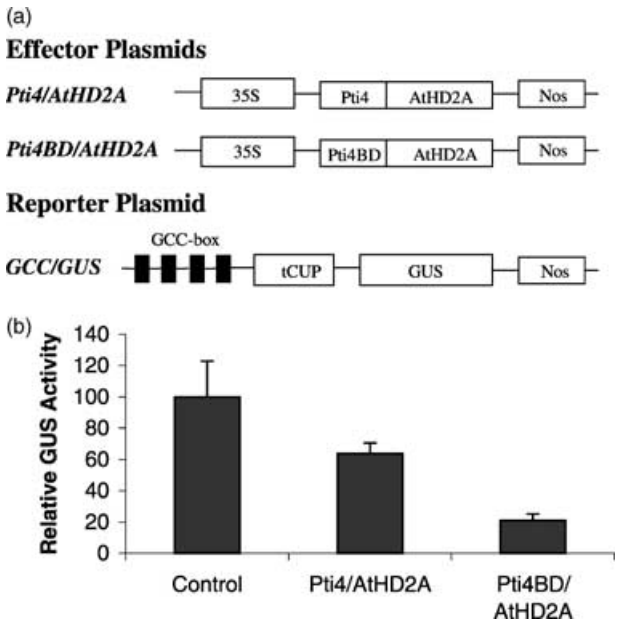

Figure 3. Repression of the GUS gene expression by Pti4/AtHD2A and Pti4BD/AtHD2 protein in transient expression assays.
(a) Schematic diagram of the effector and reporter constructs used in co-bombardment experiments. The reporter construct contains the four *GCC*-box sequences fused to the *394tCUP* promoter-*GUS* construct. The effector constructs, *Pti4/AtHD2A* and *Pti4BD/AtHD2A,* contain Pti4 protein or its DNA-binding domain (Pti4BD) fused with the AtHD2A.
(b) Repression of the GUS reporter gene by *Pti4/AtHD2A* and *Pti4BD/AtHD2A*. The reporter plasmid was co-bombarded with each effector plasmid or the control plasmid *pUC19*. GUS activity was reported as picomoles of 4-methylumbelliferone per milligram of protein per minute. Bars indicate the standard error of three replicates.

Pti4, we constructed *Pti4/AtHD2A* and *Pti4BD/AtHD2A* gene fusions coding for hybrid proteins in which AtHD2A was tethered to the Pti4 protein and its DNA-binding domain (Pti4BD), respectively (Figure 3a). As shown in Figure 3, both *Pti4/AtHD2A* and *Pti4BD/AtHD2A* inhibited the expression of the *GCC* box-containing reporter gene. *Pti4BD/AtHD2A* yielded stronger gene repression activity than *Pti4/AtHD2A*. These data indicate that AtHD2A can mediate gene repression by interacting with plant transcription factors.

A gene expression system using AtHD2A protein

In transgenic plants, we demonstrated the targeting of repression activity to the constitutive *tCUP* promoter using an upstream activating sequence (UAS_{GAL4}) specific for the yeast GAL4 protein as the target sequence (Giniger *et al.*, 1985). This was achieved by driving expression of the GAL4/AtHD2A fusion protein using tissue-specific promoters, such as napin promoter (*NAP2*; Boutilier *et al.*, 1994) to demonstrate unrestricted repression. We hoped to generate a system that would allow gene expression to be deliberately repressed by novel effector molecules and could alter the pattern of repression in a predictable manner.

The UAS_{GAL4}-*tCUP-GUS* reporter construct (Figure 2a) was transformed into *Arabidopsis* to generate reporter lines that are resistant to kanamycin. Thirteen UAS_{GAL4}-*tCUP-GUS* lines (R1–13) were generated and screened for GUS activity by histochemical assay. All of 13 UAS_{GAL4}-*tCUP-GUS* reporter lines showed intense GUS staining.

The effector lines were generated with the *tCUP-GAL4/AtHD2A* (Figure 2a) or *NAP2-GAL4/AtHD2A* genes. In *NAP2-GAL4/AtHD2A*, the napin promoter (*NAP2*) was used to replace the *tCUP* promoter in *tCUP-GAL4/AtHD2A* to drive

expression of the *GAL4/AtHD2A* fusion gene in a seed-specific fashion. A selectable hygromycin-resistance marker gene was used to permit selection of transgenic plants on the medium containing hygromycin. Six *tCUP-GAL4/AtHD2A* effector lines (ET1–6) and six *NAP2-GAL4/AtHD2A* effector lines (EN1–6) were generated. Southern analysis indicated that all the effector lines carried the *GAL4/AtHD2A* gene (data not shown). Northern analysis was carried out to identify the effector lines with strong expression of *GAL4/AtHD2A*.

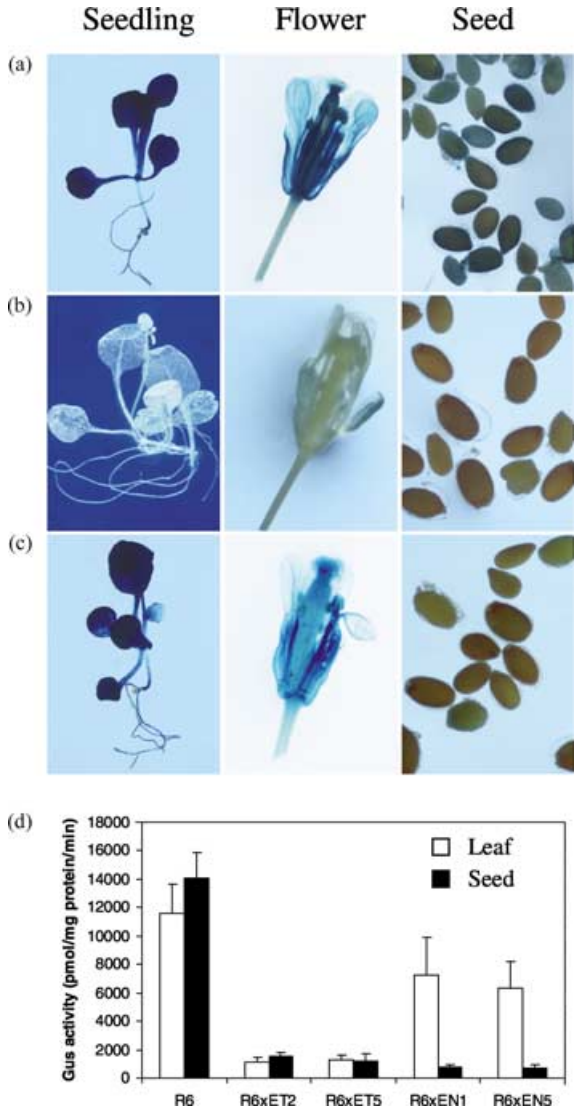

Figure 4. Evidence for the effectiveness of the transrepression system.
(a) Histochemical analysis of GUS expression in the seedlings, flowers, and seeds of the reporter line R6 transformed with *UAS$_{GAL4}$-tCUP-GUS* construct.
(b) GUS expression is repressed in the seedlings, flowers, and seeds of the F_1 progeny of the crossing between the reporter line R6 and the effector line ET2.
(c) GUS expression is repressed in the seeds, but not in the flowers and seedlings of the F_1 progeny of the crossing between the reporter line R6 and the effector line EN1.
(d) GUS expression in the F_1 progeny of the crossing reporter lines and effector lines. GUS-specific activity was analyzed in the leaves and seeds of the reporter line R6, the F_1 progeny of the crossing R6 and ET2 (R6xET2), R6 and ET5 (R6xET5), R6 and EN1 (R6xEN1), R6 and EN5 (R6xEN5). GUS activity was reported as picomoles of 4-methylumbelliferone per milligram of protein per minute. Ten to fifteen plants from each reporter line and cross line were analyzed.

GUS expression is repressed in the F_1 progeny

Two *UAS$_{GAL4}$-tCUP-GUS* reporter lines (R6 and R8) with strong GUS activity were crossed with two *tCUP-GAL4/AtHD2A* effector lines (ET2 and ET5) or two *NAP2-GAL4/AtHD2A* effector lines (EN1 and EN5) that showed strong *GAL4/AtHD2A* expression. F_1 seedlings that were resistant to both kanamycin and hygromycin were selected and revealed that expression of *GAL4/AtHD2A* was able to repress the GUS expression. As shown in Figure 4, GUS expression was repressed in the leaves, flowers, and seeds of F_1 hybrid progeny generated by crossing the reporter line R6 and the effector lines, ET2 and ET5. In contrast, GUS expression was repressed only in the seeds of the F_1 hybrid progeny produced by crossing the reporter line R6 and the effector lines, EN1 and EN5 (Figure 4c,d). Similar data were obtained when we analyzed the F_1 progeny obtained by the crossing of the reporter line R8 and these effector lines (data not shown). These data indicated the potential for deliberately silencing expression of transgenes throughout the plant or altering the specific pattern of transgene expression in transgenic plants.

We used Northern analysis to investigate whether decreased GUS activity was correlated with a decrease in the GUS transcript. As shown in Figure 5, less GUS transcript was detected in the F_1 progeny from crossing the reporter lines (R6 and R8) and the effector lines (ET2 and ET5), confirming that decreased GUS transcript in the F_1 progeny was correlated with strong *GAL4/AtHD2A* expression in these plants. Although the F_2 progeny were not examined, we observed a good correlation between repression of the reporter gene and expression of the *GAL4/HD2A* fusion gene in the F_1 progeny from the crossing of two reporter lines and four different effector lines. Our data suggested

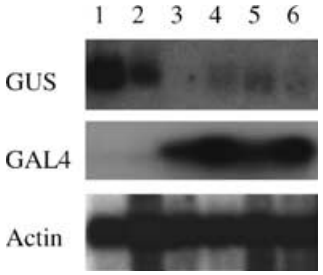

Figure 5. Northern analysis of reporter lines and F_1 progeny.
Total RNA was isolated from the leaves of the reporter lines, R6 (lane 1) and R8 (lane 2), and the F_1 progeny of the crossing R6xET2 (lane 3), R6xET5 (lane 4), R8xET2 (lane 5) and R8xET5 (lane 6). Five micrograms of total RNA was probed with GUS, *GAL4* and a loading control probe (actin).

that this repression was because of specific silencing controlled by the GAL4/AtHD2A protein rather than some general gene silencing mechanism.

Discussion

Eukaryotic HDACs can be grouped into four classes, which are all found in plants (Pandey *et al.*, 2002): (i) *Saccharomyces cerevisiae* RPD3 and RPD3-related proteins; (ii) *S. cerevisiae* HDA1 and the related proteins; (iii) yeast SIR2 and SIR2-related proteins that have NAD-dependent HDAC activities; and (iv) *Zea mays* HD2 and related proteins. HD2 shows no sequence homology to other HDAC families (Aravind and Koonin, 1998). The identification of the novel HD2 in plants suggested that deacetylases could have divergent sequences in their deacetylase domains. We also show that structural variation exists outside of the domain. Like AtHD2A, both AtHD2B and AtHD2C proteins can repress gene expression when targeted to a promoter, indicating a likely role for all the HD2 genes in the repression of gene expression. Although further studies will be needed to determine if they are functionally redundant, studies from our laboratory have revealed that HD2A and HD2B have very similar patterns of expression (Wu *et al.*, 2000a).

By fusing AtHD2A with the plant transcriptional factor Pti4, we have demonstrated that AtHD2A targeted to a *GCC*-box containing promoter will repress expression. It is therefore possible that HD2 proteins could mediate gene repression by interacting with plant transcription factors. This is consistent with the hypothesis that recruitment of HDACs to the proximity of the basal transcriptional apparatus by transcriptional factors occurs in gene repression (Thiel *et al.*, 2001).

It is believed that HDACs play a fundamental role in the repression of genes in plants and may also be linked to gene silencing involving methylation (Finnegan, 2001). Members of both the RPD3 and the HD2 families in plants can repress gene expression (Wu *et al.*, 2000a,b) and are likely to participate in these processes. The demonstration that the plant HDAC genes, combined with the DNA-binding domain of the yeast *GAL4* gene, will target HDAC activity to a promoter containing *GAL4* enhancer sequences has raised possibilities for creating new technologies for deliberately silencing genes or altering their expression pattern in a predictable manner. For instance, synthetic transcription factors that recognize specific target DNA sequences have been developed (Liu *et al.*, 2001; Zhang *et al.*, 2000), which will allow HDACs to be targeted to any selected target in the plant genome. It should therefore be possible to repress specific genes controlling important biochemical or developmental pathways to alter the plants properties. Because we have shown that we can repress transgenes that are expressed stably in plants in both a constitutive and an organ-specific manner, it is feasible to consider the modification of patterns of expression of genes residing in the genome without cloning and alteration of their sequences.

Although the range of targeting systems utilizing plant transcription factor specificities may be extensive, the range of HDAC activities or fragments that may be employed has not been fully explored. The full extent of the possibilities will be revealed once other HDACs have been more fully characterized in plant systems. Previously we have identified three distinct domains in the HD2 family of proteins: the N-terminal predicted deacetylase catalytic domain, the middle region consisting of the extended acidic domain, and the C-terminal domain (Wu *et al.*, 2000a). Deletion of the extended acidic domain and the domain containing predicted catalytic residues of AtHD2A resulted in the loss of gene repression activity, suggesting that both domains are essential for AtHD2A function. Deletion of the C-terminal domain from AtHD2A did not affect gene repression activity, indicating that this domain is not required for gene repression. This was confirmed in the study by comparing the activities of AtHD2B and AtHD2C which differ in the presence of the C-terminal zinc finger domain. We observed reduced plant growth and decreased seed set in the transgenic plants expressing *GAL4/AtHD2A* or *Pti4BD/AtHD2A*, suggesting that fusion proteins with HD2 have deleterious effects when overexpressed in transgenic plants. Truncated HD2 proteins could be used in the effector constructs because the C-terminal fragment of AtHD2A is not required for gene repression (Wu *et al.*, 2000a). It is possible that isolated domains specific for gene repression will be more efficient in the effector constructs than in the full-length HD2 proteins.

Studies on histone deacetylases are essential for understanding the regulation of gene expression in plants and present a substantial opportunity to develop new technologies for crop improvement and agricultural biosafety. We have developed a method for repression of gene expression in transgenic plants by selectively targeting an HD2 protein to specific promoters. Targeting of an HDAC protein to specific promoters using transcription factor DNA-binding domains may provide an alternative method for controlling plant physiology, composition, and development. The technology will be useful for assessing the function of unknown transcription factors by silencing genes at their target sites.

Experimental procedures

Plasmid construction

The *UAS*$_{GAL4}$*-tCUP-GUS* reporter and *tCUP-GAL4/AtHD2A* effector constructs were generated as described (Wu *et al.*, 2000a). To generate the *GCC-tCUP-GUS* reporter construct, we replaced the

35S promoter of pBI221 (Clontech, Palo Alto, CA, USA) with a truncated tCUP promoter, *−394tCUP* (Wu *et al.*, 2001), to generate the pBI-BtCUP vector. A 49 bp fragment (AGCTTAGCCGCCACTAGCCGCCGACCGAGCCGCCAAGAGCCGCCATGCA) containing four *GCC* boxes (Ohme-Takagi and Shinshi, 1995) was ligated into the *Hin*dIII and *Pst*I sites located upstream of the *−394tCUP* promoter.

To construct *tCUP-GAL4/AtHD2B* and *tCUP-GAL4/AtHD2C* plasmids, we replaced the *AtHD2A* gene in *tCUP-GAL4/AtHD2A* (Wu *et al.*, 2000a) with the *AtHD2B* and *AtHD2C* cDNA, respectively. The *NAP2-GAL4/AtHD2A* plasmid was made by replacing the *tCUP* promoter in the *tCUP-GAL4/AtHD2A* with the napin promoter (Boutilier *et al.*, 1994). To construct *Pti4/AtHD2A* and *Pti4BD/AtHD2A* plasmids, we replaced the *GAL4* gene in *tCUP-GAL4/AtHD2A* with the *Pti4* and *Pti4* DNA-binding domain, respectively (Zhou *et al.*, 1997).

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 analyses were 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 (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 *et al.*, 1989). For Northern analysis, total RNA was isolated from 100 to 200 mg *Arabidopsis* leaf tissues using Tri^{TM}Pure reagent as described by the manufacturer (Boehringer Mannheim, Mannheim, Germany). Northern blots were prepared by electrophoresis of 5–10 μg samples of total RNA through agarose gel 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, 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) and 1 mM EDTA, 0.05% SDS at 100°C for 2 min when re-probing was required. As controls, all Northern blots were also probed with an *Arabidopsis* actin cDNA.

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 three times 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 (Shaw, 1995). The *Agrobacterium*-mediated transformation of *A. thaliana* was performed as described (Clough and Bent, 1998). T_1 seeds were harvested, dried at 25°C, and germinated in a sterile medium containing 40 μg ml^{-1} kanamycin or hygromycin to select the transformants. Surviving T_1 plantlets were transferred to soil to set seeds (T_2).

GUS assays

To assay GUS activity of transgenic plants, the leaves and seeds from 4-week-old transgenic plants were harvested and frozen in liquid nitrogen. Leaf or seed tissue, 100–200 mg, was ground in an extraction buffer consisting of 50 mM sodium phosphate (pH 7.0), 10 mM 1,2-diaminocyclohexane-*N*,*N*,*N*,*N*-tetraacetic acid, 0.1% Triton X-100, and 10 mM 2-mercaptoethanol and 0.1% sodium lauryl sarcosine. The extract was sonicated two times for 20 sec in a bath-type sonicator and centrifuged for 15 min (4°C), and then the supernatant was used for GUS assays.

For fluorometric GUS assays (Jefferson *et al.*, 1987), 50 μl of the crude extract from leaf or seed tissues was incubated at 37°C with 1 mM 4-methylumbelliferyl glucuronide in 0.3 ml of GUS assay buffer (50 mM NaH_2PO_4 (pH 7.0), 10 mM EDTA, 0.1% (v/v) Triton X-100, and 10 mM β-mercaptoethanol). After 0, 0.5, 1, and 2 h of incubation, 0.1 ml aliquots were removed and added to 1.9 ml of 0.2 M Na_2CO_3 to terminate the reaction. GUS activity was expressed as picomoles of 4-methylumbelliferone per milligram of protein per minute.

For GUS histochemical assay of *Arabidopsis* plants, tissue samples were taken from both seedlings and mature plants. The GUS histochemical staining and chlorophyll removal was performed according to Jefferson *et al.* (1987).

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 a 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, B_5 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 in this medium for 1 day prior to gene delivery.

Plasmid DNA was isolated using the Qiagen Plasmid Midi Kit. 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 24 h after bombardment; GUS gene expression was determined by fluorometric assays (Jefferson *et al.*, 1987). GUS activity was measured as picomoles of 4-methylumbelliferone per milligram of protein per minute.

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