ARABIDOPSIS THALIANA HISTONE DEACETYLASE 1 (ATHD1) AND EPIGENETIC REGULATION

A Dissertation

by

LU TIAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Genetics

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ABSTRACT

Arabidopsis thaliana Histone Deacetylase 1 (AtHD1) and Epigenetic Regulation.

(May 2004)

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Epigenetic regulation is a mechanism by which heritable changes in gene expression are controlled by chromatin status rather than primary DNA sequence. Changes in chromatin structure affect accessibility of DNA elements to the transcriptional machinery and thus affect transcription activity of the gene. A key event in this process is reversible modification of core histones, which is catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDs, HDAs, or HDACs). In general, histone deacetylation is related to transcriptional gene silencing, whereas acetylation is associated with gene activation.

To study the role of histone deacetylase in plant gene regulation and development, we generated constitutive antisense histone deacetylase 1 (CASH) transgenic plants. AtHD1 is a homolog of RPD3 protein, a global transcriptional regulator in yeast. Expression of the antisense *AtHD1* caused dramatic reduction in endogenous *AtHD1* transcription, resulting in accumulation of acetylated histones. Down-regulation of histone deacetylation caused a variety of growth and developmental abnormalities and ectopic expression of tissue-specific genes. However, changes in

genomic DNA methylation were not detected in repetitive DNA sequences in the transgenic plants.

We also identified a T-DNA insertion line in exon 2 of *AtHD1* gene (*athd1-t1*), resulting in a null allele at the locus. The complete inhibition of the *AtHD1* expression induced growth and developmental defects similar to those of CASH transgenic plants. The phenotypic abnormalities were heritable across the generations in the mutants. When the *athd1-t1/athd1-t1* plants were crossed to wild-type plants, the mutant phenotype was corrected in the F_1 hybrids, which correlated with the *AtHD1* expression and reduction of histone H4 Lys12 acetylation.

Microarray analysis was applied to determine genome-wide changes in transcriptional profiles in the *athd1-t1* mutant. Approximately 6.7% (1,753) of the genes were differentially expressed in leaves between the wild-type (Ws) and the *athd1-t1* mutant, whereas 4.8% (1,263) of the genes were up- or down-regulated in flower buds of the mutant. These affected genes were randomly distributed across five chromosomes of *Arabidopsis* and represented a wide range of biological functions. Chromatin immunoprecipitation assays indicated that the activation for a subset of genes was directly associated with changes in acetylation profiles.

DEDICATION

To my parents, Qiyun Tian and Xiaolan Mao, for their endless love throughout my life. Although we are now ten thousand miles away, their support and encouragement have been always with me.

To my husband, Jiyuan Wang, for his love, understanding, and support.

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CHAPTER I

INTRODUCTION

During the past decade, the genomes from a number of organisms, including yeast (Mewes et al., 1997), *C. elegans* (Chalfie, 1998), *Drosophila* (Hodgkin, 2000), *Arabidopsis* (Arabidopsis Genome Initiative, 2000), and human (Baltimore, 2001), have been sequenced and annotated. We are able to identify every gene in these genomes and address fundamental questions in a genome-wide scale. However, this large volume of genetic information also generates many challenges, one of which is to decipher the regulatory mechanisms that allow certain genes to be turned "on" or "off" during different developmental stages or in responses to environmental stimuli (Zaret and Wolffe, 2001). Knowing the linear arrangement of DNA sequence is far from resolving this challenge. Eukaryotes have evolved molecular means to compact DNA into higher-order chromatin fibers to constrain the genome within the limited volume of the cell nucleus, but also permit dynamic changes in chromatin structures. More and more studies have provided the evidence to support the notion that beyond promoters, enhancers, and other cis-regulatory sequences, gene regulation involving chromatin remodeling or epigenetic modifications plays a key role in transcriptional switches.

This dissertation follows the format of The Plant Cell.

DNA methylation and histone modifications, together with chromatin remodeling, create epigenetic landmarks that differentiate between active and inactive chromatin structures (Nakao, 2001). Histone acetylation and deacetylation are reversible modifications on amino termini of core histones, which are catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDs, HDAs, HDACs). Histone acetylation is associated with gene activation, while deacetylation is correlated with gene repression. This study is focused on elucidating the role of histone acetylation and deacetylation in plant gene regulation and development, using *Arabidopsis thaliana* histone deacetylase 1 gene.

EPIGENETICS AND CHROMATIN REMODELING

Epigenetic processes appear to be involved in a wide range of biological functions, such as gene expression (Martienssen and Henikoff, 1999; Ohlsson et al., 2001), genomic imprinting (Joyce et al., 1997; Reik and Murrell, 2000), X-chromosome inactivation (Lyon, 1993), development (Ronemus et al., 1996; Tate et al., 1996; Wu et al., 2000a; Wu et al., 2000b; Tian and Chen, 2001), paramutation (Stam et al., 2002), transposon regulation (Singer et al., 2001), and disease resistance (Stokes et al., 2002).

As defined, epigenetics is the study of "heritable changes in gene expression that occur without a change in DNA sequence" (Wolffe and Matzke, 1999). Unlike conventional genetic regulation, epigenetic regulation provides a mechanism of gene control by which the status of gene transcription is determined by how DNA is packaged into chromatin rather than its primary nucleotide sequence and can persist for one or more generations. It now becomes evident that chromatin remodeling mechanistically

contributes to epigenetic switches and maintenance of epigenetic states (Newell-Price et al., 2000).

Chromatin basics

In eukaryotes, DNA is tightly compacted into highly ordered structures known as chromatin (Figure 1.1), which is composed of structural subunits called nucleosomes (Kornberg, 1977). Each nucleosome consists of 145-147 base pairs of DNA that wrap around an octamer containing two molecules of each of the four core histone proteins (H2A, H2B, H3 and H4) (Luger et al., 1997). Further compaction is achieved by association of histone H1 with linker DNA, leading to the formation of 30-nm fiber (Thomas, 1984). Compaction above the level of the 30-nm fiber is generally termed higher-order chromatin. When observed by transmission electron microcopy, chromatin appeared to be ranged from 100- to 300-nm in width along mitotic chromosomes (Belmont et al., 1987) versus 100-nm in width along interphase chromosomes (Belmont et al., 1989).

In addition to histones, chromatin contains other important protein components, such as high mobility group (HMG) proteins, DNA topoisomerase II, and chromatin remodeling factors (Agresti and Bianchi, 2003; Earnshaw et al., 1985). These non-histone proteins not only contribute to the higher-order levels of chromatin packaging and chromosome integrity, but also play important roles in modifying chromatin structures and gene regulation (Goodrich and Tweedie, 2002).

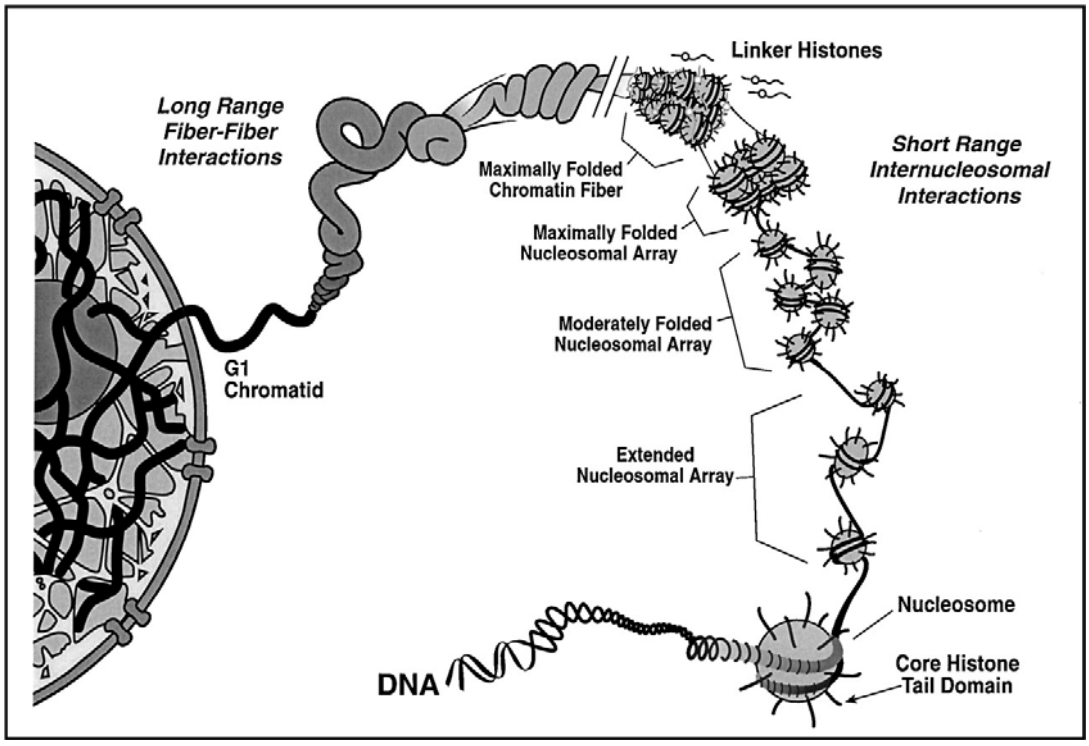

Figure 1.1. Multiple levels of chromatin fiber condensation.

DNA is packaged into chromatin by association with histones and other chromatin-associated proteins as well as some RNA species that can be subdivided into different levels of structure. Strings of nucleosomes compose the primary structural units of chromatin. Formation of 30-nm fibers through histone tail-mediated nucleosome-nucleosome interactions provides a secondary level of compaction, whereas tail-mediated association of individual fibers produces tertiary structures (such as chromonema fibers). This figure is adapted from figure 1 of Hansen (2002).

It is increasingly recognized that RNA species are also important components of chromatin. For instance, both mammalian X chromosome inactivation and *Drosophila* dosage compensation require large RNA molecules (Anderson and Panning, 2003; Wutz, 2003). These RNAs associate with chromatin over the whole chromosomes and are crucial for spreading changes in chromatin structure.

The compaction enables the genomic DNA to be constricted into the limited space of the nucleus; however, compaction of DNA into chromatin in nature also greatly impedes gene transcription (Grunstein, 1990).

Chromatin remodeling

A region of chromatin may be packaged into euchromatin that is associated with transcriptional activity. Alternatively, chromatin can be tightly packaged to form heterochromatin, which restricts the assembly of transcription factors at the promoter region of certain genes. However, the chromatin is a dynamic structure that can be altered by various epigenetic modulators (Figure 1.2), such as DNA methylation, covalent histone modifications and nucleosome repositioning in responses to cellular and environmental changes (Vermaak et al., 2003). It suggests that chromatin remodeling plays a crucial role in controlling gene transcription to ensure correct temporal and spatial development in eukaryotic organisms (Struhl, 1999). Chromatin remodeling is also critical for chromosome condensation and segregation during cell division ensuring genome integrity (Peters et al., 2001).

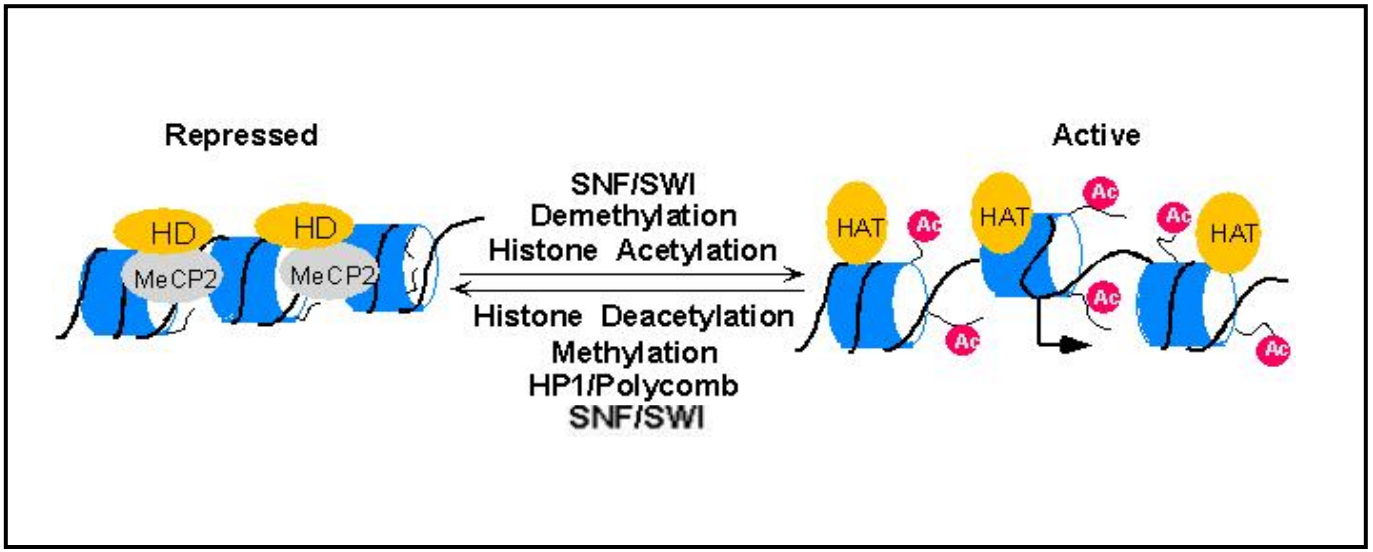

Figure 1.2. DNA methylation, histone modification and chromatin remodeling play key roles in epigenetic regulation.

DNA hypermethylation and histone deacetylation are associated with gene silencing, while DNA hypomethylation and histone acetylation is related to gene activation. SNF/SWI chromatin remodeling complexes are associated with different chromatin factors, thus to either repress or activate transcription.

A variety of regulatory proteins, including DNA methyltransferases, methyl-CpG binding proteins, histone-modifying enzymes, chromatin remodeling factors, and their multimolecular complexes are responsible for creating certain chromatin states, thus selectively activating or silencing gene transcription in eukaryotic cells (Nakao, 2001).

DNA methylation DNA methylation refers to the covalent addition of methyl groups to the 5' position of cytosine residues. The fact that DNA methylation is involved in epigenetic regulation has been extensively studied over a number of years, from plants to mammalian systems (Martienssen and Colot, 2001; Reik and Dean, 2001). Typically, the region of genomic DNA that is heavily methylated is packaged into heterochromatin and therefore associated with gene silencing (Eden and Cedar, 1994).

In plants, DNA can undergo both symmetric (CpG and CpNpG) and asymmetric DNA methylation (Lusser, 2002). Both *de novo* and maintenance methyltransferases have been described (Finnegan and Kovac, 2000). DOMAINS-REARRANGED METHYLASE 1 (DRM1) and DRM2 were identified as *de novo* methyltransferases in genome defense (Cao and Jacobsen, 2002a). CMT3 and MET1 are maintenance methyltransferases for CpNpG and CpG methylation, respectively (Kishimoto et al., 2001; Cao and Jacobsen, 2002b). Recent studies indicated that CpNpG and CpG methylation may operate in a partially redundant way to silence most plant genes (Lindroth et al., 2001). CMT3 was also shown to be responsible for asymmetric DNA methylation in both *Arabidopsis* (Lindroth et al., 2001) and maize (Papa et al., 2001).

In mammals, DNA methylation predominantly occurs within CpG dinucleotides. Three distinct types of methyltransferases, Dnmt1, Dnmt3a, and Dnmt3b, have been functionally characterized (Bestor, 2000). Dnmt1 is thought to be primarily involved in maintenance; while Dnmt3a and Dnmt3b are responsible for establishment of genomic methylation patterns (Bestor, 2000).

Methylated DNA appears to recruit methyl-DNA binding proteins, which in turn recruit histone-modifying enzymes and chromatin-remodeling factors for heterochromatin formation (Lusser, 2002). DECREASE IN DNA METHYLATION 1 (DDM1) is a member of SWI2/SNF2-type ATPases (Jeddeloh et al., 1999). It is not a DNA methyltransferase (Kakutani et al., 1995), but global methylation levels were reduced in the *ddm1* mutant (Bourc'his and Bestor, 2002). These studies have implicated the mechanistic link between chromatin remodeling and DNA methylation in both plants and animals.

DNA methylation is a stable epigenetic mark, since it can be maintained after each round of DNA replication by maintenance DNA methyltransferases. DNA demethylase has yet to be identified (Wolffe et al., 1999; Kress et al., 2001).

Histone modifications The N-terminal tail of histones is subjected to various post-translational modifications, including acetylation/deacetylation, methylation, phosphorylation, and ubiquitination (Strahl and Allis, 2000). Recent studies have suggested that histone modifications are extensively involved in epigenetic regulation, which will be discussed in detail in the next session.

ATP-dependent remodeling complexes ATP-dependent chromatin remodeling complexes use energy from ATP hydrolysis to remodel chromatin structures by changing the location or conformation of the nucleosomes (Havas et al., 2000; Gavin et al., 2001). Several underlying remodeling mechanisms have been implicated from recent studies. In the ATP-consuming reaction, chromatin remodeling factors can induce the "sliding" of the intact histone octamers to the adjacent DNA segments (Jaskelioff et al., 2000), or disrupt nucleosomes in a way that leads to histone octamers transfer to a separate segment of DNA (histone eviction) (Phelan et al., 2000). These changes alleviate the histone-DNA interaction and consequently the DNA is more accessible to transcription factors and other proteins (Gavin et al., 2001). Consistent with these activities, chromatin remodeling complexes are involved in both activation and repression of transcription (Narlikar et al., 2002).

All chromatin remodeling complexes contain an ATPase subunit that is related to the SWI2/SNF2 subfamily of the DEAD/H box supermaily of nucleic acid-stimulated ATPases (Eisen et al., 1995). These complexes can be further divided into three main classes based on whether the identity of their catalytic ATPase subunit is more related to yeast SWI2/SNF2 (SWITCH/SUCROSE NON FERMENTING) family, *Drosophila* ISWI (IMITATION SWITCH) family, or human Mi-2 family (Muchardt and Yaniv, 1999; Fry and Peterson, 2001).

The engine of chromatin remodeling complexes is an ATPase subunit, but their remodeling action is strongly associated with other protein factors (Varga-Weisz, 2001). It has been noted that ATP-dependent chromatin remodeling factors cooperate with

histone modifying enzymes such as histone acetyltransferases (HATs) and deacetylases (HDACs) to activate (Dilworth et al., 2000) and repress (Knoepfler and Eisenman, 1999) transcription, respectively. As mentioned above, chromatin remodeling complexes are also involved in DNA methylation and heterochromatin formation (Jeddeloh et al., 1999; Geiman et al., 2001). These studies indicate that these epigenetic modulators are interconnected with each other to control gene expression in a complex epigenetic regulatory network (Geiman and Robertson, 2002).

SWI2/SNF2 genes have been also isolated from plants. In the *Arabidopsis* genome, more than 40 putative SNF2-like genes have been identified (Reyes et al., 2002), although functions were characterized for only a few, namely PICKLE (PKL), DDM1, MOM and SPLAYED (SYD) (Reyes et al., 2002). Mutations in these genes resulted in various phenotypic abnormalities during different developmental stages (Reyes et al., 2002).

HISTONE CODE

Histones are basic proteins that consist of a globular domain and an N-terminal tail that protrudes from the nucleosomes. In contrast to the globular domains, the N-terminal histone tails emerging from the nucleosomes are unstructured and are not essential for maintaining the integrity of nucleosomes (Ausio et al., 1989). Instead, the N-terminal tails are thought to be responsible for making secondary and more flexible contacts with DNA and adjacent nucleosomes (Luger et al., 1997). One way for histones

to be involved in alteration of chromatin structure is achieved by being subjected to various covalent post-translational modifications, including acetylation, phosphorylation, methylation, and ubiquitination (Spotswood and Turner, 2002). Potential sites of post-translational modification on nucleosomal histones are shown in Figure 1.3. These modifications coined the term "histone code" (Jenuwein and Allis, 2001). It has been proposed that the modified histone tails provide binding sites for chromatin-associated proteins, which in turn induce alterations in chromatin structure and thereby lead to downstream transcriptional regulation (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Like the DNA code, the 'histone code' is heritable and can be translated into biological functions (Jenuwein and Allis, 2001).

Histone acetylation/deacetylation

Histone acetylation/deacetylation is the most extensively studied histone modification. Almost four decades ago, it was found that core histones could be acetylated or deacetylated by histone acetyltransferases (HATs) and histone deacetylases (HDs, HDAs, or HDACs) (Allfrey et al., 1964). There is a general correlation between acetylation and gene activity (Wade et al., 1997).

Histone acetylation occurs prior to histone incorporation into chromatin and is catalyzed by B-type histone acetyltransferases (HATs) (Verreault et al., 1998; Imhof and Wolffe, 1999). The A-type HATs are responsible for acetylation of chromosomal histones and thus are directly involved in regulating gene transcription (Carrozza et al., 2003). Histone H4 is post-translationally acetylated at lysines 5 and 12 by B-type HATs

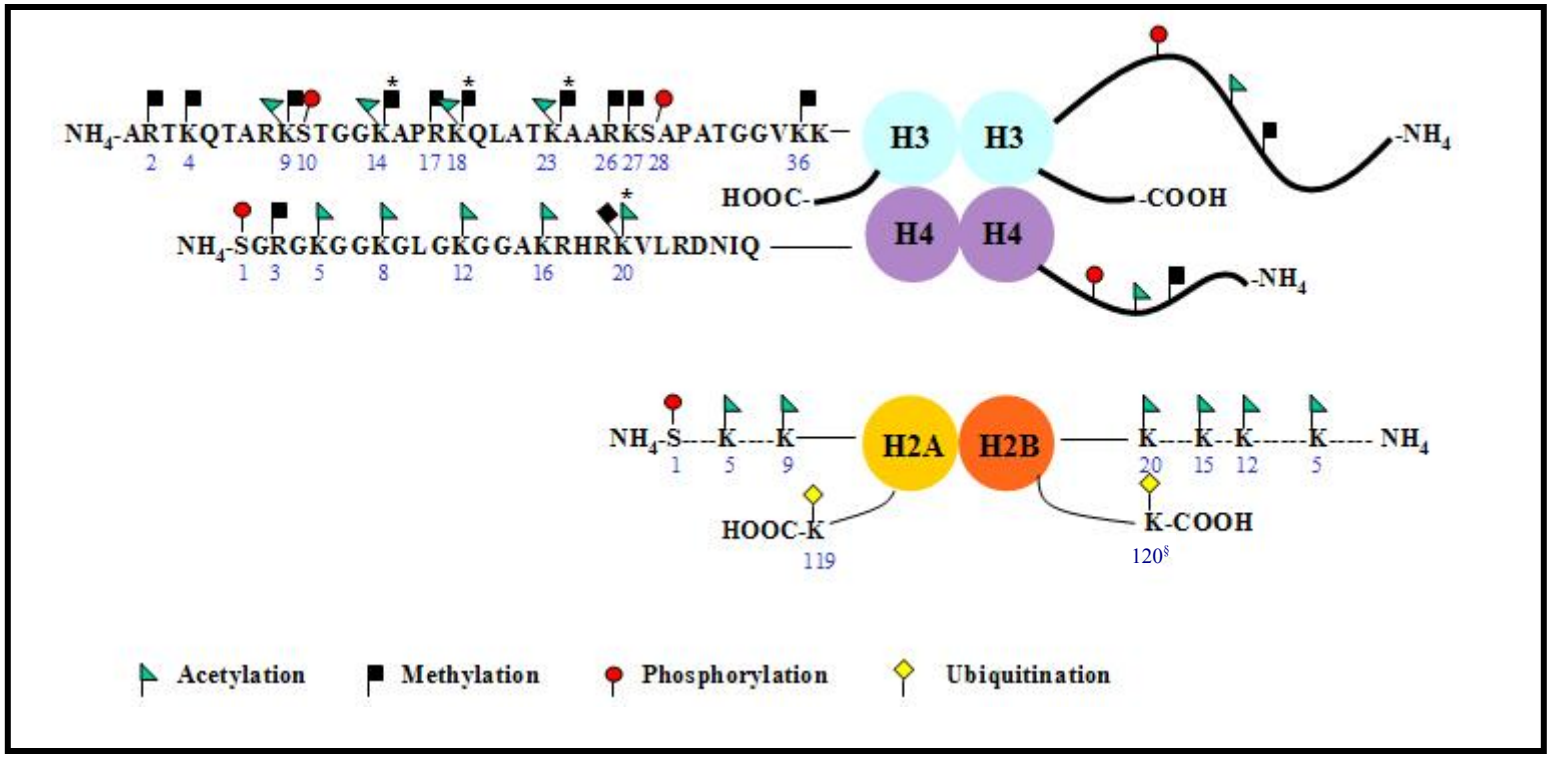

Figure 1.3. A summary of covalent post-translational modification sites on histones.

* Modifications that have been determined for plant histones only.
§ Lysine 123 of H2B is ubiquitinated in yeast, whereas in mammals lysine 120 is ubiquitinated.

Each nucleosome is formed by wrapping approximately 146 base pairs of DNA around a histone core particle, which consists of two H2A-H2B dimers and one $(H3\text{-}H4)_2$ tetramer. The modification in the N-terminal tails of histones includes acetylation on lysine residues, methylation on both lysine and arginine residues and phosphorylation on serine residues (Davie and Chadee, 1998; Jason et al., 2002; Lusser, 2002). Exceptionally, ubiquitination occurs on lysine residues of the C-terminal tails of H2A and H2B.

in cytoplasm (Verreault et al., 1998; Imhof and Wolffe, 1999). However, in heterochromatic region, the acetyl groups are removed approximately 20 minutes after DNA synthesis is completed, while in euchromatic region, histone H4 is additionally acetylated at lysines 8 and 16 (Verreault et al., 1998; Imhof and Wolffe, 1999). Therefore, acetylated histones can be recognized as a hallmark to distinguish euchromatin from heterochomatin.

Several mechanisms for histone acetylation/deacetylation associated with gene transcription have been suggested. First, histone acetylation occurs post-translationally and reversibly on the ε-NH_3^+ groups of highly conserved lysine residues in the N-terminal tails of core histones (Kuo and Allis, 1998). The acetyl group from acetyl coenzyme A is transferred to the ε-NH_3^+ groups of lysine residues by histone acetyltransferases (HATs). Histone hyperacetylation is thought to weaken DNA-histone contacts by neutralizing the positive charge of the histone tails and decreasing their affinity for negatively charged DNA (Kuo and Allis, 1998). Therefore, acetylation of the histones appears to relax the chromatin structure and be associated with gene activation. Conversely, the deacetylation process involves the removal of acetyl groups by histone deacetylases from specific lysine residues of core histones, thereby restoring positive charges on lysine residues and increasing the interactions between DNA and histones (Kuo and Allis, 1998), which further impedes accessibility of the promoter to the transcriptional machinery (Pazin and Kadonaga, 1997). Thus, hypoacetylated chromatin appears to be associated with gene silencing. Recent studies also indicate that histone acetylation might also promote transcription by preventing the folding of

nucleosomal arrays into more complex structures (Tse et al., 1998). Therefore, histone acetylation and deacetylation can regulate transcription by directly affecting higher-order structure of chromatin fibers (Horn and Peterson, 2002). Besides, acetylation and deacetylation were also implicated in gene regulation and euchromatin/heterochromatin formation by providing specific binding surfaces for the recruitment of activators and repressors. It has been shown that a number of histone acetyltransferases are associated with transcriptional activators, whereas histone deacetylases are in the repressor complexes in yeast and mammalian cells, suggesting that the enzymatic modulation of histone acetylation is an integral component of transcription regulation (Pazin and Kadonaga, 1997). For example, H3 hypoacetylation and deacetylation of H4-K16 are essential for Sir3 binding in the heterochromatic regions in yeast (Carmen et al., 2002; Kristjuhan et al., 2003). Transcription activators SRC-1 (Onate et al., 1995), p300 (Lundblad et al., 1995), ACTR (Chen et al., 1997), and PCAF (Krumm et al., 1998) have been shown to be associated with histone acetyltransferase activities, while repressors Sin3 (Heinzel et al., 1997), pRB (Brehm et al., 1998; Luo et al., 1998), YY1 (Yang et al., 1996), and NcoR (Alland et al., 1997) are associated with HDACs.

It has been demonstrated that acetylation of H3 and H4 within promoter regions is associated with gene expression in different species, including yeast, human and plants (Kuo et al., 1998; Krebs et al., 1999; Chua et al., 2001). However, it is not restricted to the promoter regions of active genes (Madisen et al., 1998; Crane-Robinson et al., 1999). Coding-region-specific acetylation and deacetylation events also occur,

which indicates that histone acetylation/deacetylation is not only involved in transcription initiation, but also plays a role in transcription elongation.

Histone acetylation is not always associated with activation and deacetylation does not necessarily lead to repression. At least one yeast histone deacetylase, Hos2p, is directly associated with gene activities rather than repression (Wang et al., 2002). One possible explanation is that Hos2p might help to restore the disrupted chromatin to its original permissive state and prepare for the next round of transcription (Wang et al., 2002). Histone deacetylase families exist in different organisms, including plants. Although different family members might act redundantly, it is more likely that each member has a distinct function. Except H2A, the core histones are acetylated at four to five lysine sites in the N-terminal tails (Figure 1.3). In particular, the highly conserved lysines 9, 14, 18 and 23 of H3 and lysines 5, 8, 12 and 16 of H4 are strongly associated with transcriptional activity (Grunstein, 1990). However, lysine 12 of H4 is preferentially acetylated within β-heterochromatin in *Drosophila* and in heterochromatin-like silent loci in yeast (Turner, 1991; Braunstein et al., 1996). Therefore, histone hyperacetylation is not always related with gene activation, which also suggests that different acetylated forms of histones and/or combinations with other histone modifications might serve as a signal for downstream regulation.

Histone acetylation/deacetylation is a conserved mechanism for transcription regulation. Histone acetyltransferases (HATs), including Hat1, Gcn5, MYST and CREB-binding protein (CBP)/p300 superfamilies, have counterparts in almost every eukaryote, including plants (Sterner and Berger, 2000; Pandey et al., 2002). HATs act

similarly in plants, yeast and mammals. For instance, *Arabidopsis* Gcn5 homolog (AtGcn5) was shown to interact with the *Arabidopsis* transcriptional adaptor proteins ADA2a and ADA2b *in vitro* (Stockinger et al., 2001). The *Arabiodopsis* transcriptional activator CBF then functions through the action of GCN5 and ADA2, to stimulate the transcription of cold-regulated genes. Recently, four *Arabidopsis* CBP homologs were identified, and at least one, PCAT2, possesses HAT activity (Bordoli et al., 2001). Besides RPD3-, HDA1- and SIR2-related histone deacetylases identified in yeasts and mammalian systems, plants also have a unique histone deacetylase family – HD2 family (Lusser et al., 1997). It is localized in nucleolus, implying that it might play a role in regulating ribosomal chromatin structure (Lusser et al., 1997). Five lysine residues (lysines 5, 8, 12, 16, 20) of H4 can be acetylated in plants (Lusser et al., 2001), while in animals and yeast, lysine 20 of H4 is not acetylated, but is methylated (Waterborg, 1992; Strahl et al., 1999).

Blocking histone deacetylase activity using chemical inhibitors for histone deacetylases can reverse the process and result in the activation of silenced rRNA genes subjected to nucleolar dominance (Chen and Pikaard, 1997). Therefore, chromatin structure could be reversibly modulated to activate or silence transcription by targeting histone acetyltransferase or deacetylase to a particular gene. The characteristics and functionalities of histone deacetylases will be further discussed later in the introduction.

Histone methylation

Although histone acetylation has been actively investigated for a number of years, histone methylation and the enzymes that catalyze this process were not studied until recently. The human chromatin protein SU(VAR)3-9 is the first identified histone methyltransferase that specifically catalyzes H3-lys9 methylation (Rea et al., 2000). The SET domain was found to be the catalytic domain of SU(VAR)3-9 (Rea et al., 2000), which is conserved in almost all the identified histone lysine methyltransferases (HKMT) (Zhang and Reinberg, 2001). Several other histone lysine methyltransferases have been characterized (Zhang and Reinberg, 2001), which are responsible for methylation at different lysine residues of histones. The lysines 4, 9, 27, 36 of H3 and lysine 20 of H4 can be mono-, di- or trimethylated (Figure 1.3). Additional methylation of lysines 14, 18, and 23 only occurs in plants (Figure 1.3). Different methylation forms are associated with either gene activation or repression. H3-K9 methylation is primarily related to heterochromatin (Lachner et al., 2001; Noma et al., 2001), whereas H3-K4 is present in transcriptionally active regions (Strahl et al., 1999; Noma et al., 2001; Nagy et al., 2002). However, methylation at H3-K4 and H3-K79 at the same time is required for silencing of gene expression near telomeric region (Krogan et al., 2003). The exact state of methylation (i.e., mono-, di- or tri-methylation) of a single lysine residue has an impact on physiological processes. For instance, dimethylation of H3-K4 occurs at both inactive and active euchromatic genes, whereas tri-methylation is present exclusively at active genes (Santos-Rosa et al., 2002). Besides, arginine residues in histone H3 and H4 can also be methylated (Davie and Dent, 2002), which is correlated with gene activation.

In the *Arabidopsis* genome, 29 SET-domain proteins were identified by a phylogenetic study (Baumbusch et al., 2001), of which KRYPTONITE (KYP) became the first H3-K9 specific histone methyltransferase to be functionally characterized in plants (Jackson et al., 2002). Methylation of H3-K9 recruits LHP1 (LIKE HETEROCHROMATIN 1), which in turn recruits the DNA methyltransferase CHROMOMETHYLTRANSFERASE (CMT3) (Jackson et al., 2002), indicating a connection between DNA methylation and histone methylation.

Histone acetylation and phosphorylation are highly dynamic processes with rapid turnover rates (Taunton et al., 1996; Hsu et al., 2000); however, histone methylation appears to be a rather static process (Jenuwein and Allis, 2001). Therefore, histone methylation is generally recognized as a stable epigenetic mark.

Histone phosphorylation

Histone phosphorylation at serine 10 of H3 has been thought to be an important modification. Chromosome condensation and transcription represent two opposite chromatin states, but H3-Ser10 phosphorylation is involved in both processes (Cheung et al., 2000; Nowak and Corces, 2000), which supports the idea that histone modification functions through providing specific "binding surfaces" to recruit other protein effectors (Jenuwein and Allis, 2001). H3-Ser10 phosphorylation does not appear to be associated with chromosome condensation in maize, suggesting that the histone code is not necessarily conserved among species (Kaszas and Cande, 2000). Although H3-Ser10 phosphorylation might have an important role in sister chromatin cohesion during

meiosis (Kaszas and Cande, 2000), its role in transcriptional regulation in plants has not been demonstrated.

The addition of negatively charged phosphate groups to histone tails neutralizes basic charges of histones and is thought to reduce their affinity for DNA. Furthermore, it was also shown that phosphorylation on Ser10 facilitates acetylation of H3-K9 and /or –K14 (Cheung et al., 2000; Clayton et al., 2000). Ser10 phosphorylation is suppressed if methylation occurs first (Rea et al., 2000). Therefore, phosphorylation may contribute to transcriptional activation through the stimulation of histone acetyltransferase activity on the same histone tail.

Histone ubiquitination

Histone ubiquitination is the least-characterized post-translational histone modification. Most histone modifications occur in the N-terminal tails, however, histone H2A and H2B are usually ubiquitinated at lysine residues in the C-terminal tails (Goldknopf and Busch, 1977, 1980; Thorne et al., 1987; Davie and Murphy, 1990). Generally, ubiquitination represents a mark on proteins that identifies them for degradation (Ciechanover et al., 2000). However, ubiquitination of histones was implicated in activation of gene expression (Davie et al., 1991; Strahl and Allis, 2000). For instance, ubiquitination of H2A was shown to be especially enriched at the 5'-end of transcriptionally active genes (Varshavsky et al., 1982). H2B is ubiquitinated when the chromatin structure is "open" during transcription (Davie and Murphy, 1990). These

studies indicate that ubiquitination of histones may impede nucleosome folding and thus facilitate transcription progress.

Intriguingly, ubiquitination of histone H2B at lysine 123 has been shown to be the prerequisite for methylation of H3-K4 and H3-K79 in yeast, which in turn regulates gene silencing at telomeric regions. This suggests that gene regulation is controlled through concerted histone modifications (Krogan et al., 2003).

HISTONE DEACETYLASES

Through genetic screens for transcriptional repressors in *S. cerevisiae*, RPD3 was first identified as a positive and negative transcriptional regulator for a subset of yeast genes (Vidal and Gaber, 1991). However, the link between histone deacetylation and transcriptional repression did not become apparent, until a mammalian histone deacetylase, HDAC1 was purified based on its affinity for the histone deacetylase inhibitor, trapoxin. HDAC1 was found to be a homolog of yeast RPD3 protein (Taunton et al., 1996). Since then, a number of histone deacetylase proteins from various species have been purified and analyzed, implying that they are responsible for different functions (Khochbin et al., 2001). Based on size, sequence characteristics and distinct association with different transcription co-factors, three distinct classes of histone deacetylases (HDACs) related to the yeast proteins – reduced potassium dependency protein 3 (RPD3), histone deacetylase 1 (HDA1) and silent information regulator protein 2 (SIR2) - have been identified in yeast, plant and animal systems (Table 1.1). Recent studies have shown that these three classes of HDACs are involved in distinct biological

Table 1.1. Summary of HDAC and HAT homologs found in yeast, plants and animals (Marmorstein, 2001; Pandey et al., 2002; Verdin et al., 2003). Abbreviations: y = yeast, At = *Arabidopsis thaliana*, Hs = *Homo sapiens*

HDAC families	Yeast	Plants* (At)	Animals (Hs)
RPD3 family	yRPD3, yHOS1, yHOS2	atHDA6, atHDA7, atHDA9, atHDA19; atHDA5, atHDA15, atHDA18; atHDA2; atHDA8,	hsHDAC1, hsHDAC2, hsHDAC3 hsHDAC8, hsHDAC11
HDA1 family	yHDA1, yHOS3	atHDA14	hsHDAC4, hsHDAC5, hsHDAC6, hsHDAC7, hsHDAC9, hsHDAC10
Sir2 family	ySir2, yHST1, yHST2, yHST3, yHST4	SRT1, SRT2	hsSIRT1, hsSIRT2, hsSIRT3, hsSIRT4, hsSIRT5, hsSIRT6, hsSIRT7
HD2 family	-	HDT1, HDT2, HDT3, HDT4	-

* In *Arabidopsis*, RPD3-like and HDA1-like HDACs are grouped into RPD3/HDA1 superfamily (Pandey et al., 2002). Members of this superfamily are subdivided into three classes. The atHDA6, atHDA7, atHDA9, atHDA19 are class I proteins; the atHDA5, atHDA15, atHDA18 are class II proteins; and the atHDA2 constitutes the new class (class III) of this superfamily, which also includes recently identified HDAC11 in human (Pandey et al., 2002).

processes but also bear some overlapping functions (Bernstein et al., 2000; Hughes et al., 2000; Robyr et al., 2002). The fourth class of HDACs was first discovered in maize and appears to be a plant-specific histone deacetylase (Lusser et al., 1997).

Class I - The RPD3-like proteins

Members of the class I HDACs share a high degree of sequence homology to yeast RPD3 protein (yRpd3p) and are referred to as RPD3-like proteins. Generally, class I HDACs have a N-terminal histone deacetylase domain (Marmorstein, 2001). Many members of class I HDACs have been shown to be sensitive to small molecule inhibitors, such as trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA) and trapoxin (TPX) (Weidle and Grossmann, 2000). Moreover, members of the class I HDACs have been shown to be tightly associated with several other protein subunits, such as Sin3 and N-CoR, to mediate histone deacetylation and transcriptional corepression *in vivo* (Knoepfler and Eisenman, 1999; Ng and Bird, 2000).

Using a potent histone deacetylase inhibitor, trapoxin, two nuclear proteins were co-purified from human and bovine (Taunton et al., 1996). One of the trapoxin-interacting proteins, HDAC1 (initially termed HD1), is highly homologous to the yeast RPD3 protein; the other protein is RbAp48 (Rb-associated protein 48). Almost concurrently, another mammalian RPD3 homologue, HDAC2, was identified as a protein that bound to the transcriptional repressor YY1 using the yeast two-hybrid system (Yang et al., 1996). These findings provided direct experimental evidence linking histone deacetylation to transcriptional control.

Mammalian HDAC1 and HDAC2 are in large multiprotein complexes, mSin3A and NuRD. The mSin3A complex contains mSin3, N-CoR or SMRT, SAP18, Sap30, RbAp48, RbAp46, and c-Ski (Nomura et al., 1999); while the NuRD (nucleosome remodeling histone deacetylase complex) consists of N-CoR, MTA2, Mi2, RbAP46/48, and MBD2 (methyl-CpG-binding domain-containing protein), and has both ATP-dependent chromatin remodeling and HDAC activities (Zhang et al., 1999). Another member, HDAC3 is not contained in mSin3A or NuRD complexes (Knoepfler and Eisenman, 1999). Instead, it has been shown to form a complex with N-CoR, and this corepressor complex inhibits JNK activation through an integral subunit, GPS2 (Zhang et al., 2002). HDAC1, HDAC2, HDAC8, HDAC11 are localized in the nucleus (Hu et al., 2000; Gao et al., 2002), while HDAC3 is the only member in this family to shuttle between the nucleus and the cytoplasm (Takami and Nakayama, 2000). Similarly, yeast Rpd3p is also in a large multiprotein complex that consists of Sin3p, Sap30p, Sds3p, Pho23p and Ume1p, and several other uncharacterized factors (Kurdistani and Grunstein, 2003). In plants, a maize RPD3-type histone deacetylase (ZmRpd3I) and the retinoblastoma-related (ZmRBR1) homologues were found to physically interact *in vivo* and cooperate in repressing gene expression (Rossi et al., 2003). Recently, *FLOWERING LOCUS D* (*FLD*), one of six genes in the autonomous floral-promotion pathway, was determined to be a plant homolog of a human protein, which is a component of HDAC1/HDAC2 complex in mammalian systems (He et al., 2003). Lesions in *FLD* cause extremely hyperacetylation of histones and extremely delayed

flowering (He et al., 2003), suggesting that histone deacetylases in plants also work in a multiprotein complex.

Class II – The HDA1-like proteins

The founding member of the class II HDACs is yeast Hda1p (Rundlett et al., 1996). Members of this family share sequence homology with class I proteins in their catalytic domain, and are also sensitive to histone deacetylase inhibitors (Marmorstein, 2001). However, they have distinct structural and functional features (Wade, 2001). In human, HDAC4, 5, 6, 7, 9, 10 belong to this family (Fischle et al., 1999; Grozinger et al., 1999; Zhou et al., 2001; Fischer et al., 2002). These HDACs are larger, almost twice the size of class I family members. Most of them have a COOH terminus catalytic domain, except for HDAC6, which has a second catalytic domain in the NH_2 terminus (Grozinger et al., 1999). HDAC10 has an NH_2 terminus catalytic domain and a COOH terminus pseudo-domain (Fischer et al., 2002). The class II HDACs are cytoplasmic and are shuttled to the nucleus when they are needed (Verdin et al., 2003). An exception is that HDAC10 is primarily cytoplasmic but shows significant nuclear localization in several cell lines (Fischer et al., 2002; Guardiola and Yao, 2002; Kao et al., 2002; Tong et al., 2002).

Class II HDACs are also included in large multiprotein complexes and interact with other protein factors. A common NH_2 terminal extension in HDAC4, 5, and 7 allows them to interact with the MEF2 transcription factors after they translocate from the cytoplasm to the nucleus (Miska et al., 1999; Lemercier et al., 2000; Dressel et al.,

2001). HDAC4, 5 and 7 were shown to interact with HDAC3 (Grozinger et al., 1999; Grozinger and Schreiber, 2000; Dressel et al., 2001; Fischle et al., 2001) and form a complex with N-CoR and SMRT (Huang et al., 2000; Fischle et al., 2001). This association is regulated by 14-3-3 protein (Grozinger and Schreiber, 2000; Wang et al., 2000; Kao et al., 2001). Interaction of HDAC4 or 5 with the 14-3-3 protein restricts the protein in the cytoplasm. When this interaction is lost, HDAC4 and 5 enter the nucleus, bind with HDAC3, and repress gene expression (Grozinger and Schreiber, 2000). Therefore, it is possible that HDAC4, 5 and 7 participate, with the class I members, in nuclear-receptor-mediated silencing (Huang et al., 2000; Kao et al., 2000). The recently identified HDAC10 also interacts with SMRT as well as with HDAC2 (Fischer et al., 2002).

In yeast, Hda1p is recruited to its target promoters through the Tup1 repressor (Wu et al., 2001). Both Hda1p and Rpd3p deletions increase acetylation levels *in vivo* at all sites examined in both core histones H3 and H4 (Rundlett et al., 1996). RPD3 has a broader range of substrates with greater impact on histone H4 lysine 5 and 12 (Rundlett et al., 1996); while the Hda1p complex only deacetylates lysine residues in histone H3 and H2B (but not the H4 or H2A) amino-terminal tails (Wu et al., 2001).

In plants, HDA1-related protein was purified from maize and was shown to preferentially deacetylate H3 and H2B proteins (Brosch et al., 1992; Brosch et al., 1996). However, the function of HDA1-related HDAC in plants is still not clear.

Class III – SIR2-like proteins

Sir2p, a yeast repressor of transcription was shown to have an *in vitro* NAD-dependent HDAC activity (Imai et al., 2000; Landry et al., 2000a; Smith et al., 2000). It has been implicated to play roles in chromatin silencing, cellular metabolism, and aging (Guarente, 2000). The members in this family have shown significant sequence and functional divergence from RPD3-like proteins and HDA1-like proteins (Marmorstein, 2001) and have different responses to class I and II histone deacetylase inhibitors (Kyrylenko et al., 2003).

Computational analysis revealed a large family of SIR2-like proteins broadly conserved in higher eukaryotes (Frye, 2000). The distinct function of the SIR2-like proteins is that their deacetylase activity is NAD-dependent (Landry et al., 2000b), which suggests a direct link between their deacetylase activity and cellular metabolism. A large fraction of the yeast Sir2p is found within the nucleolus (Guarente, 2000), associated with the tandem rDNA repeats, implying a role in rDNA silencing (Cioci et al., 2002). Several other Sir2p-related proteins have also been found in the cytoplasm (Perrod et al., 2001). Interestingly, Sir2p in yeast was shown to be responsible for deacetylation at H4 Lys16 and maintenance of the boundary at telomeric heterochromatin region (Kimura et al., 2002; Suka et al., 2002). Besides the HDAC activity, another enzymatic activity, ADP-ribosyltransferase (another histone modifying enzyme), is associated with Sir2p (Imai et al., 2000). These data suggest that SIR2-like proteins constitute a functionally distinct class of HDACs.

SIR2-like proteins are not contained in the class I or class II multiprotein complexes. At telomeres and the mating-type loci, yeast Sir2p was found in a multiprotein complex with Sir3p and Sir4p (Moazed, 2001). The Sir2p complex contributes to the stability and maintenance of telomeric repeats (Palladino et al., 1993). At rDNA, the Sir2p complex is associated with both the Net1p protein (Straight et al., 1999), which tethers the Sir2p complex to rDNA, and Cdc14p (Shou et al., 1999), a protein phosphatase involved in cell-cycle control. Recently, one of human SIR2-like proteins, SIRT2, was shown to be predominantly colocalized with microtubules. It also colocalizes and interacts *in vivo* with HDAC6, another tubulin deacetylase (North et al., 2003). It suggests that SIRT2 and HDAC6 are part of a single multiprotein complex for tubulin deacetylation. Another human Sir2 homolog, SIRT1 was shown to specifically deacetylate p53 protein, thereby repressing p53-mediated transcriptional activation. This prevents growth inhibition or apoptosis in response to DNA damage (Luo et al., 2000a; Luo et al., 2001). The data suggest that histones are not the only substrates of SIR2-like HDACs. Similarly demonstrated in mouse, Sir2α (SIRT1) can deacetylate the TAFI68 component of the TATA-box-binding-protein-containing factor (Muth et al., 2001), thereby repressing RNA polymerase I transcription *in vitro*. Phylogenetic analysis indicates the presence of SIR2-like proteins in plants, including *Arabidopsis*, maize and rice; however, none of them has been genetically or biochemically identified and characterized (Pandey et al., 2002).

Plant-specific HDAC family – HD2-like proteins

HD2-like HDACs form multigene families within the plant kingdom (Pandey et al., 2002). HD2 was first identified in maize, and shown to be unrelated to other classes of HDACs (Lusser et al., 1997; Aravind and Koonin, 1998). Instead, HD2 proteins share some sequence similarity with the FKBP (FK506 binding protein)-type PPIases (pepetidylprolyl *cis-trans* isomerases) (Aravind and Koonin, 1998). However, unlike RPD3-like and HDA1-like proteins, little is known about the mechanism of HD2 function. In maize, HD2 was shown to be tightly chromatin-bound, localized in the nucleolus, and homologous to acidic nucleolar phosphoproteins. It suggests that HD2 may be involved in regulation of ribosomal chromatin structure and may function by deacetylating nucleolar core histones (Lusser et al., 1997). Antisense silencing of *AtHD2A* expression results in aborted seed development in transgenic *Arabidopsis* plants, suggesting that *AtHD2A* is critical in reproductive development of *Arabidopsis thaliana* (Wu et al., 2000b). Functional analysis of the *Arabidopsis* homologue *AtHD2A, AtHD2B, AtHD2C* revealed that it can repress transcription when targeted to a reporter gene *in vivo* (Wu et al., 2000b; Wu et al., 2001; Wu et al., 2003), a feature shared with other HDACs.

HDACs in plants

Over the past decade, the function of histone deacetylases in yeast and animals have been extensively characterized (Peterson, 2002; Thiagalingam et al., 2003). In contrast, much less is known about the function of HDACs in plants. Biochemical

studies revealed the presence of multiple HDAC activities in maize (Kolle et al., 1999). The *Arabidopsis* genome bears ten RPD3/HDA1 superfamily members, two SIR2-like HDACs and four HD2 homologs (Pandey et al., 2002). A plant RPD3 homolog was first identified in maize, which was shown to complement the phenotype of the *rpd3* null mutant of *S. cerevisiae* (Rossi et al., 1998). A recent study revealed that ZmRBR1, a retinoblastoma-related protein in maize, recruited ZmRpd3I, to control gene transcription (Rossi et al., 2003). Several yeast RPD3 homologs, AtHD1 (also called AtRPD3A, AtHDA19), AtHDA6 (AtPRD3B) were also characterized in *Arabidopsis* (Wu et al., 2000a; Murfett et al., 2001; Tian and Chen, 2001). Expression of the antisense *AtHD1* resulted in accumulation of acetylated histones and various developmental abnormalities (Tian and Chen, 2001). Some of the phenotypes may be attributed to ectopic expression of tissue-specific genes (e.g., *SUPERMAN*) in vegetative tissues (Tian and Chen, 2001). Several *AtHDA6* mutants were shown to de-repress the expression of transgenes, although none of the endogenous genes was affected and no obvious developmental defects were observed (Murfett et al., 2001). Moreover, AtHDA6 was suggested to be involved in homologous DNA methylation directed by introduction of double stranded RNA (Aufsatz et al., 2002). These data indicate that histone deacetylases play an important role in plant gene regulation and genomic modifications. No information is available about the function of the HDA1-like and SIR2-like HDACs in plants.

CROSSTALK BETWEEN HISTONE ACETYLATION/DEACETYLATION AND OTHER EPIGENETIC MODULATORS

It is increasingly clear that DNA methylation, histone modifications, and nucleosome repositioning play important roles in chromatin remodeling to regulate gene transcription without changing DNA sequence. These epigenetic modulators do not just act independently. Instead, they function through interrelated regulatory networks. Histone acetylation and deacetylation are reversible histone modifications, which have been extensively studied among different species and implicated in many important biological processes. In this section, I will discuss the relationship between histone acetylation/deacetylation and other epigenetic modulators.

Crosstalk between histone deacetylation and DNA methylation

Densely methylated DNA is associated with transcriptionally repressive chromatin in which histones are deacetylated (Antequera et al., 1990; Eden et al., 1998). In mammalian systems, two independent pathways connect DNA methylation to histone deacetylation (Figure 1.4). Both pathways operate through recruiting histone deacetylases. The players in the first pathway are methyl-CpG-binding proteins, MeCP, or MBD (methyl binding domain). The MeCP protein, or MBD, appears to be in a complex with histone deacetylase activity (Jones et al., 1998; Nan et al., 1998). MeCP2 can mediate formation of transcriptionally repressive chromatin on methylated promoter templates *in vitro*, and this process can be reversed by trichostatin A (TSA), a chemical inhibitor of histone deacetylases (Yoshida et al., 1995; Jones et al., 1998; Nan et al.

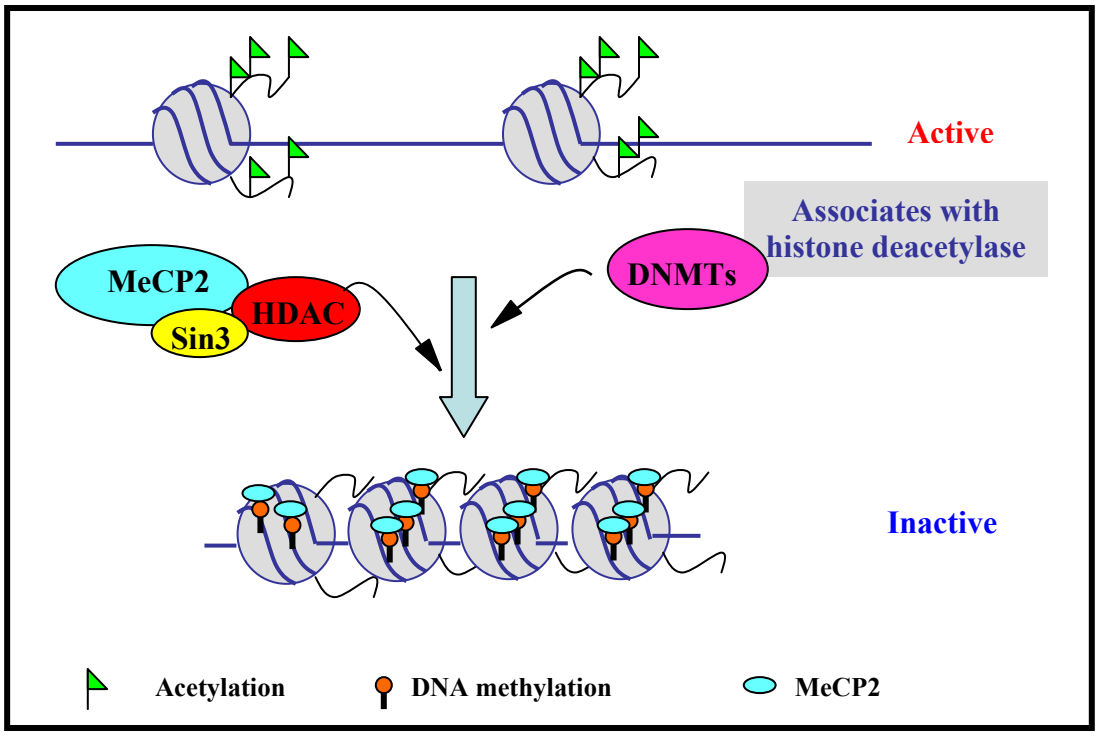

Figure 1.4. DNA methylation and histone deacetylation are connected in remodeling chromatin structures.

Open chromatin is transcriptionally active, which is featured with hypomethylated DNA and histones with acetylated tails (green triangles). In mammals, both DNA methyltransferase (DNMT) and methyl-CpG-binding protein (MeCP) associate with histone deacetylase activities. Therefore, DNA methyltransferase activity not only results in the methylation of DNA (orange circles), but also leads to the deacetylation of histones. As a result, the chromatin is compacted into an inactive state that is inaccessible to transcription factors.

1998). In the second pathway, the mammalian DNA methyltransferases, Dnmt1, Dnmt3a and Dnmt3L associates with histone deacetylase activities (Fuks et al., 2000; Fuks et al., 2001; Deplus et al., 2002). In *Neurospora*, the HDAC inhibitor TSA leads to cytosine hypomethylation at specific sequences (Selker, 1998). Similar effects were also found in mammalian cells (Cervoni and Szyf, 2001).

The link between methylation and histone deacetylation in plants was first observed in the study of nucleolar dominance in *Brassica* (Chen and Pikaard, 1997). Silencing of underdominant rRNA genes can be derepressed by treatment with the methyltransferase inhibitor 5-aza-2'-deoxycytosine (aza-dC) and/or by the HDAC inhibitor trichostatin A (TSA), suggesting that both DNA methylation and histone deacetylation function in the same pathway. Recently, 12 putative MBD proteins were identified in *Arabidopsis* (Berg et al., 2003; Ito et al., 2003; Zemach and Grafi, 2003). Among those proteins, AtMBD6 and AtMBD7 were shown to specifically bind to symmetrically methylated CpG sites (Zemach and Grafi, 2003). AtMBD5 not only binds to symmetrically methylated CpG dinucleotides, but also efficiently binds to methylated CpNpN (N is A, T, or C) sequences (Ito et al., 2003; Zemach and Grafi, 2003). Intriguingly, it has been demonstrated that AtMBD6 precipitates histone deacetylase activity from leaf nuclear extracts, implying that DNA methylation is linked to histone deacetylation (Zemach and Grafi, 2003), to silence gene expression in plants.

Crosstalk between histone acetylation/deacetylation and other histone modifications

It is not always true that histone acetylation is associated with gene activation and histone deacetylation with gene silencing. Increasing evidence suggests that histone acetylation and deacetylation do not regulate gene expression independently, but rather work with other histone modifications to provide distinct "histone surfaces" for recruiting specific protein effectors, as proposed in the "histone code" hypothesis.

It was first demonstrated in *Drosophila* embryo that SU(VAR)3-9 (H3-K9 specific histone methyltransferase) and HDAC are physically and functionally associated *in vivo*, suggesting the concerted action of histone methylation and histone deacetylation in setting a permanent silencing pattern of gene transcription (Czermin et al., 2001). On the other hand, H3-K4 methylation is a mark of transcriptionally active region, which was recently discovered to reside in the coding regions of active and promoter-acetylated genes (Bernstein et al., 2002). Since H3-K4 methylation precludes recruitment of the mammalian HDAC complex NuRD (Nishioka et al., 2002a; Zegerman et al., 2002), it has been suggested that H3-K4 methylation may play a role in gene activation partly by protecting active coding regions from deacetylation (Bernstein et al., 2002). Lysine 9 of H3 is a modifiable position that both histone methylation and histone acetylation compete for (Figure 1.5). Methylation of H3-K4 promotes acetylation of H3-K9, but inhibits methylation at the same position (Figure 1.5), which provides a regulatory switch (Wang et al., 2001a). The crosstalk between histone acetylation/deacetylation and histone methylation is not limited to the N-terminal tails of H3, but also occurs on

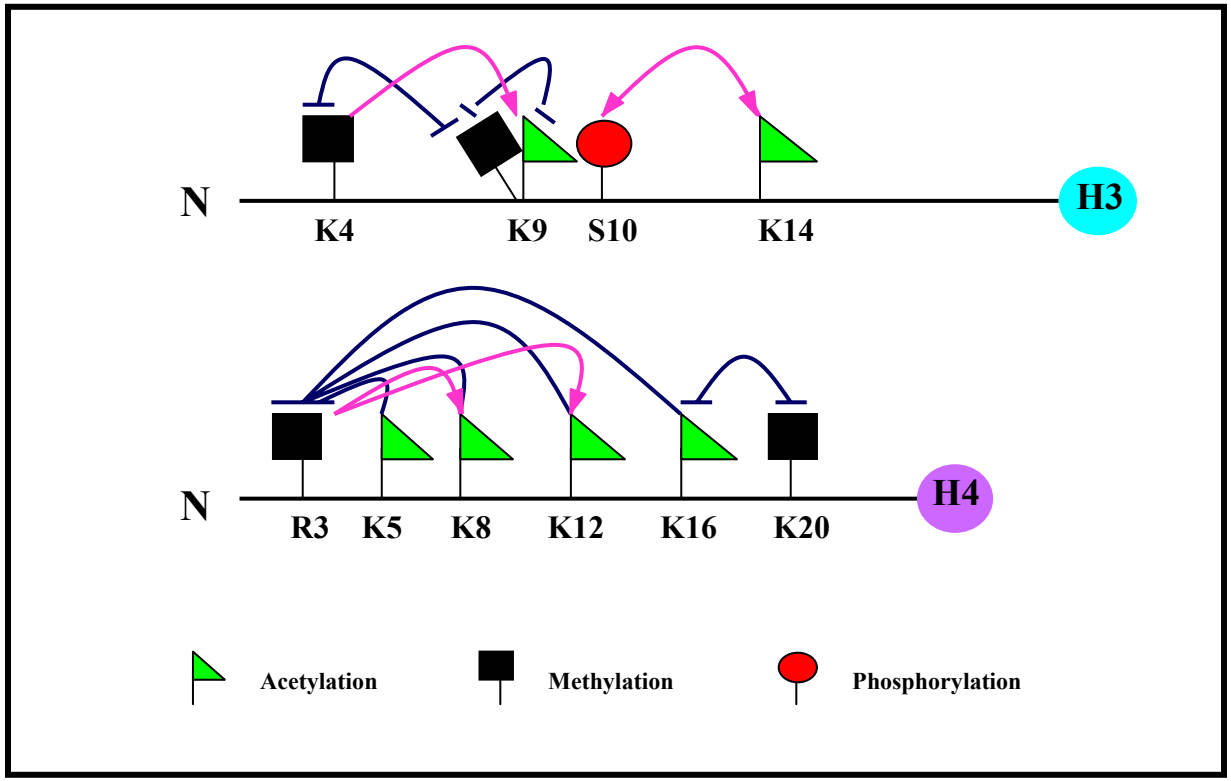

Figure 1.5. Crosstalk between histone acetylation/deacetylation and other histone modifications on N-terminal tails of histone H3 and H4.

The pink arrows indicate that one modification facilitates the occurrence of another modification (e.g., methylation of H3 Lys4 facilitates acetylation of H3 Lys9). The blue blocked arrows indicate that the presence of one modification inhibits the occurrence of another modification (e.g., methylation of H3 Lys9 and acetylation of H3 Lys9 are mutually exclusive). Modification abbreviations: Ac, histone acetylation; M, histone methylation; P, histone phosphorylation. Major amino acid residues involved in crosstalk are: K (lysine) 4, 9, 14 of H3; S (serine) 10 of H3; K (lysine) 5, 8, 12, 16, 20 of H4; R (arginine) 3 of H4.

H4. Methylation of H4-R3 is heavily impaired by acetylation of H4 on K5, K8, K12, and K16 (Wang et al., 2001b; Figure 1.5). Conversely, acetylation of H4 on K8 and K12 by the HAT p300 is elevated after methylation of H4-R3 (Wang et al., 2001b; Figure 1.5). It has been suggested that methylation of H4-K20 and acetylation of H4-K16 are mutually exclusive (Nishioka et al., 2002b; Figure 1.5).

Histone phosphorylation also interacts with histone acetylation. Snf1 and Gcn5 are two enzymes that are responsible for H3-S10 phosphorylation and H3-K14 acetylation in yeast, which appear to function synergistically to mediate gene activation (Lo et al., 2001; Figure 1.5). Remarkably, a cascade of distinct histone modifications, including histone acetylation, histone H3-K4 di- and trimethylation and histone H3-S10 phosphorylation, are reported to be involved in activation of collagenase gene activation (Martens et al., 2003).

Finally, recent evidence indicates that histone ubiquitination induce gene expression via histone acetylation. It is suggested that ubiquitination of H2B at the beginning of gene activation, followed by sequential deubiquitination, is required to optimally induced transcription (Henry et al., 2003). H2B is deubiquitinated by Ubp8, a stable component of SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex, in collaboration with histone acetyltransferase Gcn5.

Crosstalk between histone acetylation/deacetylation and chromatin remodeling proteins

It has been shown that ATP-dependent chromatin remodeling factors cooperate with histone acetyltransferases (HATs) and histone deacetylases (HDACs) in gene regulation. The functional link between ATP-dependent remodeling and histone acetylation was first suggested from genetic studies in yeast (Pollard and Peterson, 1997). Mutations in Gcn5 of the SAGA complex in combination with mutations in the SWI/SNF complex resulted in lethal phenotypes, indicating a concerted interaction between components of these two complexes. It is suggested that SWI/SNF-dependent remodeling occurs prior to Gcn5-dependent histone acetylation (Krebs et al., 2000), and is required for acetylation to occur (Figure 1.6). This observation implies that histone acetyltransferase complexes cannot penetrate the compact chromatin without remodeling. Alternatively, histone acetylation may occur followed by ATP-dependent remodeling (Figure 1.6), thereby altering chromatin structure for general transcription factor binding (Agalioti et al., 2000), or providing a better surface for stabilization of ATP-dependent remodeling complexes (Hassan et al., 2001).

Isw2p is an ISWI family ATP-dependent remodeling complex in yeast. Although Isw2p and Sin3p-Rpd3p histone deacetylase complex have unique biochemical activities, genetic studies indicate that Isw2p and Sin3p-Rpd3p function synergistically to regulate gene repression (Fazzio et al., 2001; Figure 1.6).

Both nucleosome remodeling ATPase Mi-2 and histone deacetylases (HDAC1/2 in mammals and RPD3 in *X. laevis*) reside in the NURD (nucleosome remodeling and

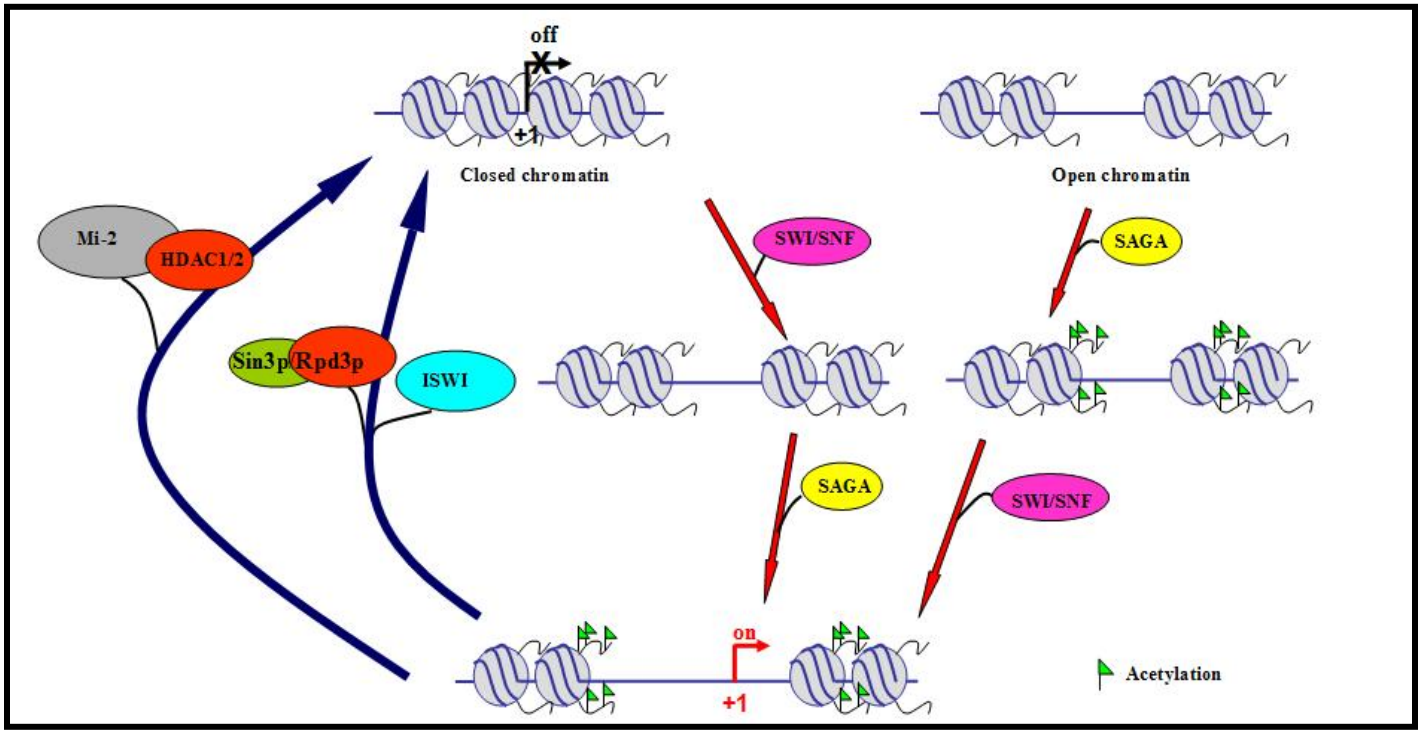

Figure 1.6. Crosstalk between histone acetylation/deacetylation and ATP-dependent chromatin remodeling factors in transcriptional regulation.

SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex and ATP-dependent SWI/SNF family of chromatin remodeling enzyme complexes are coordinated in transcriptional activation, as indicated by red arrows in the diagram. Alteration of chromatin structure can either precede or follow histone acetylation. When chromatin is condensed, SWI/SNF-dependent remodeling occurs before Gcn5-depedent histone acetylation, and is required for acetylation to occur. Alternatively, for certain inducible genes, histone acetylation occurs before ATP-dependent remodeling, and acetylated histones provide a better surface for stabilization of SWI/SNF remodeling complex.

On the contrary, ATP-dependent ISWI complex and NURD complex may play roles in reversion of chromatin structure back to "closed" states, as depicted by black arrows. ISWI complex and Sin3p-Rpd3p complex may function synergistically to repress gene expression. Both nucleosome remodeling ATPase Mi-2 and HDAC1/2 reside in NURD complex to facilitate chromatin remodeling and gene repression.

deacetylation) complexes (Figure 1.6), implying that histone modification (deacetylation) and ATP-dependent remodeling are synergistic actions in chromatin remodeling (Knoepfler and Eisenman, 1999; Guschin et al., 2000). The ATPase activity of Mi-2 was shown to be able to increase the efficiency of histone deacetylation by NURD complexes (Tong et al., 1998; Xue et al., 1998; Guschin et al., 2000).

In plants, there is no direct evidence for a connection between histone deacetylation and chromatin remodeling factors. However, recent studies imply that histone deacetylases might interact with other chromatin remodeling proteins to control gene expression in plants. Chromodomain-Helicase-DNA-binding proteins (CHD) are members of the SWI2/SNF2 subfamilies, components of higher-order chromatin remodeling machinery (Kingston and Narlikar, 1999). Mutations in one of *Arabidopsis* CHD proteins, PICKLE, result in various phenotypic effects including derepression of a transcription factor implicated in embryo development (Ogas et al., 1999). *Drosophila* and mammalian members of this subfamily act in complexes with histone deacetylases to repress transcription (Wade et al., 1998; Zhang et al., 1998a). Recently, the involvement of the Polycomb group (PcG) proteins in plant development has been demonstrated (Chaudhury et al., 1997; Grossniklaus et al., 1998; Luo et al., 2000b). PcG proteins are chromatin silencing factors known to regulate early development in animals. Some PcG proteins mediate gene expression through histone deacetylation (Pirrotta, 1998; van der Vlag and Otte, 1999).

OUTLINE OF DISSERTATION

Despite extensive studies on the role of histone deacetylases in yeast and mammalian cells, the effects of histone deacetylation on plant gene regulation are poorly understood. Further analysis of the function of histone deacetylases in *Arabidopsis* will help to elucidate regulatory network in plant development. This dissertation features the molecular, biochemical, and genomic studies of *Arabidopsis* AtHD1, a putative histone deacetylase sharing the highest protein sequence identity with yeast RPD3 protein.

Study of the general role of histone deacetylation in plant gene regulation and development is described in Chapter II. By generating constitutive antisense *AtHD1* (CASH) transgenic plants, the expression of endogenous *AtHD1* is dramatically reduced, resulting in various developmental abnormalities and ectopic expression of tissue-specific genes in association with accumulation of acetylated histones. No changes in genomic DNA methylation are detected in CASH plants. These results suggest that AtHD1 is a global regulator, which controls gene expression independent of DNA methylation.

In the transgenic plants, it is difficult to control the expression levels of antisense *AtHD1*. Overexpressing antisense *AtHD1* may also suppress other *AtHD1* homologs in the genome. To study the specific role of *AtHD1* in plant gene regulation and development, we obtained a line with a T-DNA insertion in the second exon of *AtHD1* (*athd1-t1*). This line was a generous gift from Dr. Stanton Gelvin at Purdue University. Chapter III and Chapter IV mainly focus on analysis of the *athd1-t1* line.

In Chapter III, we investigated the genetic control of developmental changes induced by disruption of *AtHD1* expression in *Arabidopsis*. T-DNA insertion causes a complete suppression of endogenous *AtHD1* expression, resulting in a null allele (*athd1-t1*). The homozygous (*athd1-t1*/*athd1-t1*) mutant plants display similar developmental abnormalities as observed in the CASH plants, which are inheritable across generations. The developmental abnormalities were expunged immediately in the F1 hybrids from hybridization of the *athd1-t1/athd1-t1* plants with wild-type plants. F_1 hybrids exhibited simultaneous restoration of *AtHD1* expression and reduction of histone H4-K12 acetylation. Unlike the stable epigenetic code – DNA methylation and histone methylation, histone acetylation/deacetylation represents a reversible "histone code".

Chapter IV describes the genome-wide analysis of histone deacetylase 1 in *Arabidopsis* (AtHD1). Differentially expressed genes in leaves or flower buds of Wild type (Ws) and the *athd1-t1* mutant were detected using oligo-gene microarray techniques. Furthermore, histone acetylation and methylation status in the vicinity of reactivated or silenced genes in the histone deacetylase mutants were monitored by chromatin immunoprecipitation assay, providing evidence for direct or indirect effects of down-regulation of histone deacetylase 1 on plant gene regulation.

CHAPTER II

BLOCKING HISTONE DEACETYLATION IN *ARABIDOPSIS* INDUCES PLEIOTROPIC EFFECTS ON PLANT GENE REGULATION AND DEVELOPMENT *

OVERVIEW

Histone acetylation and deacetylation play essential roles in eukaryotic gene regulation. Reversible modifications of core histones are catalyzed by two intrinsic enzymes, histone acetyltransferase (HAT) and histone deacetylase (HD). In general, histone deacetylation is related to transcriptional gene silencing, whereas acetylation correlates with gene activation. We produced transgenic plants expressing the antisense *Arabidopsis* HD (AtHD1) gene. AtHD1 is a putative homolog of human HD1 and RPD3 global transcriptional regulator in yeast. Expression of the antisense *AtHD1* caused dramatic reduction in endogenous *AtHD1* transcription, resulting in accumulation of acetylated histones, notably tetra-acetylated H4. Reduction in *AtHD1* expression and AtHD1 production and changes in acetylation profiles were associated with various developmental abnormalities, including early senescence, ectopic expression of silenced

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* This chapter is reformatted from "Blocking histone deacetylation in *Arabidopsis* induces pleiotropic effects on plant gene regulation and development" by Tian, L., and Chen, Z. J. (2001). *Proc. Natl. Acad. Sci. USA* vol. 98, pp. 200-205.

genes, suppression of apical dominance, homeotic changes, heterochronic shift toward juvenility, flower defects, and male and female sterility. Some of the phenotypes could be attributed to ectopic expression of tissue-specific genes (e.g., *SUPERMAN*) in vegetative tissues. No changes in genomic DNA methylation were detected in the transgenic plants. These results suggest that AtHD1 is a global regulator, which controls gene expression during development through a DNA-sequence independent or epigenetic mechanism in plants. In addition to DNA methylation, histone modifications may be involved in a general regulatory mechanism responsible for plant plasticity and variation in nature.

INTRODUCTION

Core histones can be acetylated or deacetylated through intrinsic activities of histone acetyltransferases or deacetylases (Allfrey et al., 1964). The acetylation state often relates to gene activity, whereas the deacetylation state is typically associated with inactivity (Turner, 1991). The deacetylation process involves the removal of acetyl moieties by deacetylases from specific lysine residues of core histones, thereby restoring positive charges on the lysine residues. The interaction between positively charged lysines and negatively charged DNA reduces nucleosome mobility on DNA, hindering accessibility of the promoter to the transcriptional machinery. Inhibition of histone deacetylase (HD) activity can reverse the process and result in gene activation. In eukaryotic organisms that use both DNA and histone modifications, HDs are recruited by DNA methyl-binding proteins (e.g., MeCP2, MBD2) (Jones et al., 1998; Nan et al.,

1998; Ng et al., 1999), DNA methyltransferase (Dnmt1) (Fuks et al., 2000), or sequence-specific DNA-binding proteins (Heinzel et al., 1997; Pazin and Kadonaga, 1997; Brehm et al., 1998; Nicolas et al., 2000) to silence genes.

RPD3, an HD in yeast, is a global transcriptional regulator (Vidal and Gaber, 1991). RPD3-deletion mutants both up- and down-regulate gene expression in yeast (Vidal and Gaber, 1991; Rundlett et al., 1996; Kadosh and Struhl, 1998b) and enhance position-effect variegation in *Drosophila* (De Rubertis et al., 1996). Mouse *HD1*, and *RPD3* homolog, is identified as a growth factor-inducible gene (Bartl et al., 1997). Overexpression of mouse *HD1* in stable transfected mammalian cells causes a remarkable reduction in the growth rate and severe delay during the G2/M phases of the cell cycle, implying a role of histone acetylation in cell cycle progression.

Despite extensive studies on the role of histone modifications in eukaryotic gene regulation, the effects of histone deacetylation on plant gene regulation and development are unclear. It has been demonstrated that both histone deacetylation and DNA methylation are involved in silencing one parental set of rRNA genes in allotetraploid *Brassica* (Chen and Pikaard, 1997), a close relative of *Arabidopsis*. In this study, we used an antisense inhibition approach to down-regulate antisense *Arabidopsis* HD gene (*AtHD1*) expression. Antisense-*AtHD1* transgenic plants had reduced levels of the HD and increased levels of tetra-acetylated histone H4. As a result, these plants displayed ectopic expression of tissue-specific genes [e.g., *SUPERMAN*, (*SUP*)] (Sakai et al., 1995) and various types of aberrant phenotypes. Some phenotypes were present in the subsequent selfing generations. Changes in DNA methylation in the repetitive and

single-copy DNA sequences were not detected in the transgenic plants. We conclude that besides DNA methylation, histone deacetylation plays an essential role in plant gene regulation and development.

RESULTS

Characterization of HD genes in *Arabidopsis*

The nucleotide sequence of *AtHD1* (AF014824) encodes 501 amino acids, with 56 and 55% amino acid sequence identify, respectively, to HD1 in mammals (Taunton et al., 1996) and RPD3 in yeast (Vidal and Gaber, 1991). Thus, the *Arabidopsis* gene is named *AtHD1*. HD1 homologs are highly conserved with 55-96% overall identify among *Arabidopsis*, yeast (Vidal and Gaber, 1991), *Drosophila* (De Rubertis et al., 1996), maize (Rossi et al., 1998), and human (Taunton et al., 1996).

The copy number of *AtHD1* was examined by DNA blot analysis. Using the 3' region of the cDNA fragment as a probe (Figure 2.1A), we detected only single fragments in the genomic DNA digested with four restriction enzymes (Figure 2.1B). Thus, *AtHD1* is a single-copy gene. Genomic sequence of the gene is located on the short arm of chromosome 4 and tagged by the DNA marker mi390 (Mayer et al., 1999). Another gene (*HD2*) has been identified in maize (Lusser et al., 1997) and *Arabidopsis* (Wu et al., 2000b). Two *AtHD2* homologs (*AtHD2A* and *AtHD2B*) are primarily expressed in flowers and young siliques (Wu et al., 2000b). Expression of *AtHD1* (≈ 2 kb) was high in leaves in *A. thaliana* and its related species, *Cardaminopsis arenosa* and *A. suecica* (Figure 2.1C).

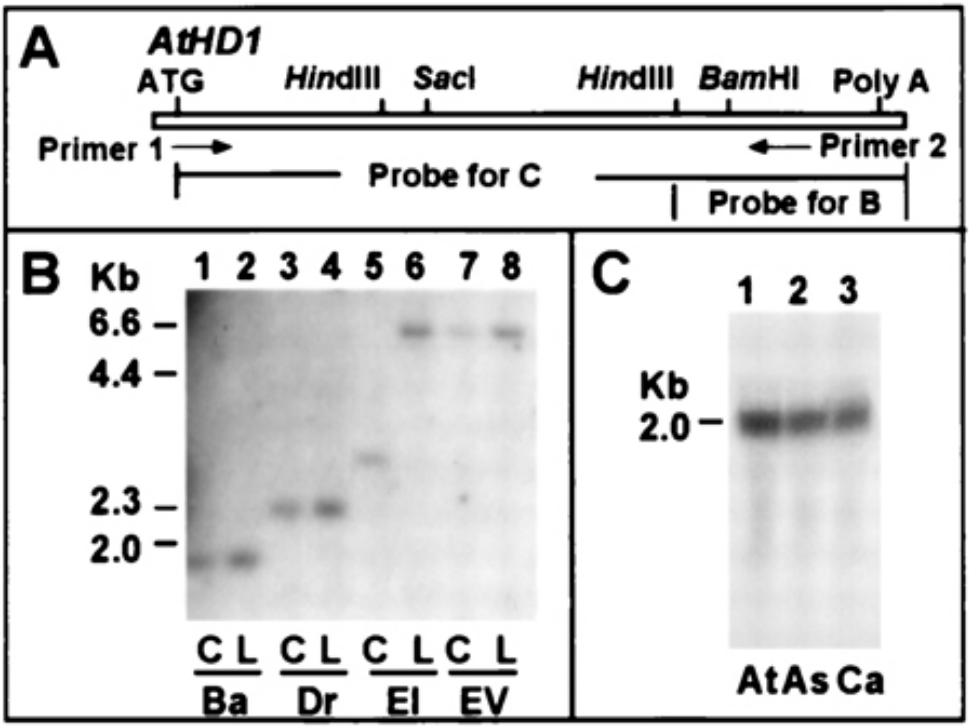

Figure 2.1. AtHD1 is a single-copy gene and is expressed in *Arabidopsis*.

(A) A simplified restriction map of *AtHD1*. Arrows indicate the primers used to amplify a 1.5-kb reverse transcription-PCR fragment. The fragments used as DNA probes are indicated below the diagram.

(B) Autoradiogram showing a DNA blot containing genomic DNA from Columbia (C) and Lansberg (L), which was hybridized with the 3' region (≈ 500bp) of *AtHD1* (A). Ba, *Bam*HI; Dr, *Dra*I; EI, *Eco*RI; EV, *Eco*RV.

(C) An RNA blot was hybridized with a full-length cDNA fragment as a probe (A). At, *A. thaliana*; Ca, *Cardaminopsis arenosa*; As, *A. suecica*.

Yeast has five related genes, *RPD3*, *HDA1*, *HOS1*, *HOS2*, and *HOS3* (Rundlett et al., 1996), in addition to *Sir2*, that encodes an NAD-dependent HD (Imai et al., 2000). On the basis of BLAST/N analysis, *Arabidopsis* has *Sir2*-like and other HD genes, including a single-copy *AtHD1* or *RPD3* (Figure 2.1B), two copies of *RPD3*-related *AtHDA1*, at least two copies of *AtHD2*, and several homologs of *HOS1*, *2*, and *3*. In yeast, both RPD3 and HDA1 are purified (Rundlett et al., 1996). RPD3 and HDA1 have slightly different functions in deacetylating histones. RPD3 has greater effects than other homologs on deacetylating lysine residues 5 and 12 on histone H4 and plays a role in both heterochromatic gene silencing and inducible gene activation (Vidal and Gaber, 1991; Rundlett et al., 1996).

The AtHD1 has two components. The N terminus of the protein (201 amino acids) is homologous to yeast RPD3; the C terminus (300 amino acids) is highly hydrophobic and specific to multicellular eukaryotic organisms, including plants and mammals (Figure 2.2A). The histidines at positions 148/149 and 186/187 are conserved catalytic sites for deacetylation activity in yeast (Kadosh and Struhl, 1998b).

Production of antisense and sense *AtHD1* transgenic plants

The antisense p35S::AtHD1 construct and transgenics are referred to as CASH (constitutive *a*ntisense histone deacetylase 1) (Figure 2.2A). The presence of the transgene in 157 CASH plants was screened by PCR amplification. A subset of the PCR results is shown in Figure 2.2B. Of the 157 plants tested, 151 contained at least one insert; only 6 plants had no insert. Consistent with the PCR results, DNA blot analysis

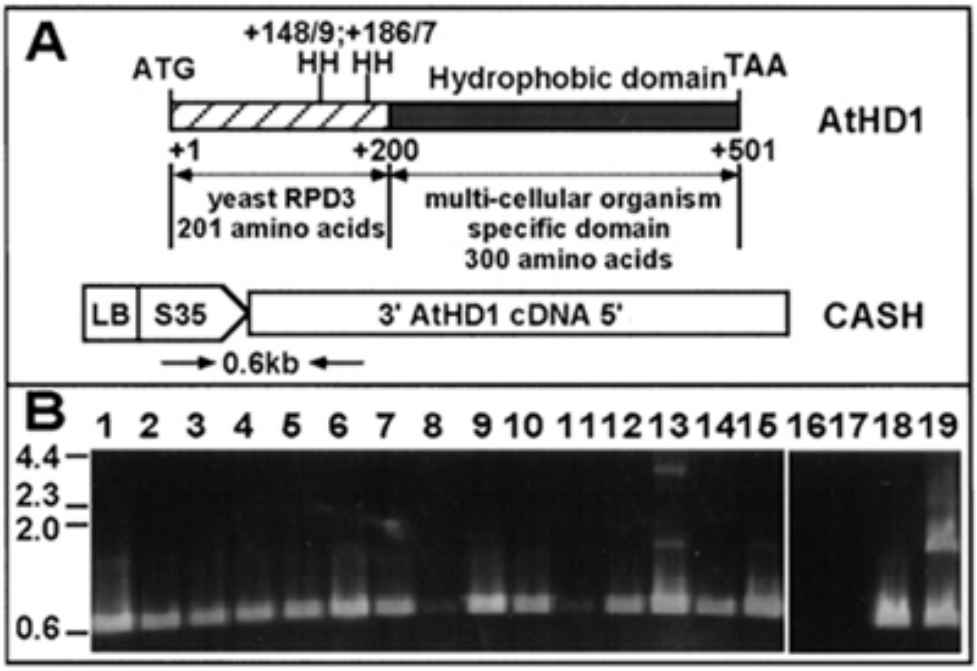

Figure 2.2. AtHD1 construct and transgene detection in transgenic plants.

(A) The diagram of AtHD1 and CASH construct. N terminus of AtHD1 (201 amino acids) is homologous to yeast RPD3; the C terminus (300 amino acids) is highly hydrophobic and specific to multicellular organisms. The CASH construct is shown below the AtHD1 diagram. The primers used for amplify a ≈ 600-bp fragment in CASH plants are shown.

(B) Ethidium bromide-stained agarose gel showing PCR-amplified fragments in CASH plants. Controls were a transgenic plant transformed with the vector only (lane 16) and a CASH plant in a PCR reaction without primers (lane 17). More than one insert was present in some plants (lane 13 and 19); low amplification was found in others (lanes 8 and 11).

by using the kanamycin gene as a hybridization probe confirmed that all 50 plants analyzed have the transgene (data not shown). Eleven of them with one to three copies of the transgene were selected for further study.

Antisense *AtHD1* expression down-regulates endogenous *AtHD1* expression

Expression of antisense *AtHD1* transcripts was analyzed in CASH plants and a control plant (Figure 2.3). Except as noted otherwise, "control" refers to plants transformed with the vector only. The antisense transcripts were highly expressed in 11 CASH plants (Figure 2.3A, lanes 2-12) but were absent in the control (lane1). As a result, endogenous *AtHD1* transcripts were dramatically reduced, ranging from <5% (Figure 2.3B, lanes 2, 3, and 12) to 40% (lane 8) of the level in the control (lane1). Only trace amounts of *AtHD1* transcripts were detected in three CASH plants (lanes 2, 3 and 12), most of which had severe phenotypes.

Although there was little variability in the levels of antisense *AtHD1* overexpression (Figure 2.3A), the levels of endogenous *AtHD1* transcripts in the CASH plants varied from 5 to 40% of the level in the wild type (Figure 2.3B). Gradient reductions in *AtHD1* transcription in multiple independent CASH plants were correlated with stochastic effects of inhibiting *AtHD1* expression on plant development (see below).

Ectopic expression of *SUPERMAN* (*SUP*) in the CASH transgenic plants

Normal control over gene expression was obviously disrupted in some CASH plants, in which ectopic expression of *SUP* (Figure 2.3B, lanes 2, 4, and 6) and delay of

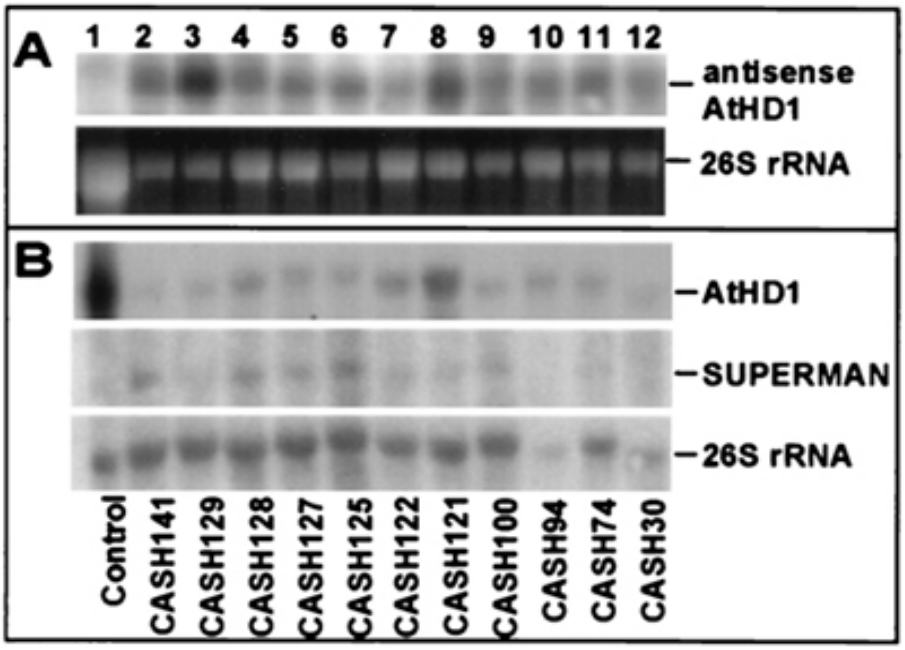

Figure 2.3. Gene expression patterns in the control and CASH transgenic plants.

Total RNA (20 μg) from the control and CASH plants was subjected to electrophoresis in a 1.2% agarose gel containing 2% formaldehyde and transferred onto Hybond-N+ membrane (Amersham Pharmacia). The blot was then hybridized with a radiolabeled probe with the use of either an *in vitro* transcript kit (for *AtHD1* and *SUP*) or a random priming method (for 26S rDNA).

(A) The membrane was probed with radiolabeled single-stranded RNA of sense *AtHD1*. An ethidium bromide-stained RNA gel was used as the RNA loading control.

(B) The membrane was probed with radiolabeled single-stranded RNA of antisense *AtHD1*, antisense *SUP*, or 26S rDNA.

Table 2.1. Phenotypic variation in 151 independent transgenic plants containing CASH

Genotypes:	Columbia (CASH)		Columbia (vector)		Columbia	
	No. of plants, %		No. of plants, %		No. of plants, %	
Phenotypes*						
1. Early senescence	14	9.2	0		0	
2. Serrated leaves	25	16.6	1[a]	4	0	
3. Rosettes on early nodes	20	13.2	0		0	
4. Combined phenotypes of 2 and 3	15	9.9	0		0	
5. Homeotic transformation	6	4.0	0		0	
6. Flower defects and infertility	3	2.0	0		0	
7. Flowering time, days[b]	35-70		35 (±3)		35 (±2)	
8. Normal phenotypes	68	45.0	24	96.0	83	100.0
Total	151	100.0	25	100.0	83	100.0

* Only distinct phenotypes were shown.
[a] With a few serrated rosette leaves.
[b] The frequencies of flowering time were as follows: 30 plants flowered at 35-40 days, 81 at 41-50 days, and 2 at 61-70 days after germination. The total number of the plants did not add up to 151 because of early senescence of some CASH plants.

flowering were frequently observed. One of the plants (CASH125) developed abnormal flowers without sepals and petals but with extra stamens. This phenotype is different from SUP, which has localized effects on floral whorl boundaries (Sakai et al., 1995). It is likely that inhibiting histone deacetylation affects not only *SUP* expression but also expression of additional genes (e.g., B function genes) required for floral (sepal and petal) development. Weak expression of *SUP* was detected in three other CASH plants (lanes 5, 7, and 9), none of which developed SUP phenotypes. However, two of them (CASH100 and 127) developed homeotic changes, suggesting a role of histone deacetylation in plant development.

Down-regulation of AtHD1 induces developmental pleiotropy in *Arabidopsis*

About 55% of the 151 multiple independent CASH plants showed visibly aberrant phenotypes (Table 2.1), including early senescence (9%), serration (17%), aerial rosette formation (23%), homeotic changes (4%), floral abnormalities (2%), and delay of flowering. A gradient of phenotypic changes existed in each category. It is notable that, with the exception of one plant that was transformed with vector only and displayed very weakly serrated leaves, none of the 25 control or 83 wild-type plants showed phenotypic abnormalities. This lack of abnormalities reduces the possibility that transformation artifacts were responsible for the observed phenotypic changes in the CASH plants.

In *Arabidopsis*, development of the first two leaves is symmetrical and is initiated during embryogenesis. In some CASH plants, the first and second pairs of

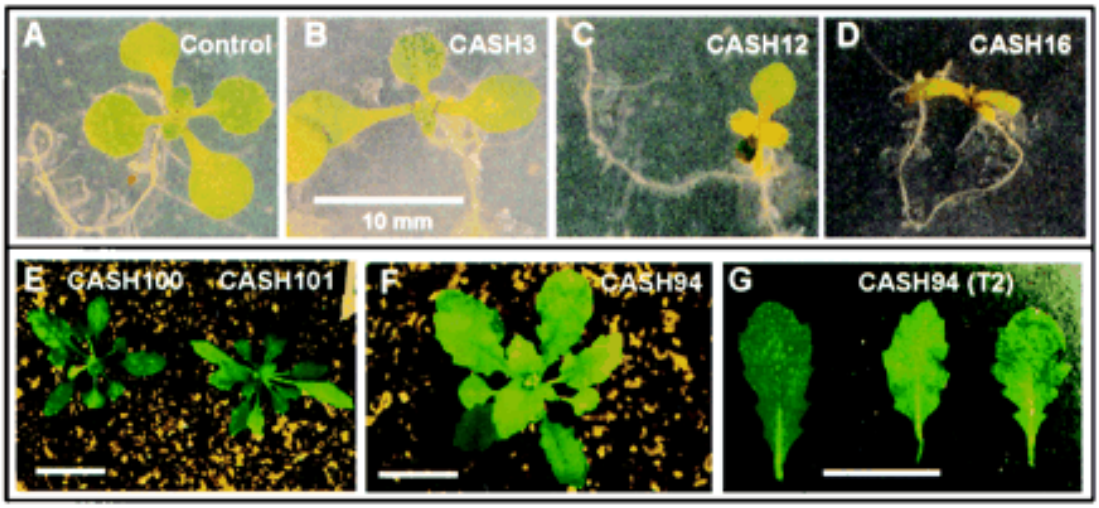

Figure 2.4. Phenotypic variation at early developmental stages in CASH plants.

(A-E) Photographs of 8-day-old seedlings, showing development of asymmetric leaves in CASH3 (B) and CASH12 (C), narrow and elongated leaves in CASH16 (D), and spade-shaped leaves in CASH100 and 101 (E). The control seedling is shown in A.

(F) Photograph of a serrated plant (CASH94).

(G) The picture of leaves collected at rosette positions 4, 6, and 8 from the same plant after two generations of selfing. (Bars = 10mm. The same scale is used from A to D.)

leaves developed asymmetrically (Figures 2.4B and 2.4C). In two plants, the first two leaves were elongated with little expansion and developed into "needle-like" structures (Figure 2.4D). Approximately 9% of the transgenic plants exhibited early developmental abnormalities, and the majority died within about 2 weeks on media. As a result, we could not examine gene expression patterns in these plants. However, the data suggest that histone deacetylation is required for coordinated gene expression during early development, including embryogenesis. The surviving seedlings developed into various phenotypes, depending on the stages of gene expression affected.

Serration of leaf margins is controlled by a single gene, *SERRATE*, in *Arabidopsis* (Clarke et al., 1999). *SERRATE* encodes a zinc-finger protein that may be involved in transcriptional regulation. Wild-type *Arabidopsis* leaves are round with little or no serration in early rosette leaves. In some CASH plants (e.g., CASH94), the early leaves were heavily serrated (Figure 2.4F). Moreover, *SERRATE* was present in the subsequent generations (Figure 2.4G) in the plants homozygous for the *CASH* transgene. The data suggest that ectopic expression of *serrate* alleles is induced by blocking histone deacetylation (Figure 2.3, lane 11). Alternatively, a repressor that is turned on by inhibiting histone deacetylation indirectly activates the *serrate*.

Bract formation is a transition from vegetative to reproductive (flower) development; cauline leaves and inflorescence are developed from bracts. Some CASH plants (e.g., CASH127) developed aerial rosette-like structures, usually in the first two nodes (Figure 2.5A). The secondary aerial rosettes behaved like the primary rosettes and developed into stem and inflorescence structures in late development stages. This

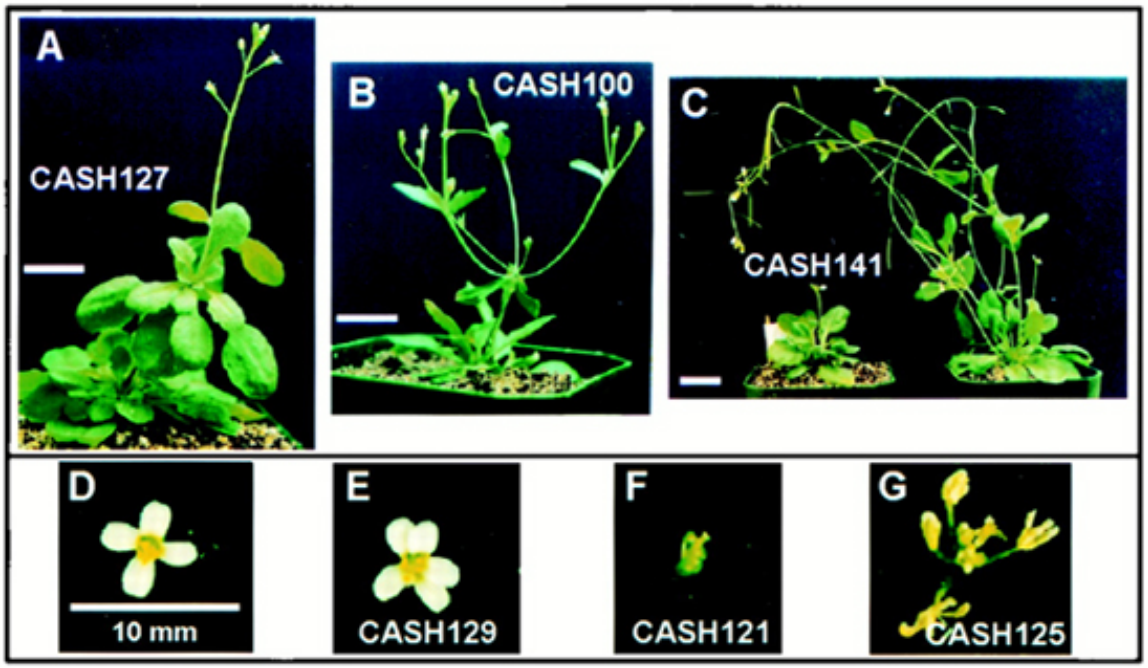

Figure 2.5. Phenotypic variation at late developmental stages in CASH plants.

(A) Photograph of the CASH127 plant, showing the development of aerial rosettes in the first node.

(B) Photograph of the CASH100 plant, showing no apical dominance and developing four inflorescence branches at the first node.

(C) Photograph of a dwarf and late flowering plant (CASH141, left) and a normal control plant (Right).

(D-G) Photographs of various flower phenotypes, including a flower with five petals (E), no petals (F), and no petal and sepal (G). A normal *Arabidopsis* flower is shown in D. (Bars = 10mm. The same scale is used from D to G.)

phenomenon resembles homeotic transformation, as initially described in *Drosophila* (Morata and Kerridge, 1981). CASH100 and 101 developed narrow rosette leaves (Figure 2.4E), and homeotic changes occurred as time progressed. CASH100 had elongated internodes with no apical dominance, which eventually led to the development of four equally growing branches (Figure 2.5B). This plant possessed severe developmental abnormalities, including male and female sterility, reduced *AtHD1* expression, and ectopic expression of *SUP* (Figure 2.3B, lane 9).

The developmental changes observed included a delay of the phase transition from the vegetative to the reproductive stage. Under 14h/day of illumination, the Columbia strain typically flowered at about 35 days after germination. The CASH plants displayed prolonged juvenile stages (Figure 2.5C *Left*), with the majority flowering at 41-70 days (Table 1). Delay of the phase transition was also observed in DNA methylation mutants (Kakutani et al., 1996) and antisense methyltransferase plants (Finnegan et al., 1996; Ronemus et al., 1996). Thus, like DNA methylation, histone deacetylation plays a role in orchestrating gene expression during reproductive development.

Phenotypic changes in CASH transgenic plants are associated with AtHD1 reduction and histone hyperacetylation but not with DNA methylation

Changes in AtHD1 production and histone acetylation profiles were examined in a plant that showed a dramatic reduction in *AtHD1* transcription. CASH141 had approximately 10% of AtHD1 protein produced compared with a control plant (Figure

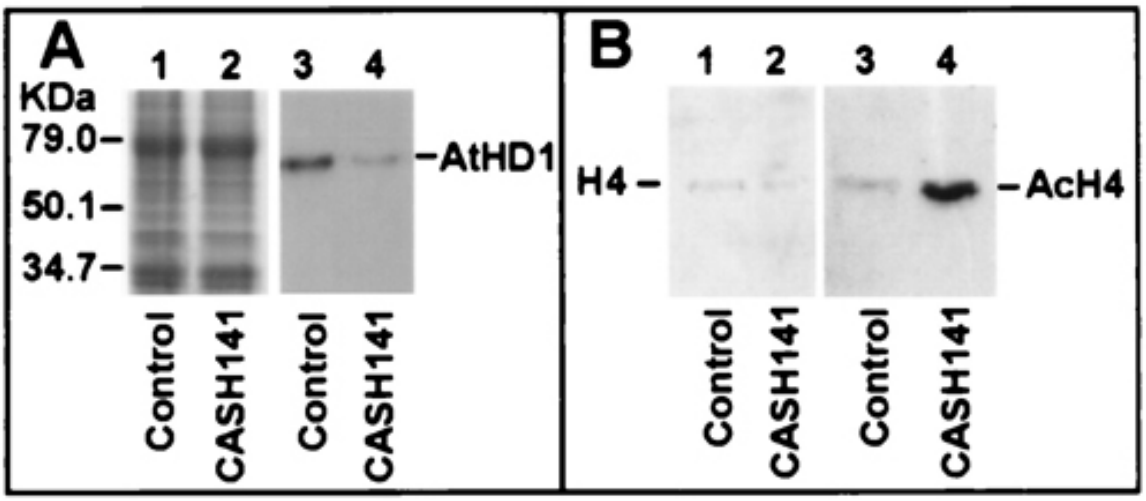

Figure 2.6. AtHD1 reduction and histone H4 hyperacetylation in CASH141.

Crude protein extracts (25 µg) or histone fractions (2 µg) were loaded onto a SDS/PAGE gel and then immunoblotted onto immobilon-P (Milipore) or Hybond-ECL (Amersham Pharmacia). The membrane was probed with antibodies against the N terminus of AtHD1 and antibodies specific for nonacetylated (H4) or tetra-acetylated histone H4 (AcH4).

(A) AtHD1 in CASH141 was reduced to 10% (lane4) of the level in a control plant (lane 3). Protein loading control is shown in an 8% SDS/PAGE stained with Coomassie blue (lanes 1 and 2).

(B) Histone H4 was hyperacetylated ≈ 10-fold in the CASH141 (lane 4) compared with the wild-type plant (lane3). An equal amount of nonacetylated histone H4 was detected (lanes 1 and 2).

2.6A, lanes 3 and 4). As a result, the transgenic plant had an elevated level (≈ 10-fold) of tetra-acetylated histone H4 (Figure 2.6B, lanes 3 and 4). Histone H4 hyperacetylation was previously found in the yeast *rpd3* null mutant (Rundlett et al., 1996). Thus, the *AtHD1* shares a similar deacetylation function with *RPD3* in yeast (Vidal and Gaber, 1991; Rundlett et al., 1996) and *HD1* in mammals (Taunton et al., 1996). This claim is supported by the evidence that the *HD1* homolog in maize functionally complements the *rpd3* mutation in yeast (Rossi et al., 1998).

Biochemical studies suggest that DNA methylation is involved in the repression of gene transcription by recruiting HDs (Jones et al., 1998; Nan et al., 1998). However, inhibiting HDs by trichostatin A may also induce changes in DNA methylation at some specific loci (Selker, 1998). To determine whether reduction in AtHD1 has any effects on changes in DNA methylation, we examined methylation status in the repetitive DNA sequences (rDNA and centromere) and a specific locus (*SUP*). The *Hpa*II restriction enzyme has a nucleotide-recognition site (CCGG); however, it does not digest the DNA if the inner cytosine is methylated. Among the seven transgenic plants analyzed, the methylation patterns in the rDNA (Figure 2.7A, lanes 3-9) and centromere repeats (Figure 2.7B, lanes 3-9) were similar to those in the control plant (Figure 2.7, lane 1). In contrast, demethylation was observed in an antisense *MET1* plant (Figure 2.7, lanes2). In addition, we failed to detect any changes in DNA methylation in the *SUP* locus (data not shown) with the use of *Mob*I and *Sau*3A, which could distinguish methylation status between wild-type and *SUP* alleles (Jacobsen and Meyerowitz, 1997).

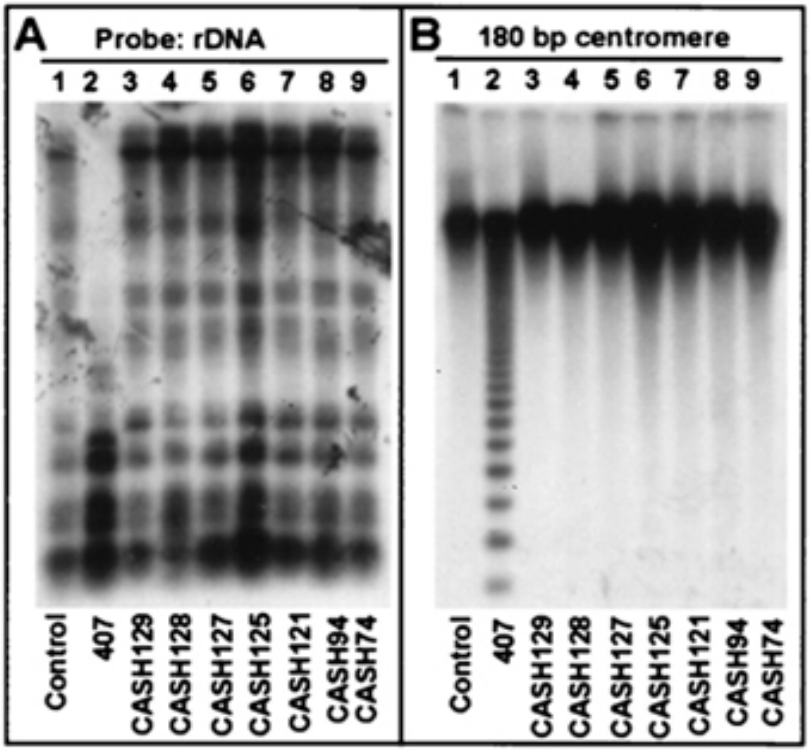

Figure 2.7. DNA blot analysis of the genomic DNA methylation in the CASH plants and an antisense MET1 plant.

Total genomic DNA (2 µg) was digested with *Hpa*II and transferred onto Hybond-N+ membrane.

(A) The blot was probed with intergenic spacer probe of the 26S rDNA (Chen and Pikaard, 1997).

(B) The blot was hybridized with a 180-bp centromere repeat (Kakutani et al., 1996).

DISCUSSION

Histone deacetylation is involved in epigenetic silencing in *Arabidopsis*

The role of histone deacetylation in plant gene regulation and development is unclear. Trichostatin A, a HD inhibitor, could induce transient phenotypes in *Arabidopsis*; the phenotypes were fewer and less severe than what was observed in the CASH plants (data not shown). However, blocking histone deacetylation by trichostatin A derepresses one parental set of rRNA genes that is normally silenced in a *Brassica* allotetraploid (Chen and Pikaard, 1997), indicating that histone modifications are involved in epigenetic control of gene expression in plants as in other eukaryotes. This notion has been supported by our data in the CASH plants that display developmental pleiotropy (Figures 2.4 and 2.5). Down-regulation of *AtHD1* expression changes gene expression and phenotypes at various stages of plant development from embryogenesis to flower development and seed production. Some changes are associated with epigenetic reactivation of genes that are normally silenced, for example, ectopic expression of *SUP* and phenotypic expression of serrated leaves, which is reminiscent of the lethal phenotypes observed in early developmental stages and male and female sterility at late stages. Many CASH plants have reduced fertility, ranging from 0 to 90% of the seed sets of wild-type plants. Seed abortion is also observed in antisense *AtHD2* plants (Wu et al., 2000b). *AtHD1* suppression appears to have more effects than *AtHD2* on plant development. Multiple HDs may be involved in various biological processes. For example, HD2 is localized in nucleoli (Lusser et al., 1997), suggesting a role in

rDNA chromatin organization. Sir2 protein is an NAD-dependent HD and is involved in transcriptional silencing in yeast (Imai et al., 2000).

Overexpressing antisense *AtHD1* induces a wide range of phenotypes, which is likely caused by the various levels of endogenous *AtHD1* suppression (Figure 2.3). The levels of AtHD1 production from representative CASH plants in six groups (Table1, phenotypes 2-7) are 90, 90, 95, 85, 40, and 10% of the control levels. Compared with the *AtHD1* suppression, the levels of AtHD1 reduction correlate with the severity of phenotypes in the plants with severe abnormalities in the last two groups but not in others (Table 1 and Figure 2.3B). One possibility is that the AtHD1-N antibodies crossreact with related proteins, whereas the RNA probe is more specific, so that the correlation at the RNA level is more reliable (Figure 2.3). Alternatively, a defective AtHD1 (RPD3 homolog) may not be compensated for by other related proteins. Indeed, yeast RPD3 and HDA1 have slightly different effects on the specificity of deacetylating lysine residues (Rundlett et al., 1996).

In our previous studies, neither 5'-aza-2'-deoxycytidine nor trichostatin A induced transmissible changes in gene expression (Chen and Pikaard, 1997), which was different from results in maize, in which the phenotypes induced by 5'-aza-2'-deoxycytidine were inherited for a few generations (Ronchi et al., 1995). In this study, some phenotypic changes induced by antisense *AtHD1* expression were similar to those induced by chemical treatments, i.e., they were not heritable. However, some phenotypes (e.g., serrate) were transmitted through meiosis (Figure 2.4G) when CASH transgene was present. We postulate that some changes induced by blocking histone

deacetylation either are transient or are corrected by other chromatin factors, such as DNA methylation, whereas others are heritable by maintaining specific chromatin states, as observed in mating-type and telomeric loci in yeast (Braunstein et al., 1993) and position-effect variegation in *Drosophila* (De Rubertis et al., 1996) (see below). We have recovered some plants that carry silenced transgenes (sensitive to kanamycin) but maintain abnormal phenotypes, implying that changes induced by epigenetic modifications on histones may be selected and maintained.

The role of histone deacetylation and DNA methylation in plant gene regulation and development

HDs are involved in several pathways for transcriptional repression (Pazin and Kadonaga, 1997; Kadosh and Struhl, 1998b). The HD repressor complex, including Sin3, can be recruited to mediate gene silencing or transcriptionally competent states by sequence-specific DNA-binding proteins, such as N-CoR, SMRT, Mad/Max, and E2F-Rb (Heinzel et al., 1997; Pazin and Kadonaga, 1997; Brehm et al., 1998; Nicolas et al., 2000). Alternatively, acetylated lysine residues can serve as signals for transcriptional silencing or activation (De Rubertis et al., 1996). In organisms that have both DNA and histone modifications, HDs either exist within a complex containing MeCP2 or MBD2 (Nan et al., 1998; Ng et al., 1999) or directly interact with Dnmt-1 (Fuks et al., 2000), implying that DNA methylation represses gene activity through changes in histone deacetylation. Four lysine residues, 5, 8, 12, and 16 can potentially be modified. It is interesting to note that hyperacetylation of lysine 14 on H3 and lysine 5 on H4 is

associated with gene activation, whereas acetylation of lysine 12 on H4 is correlated with silencing of some genes (Turner, 1991; De Rubertis et al., 1996; Pazin and Kadonaga, 1997). An increase in H4 hyperacetylation induced by antisense *AtHD1* expression (Fig 2.6) may disrupt both negative and positive circuits of gene regulation in the CASH plants, resulting in pleiotropic effects on plant gene regulation and development.

Although CASH plants display many changes, genomic DNA methylation is maintained at the same levels as in the control plants in the rDNA, centromeres, and *SUP* (Figure 2.7). Our data support the notion that histone deacetylation is directly involved in gene silencing, whereas DNA methylation may recruit HDs to silence genes or act upstream of histone deacetylation.

Reduction in the type and amount of HDs produced affects stochastic interactions with transcriptional factors or chromatin proteins and results in gene activation. The onset of a series of phenotypes during CASH plant development may reflect this effect on the multiple independent transgenic plants. In some plants, e.g., CASH74, aberrant phenotypes were observed during selfing, implying that inhibiting histone deacetylation might reactivate some factors, such as transposable elements, causing unstable mutations. Alternatively, if the antisense *AtHD1* transgene is not silenced in subsequent generations or segregated away from a particular phenotype, it may induce additional epimutations (Kakutani et al., 1996). Collectively, current data suggest that epigenetic regulation involving histone modifications plays an important role in morphogenesis and possibly evolution of plant form and function.

Evidence supports that a natural variation of asymmetrical flower development in *Linaria vulgaris* (Cubas et al., 1999) is because of epimutation in genomic DNA methylation in *Cycloidea* (*Lcyc*), a gene encoding a transcriptional factor. Also, suppression of mutations in *SUP* alleles in *Arabidopsis* is correlated with DNA methylation (Jacobsen and Meyerowitz, 1997). However, MOM, a protein predicted with chromatin remodeling motifs, is associated with transcriptional gene silencing independent of DNA methylation (Amedeo et al., 2000). It will be interesting to know how the methylated alleles cause phenotypic changes and whether histone deacetylation is involved in the process of these epimutations.

METHODS

Cloning of HD gene in *Arabidopsis*

PCR was used to amplify *AtHD1* fragments from total cDNA of *Arabidopsis thaliana* (Columbia) with the use of primers designed from human *HD1* (Taunton et al., 1996) (Figure 2.1A). Sequences of three cDNA fragments matched an expressed sequence tag (G11C3T7) and *AtHD1* (AF014824) in the *Arabidopsis* database. The insert was sequenced, and the expressed sequence tag clone was designated pAtHD1-2.

Production of constitutive antisense HD (CASH) transgenic plants

A full-length cDNA fragment released from the pAtHD1-2 was ligated into the *Xba*I-*Kpn*I sites of the pMON10098 vector (Klee et al., 1991). The expression of the antisense transgene is driven by a 35S cauliflower mosaic virus constitutive promoter.

Agrobacterium-mediated transformation (Bechtold and Pelletier, 1998) was used to produce transgenic *A.thaliana* (Columbia). About 30,000 seeds (T0) were sterilized and germinated on Murashige/Skoog medium (Sigma) containing 50 mg/liter kanamycin. A total of 157 resistant seedlings (T1) were obtained and screened for the presence of the transgene by PCR. The primers were as follows: 35S: 5'-TGA CGC ACA ATC CCA CTA TCC TTC GCA-3'; AtHD1-A3: 5'-CCT GAT ACA GAG ACT CCC GAG GTT GAT-3'. Eleven homozygous CASH plants (T2 and T3) were further characterized in this study. Antisense DNA-methyltransferase (MET1) transgenic plants were also produced with the use of a full-length cDNA (Finnegan and Dennis, 1993) and the same vector. The detailed characterization of 105 antisense-MET1 plants was omitted. One plant (no.407) was used as a demethylation control in the DNA blot analysis. All plants were grown in vermiculite and mixed with 10% soil in a growth chamber with growth conditions of 18/22ºC (day/light) and 14 h of illumination per day. Photographs were taken with a Pentax (Lyndhurst, NJ) camera with a macro lens.

Nucleic acid isolation and detection

RNA and DNA were isolated from at least five leaves of each plant in the same developmental stages as previously described (Chen and Pikaard, 1997). Total RNA (20 μg) or DNA (2 μg) was subjected to electrophoresis in an agarose-formaldehyde or agarose gel and blotted onto Hybond-N+ membrane (Amersham Pharmacia). To make antisense or sense RNA probe, the plasmid pAtHD1-2 was linearized by digestion with *Xho*I or *Xba*I, respectively, and was used as a template in separate reactions to generate

single-stranded RNA with *in vitro* synthesis kits (Promega). Sense and antisense *AtHD1* was synthesized by the T3 and T7 RNA polymerases, respectively. The antisense *SUP* was synthesized with the use of SP6 RNA polymerase as previously described (Sakai et al., 1995). The DNA probe was prepared by a random priming method. Hybridization was performed following the method of Church and Gilbert (Church and Gilbert, 1984). The DNA and RNA blots were washed at 65ºC in 2x SSC/0.2% SDS for 30-60 min (1x SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), and hybridization signals were detected by digital imaging analysis or exposure to Kodak X-ray film.

Antibody production, histone isolation, and immunoblotting

The cDNA fragment containing the N terminus of AtHD1 (1-199 amino acids) was subcloned in frame in the pET21a expression vector (Novagen), which was then transformed into *Escherichia coli* (strain BL21). Recombinant AtHD1 protein was induced with isopropyl-β-D-thiogalactoside, purified by column chromatography, and sent to Cocalico Biologicals (Reamstown, PA) for polyclonal antibody production in rabbit. The antisera were used in 1:500 dilution for immunoblotting. The antibodies detected a major band (≈ 25 KDa) and a minor band (≈ 70 KDa) in recombinant protein (data not shown) but a single band (≈ 60 KDa, expected sizes) in crude protein extracts. The minor band detected in recombinant proteins may be caused by the presence of a small amount of nonspecific protein retained after histidine affinity purification. Antibodies against tetra-acetylated histone H4 or nonacetylated histone H4 (H4) were purchased from Serotec and Upstate Biotechnology (Lake Placid, NY). Protein crude

extracts and histone fractions were prepared as previously described (Chen and Pikaard, 1997) and subjected to electrophoresis in 8 and 15% SDS/PAGE, respectively. The immunoblots were probed with antisera AtHD1, tetra-acetylated histone H4, and H4; antibody binding was then detected by enhanced chemiluminescence (Amersham Pharmacia).

CHAPTER III

GENETIC CONTROL OF DEVELOPMENTAL CHANGES INDUCED BY DISRUPTION OF *ARABIDOPSIS* HISTONE DEACETYLASE 1 (*ATHD1*) EXPRESSION *

OVERVIEW

Little is known about the role of genetic and epigenetic control in the spatial and temporal regulation of plant development. Overexpressing antisense *Arabidopsis thaliana HD1* (*AtHD1*) encoding a putative major histone deacetylase induces pleiotropic effects on plant growth and development. It is unclear whether the developmental abnormalities are caused by a defective *AtHD1* or related homologs and are heritable in selfing progeny. We isolated a stable antisense *AtHD1* (CASH) transgenic line and a T-DNA insertion line in exon 2 of *AtHD1*, resulting in a null allele (*athd1-t1*). Both *athd1-t1* and CASH lines display an increased level of histone acetylation and similar developmental abnormalities, which are heritable in the presence of antisense *AtHD1* or in the progeny of homozygous (*athd1-t1/athd1-t1*) plants. Furthermore, when the *athd1-t1/athd1-t1* plants are crossed to wild-type plants, the

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* This chapter is reformatted from "Genetic control of developmental changes induced by disruption of *Arabidopsis* histone deacetylase 1 (*AtHD1*) expression" by Tian, L., Wang, J., Fong, M., K., Chen, M., Cao, H., Gelvin, S. B., and Chen, Z. J. (2003). *Genetics* Vol. 165, pp. 399-409.

pleiotropic developmental abnormalities are immediately restored in the F1 hybrid, which correlates with *AtHD1* expression and reduction of histone H4 Lys12 acetylation. Unlike the situation with the stable code of DNA and histone methylation, developmental changes induced by histone deacetylase defects are immediately reversible, probably through the restoration of a reversible histone acetylation code needed for the normal control of gene regulation and development.

INTRODUCTION

Plant development is plastic and affected by genetic, epigenetic, and environmental factors. Vegetative and reproductive (inflorescence) development is initiated at shoot apical meristems and/or axillary meristems that can be induced by internal and external signals (Bernier, 1986; Walbot, 1996; Bleecker and Patterson, 1997; Meyerowitz, 1997). The molecular mechanisms underlying the plastic nature of plant development are largely unknown. Both genetic and epigenetic changes may contribute to the developmental plasticity of plants (Walbot, 1996; Meyerowitz, 1997; Srinivas, 2000; Habu et al., 2001; Martienssen and Colot, 2001).

Epigenetic regulation is a major aspect of gene control by which heritable changes in gene expression occur without an alteration in DNA sequence. Changes in chromatin structure may affect accessibility of promoter elements to the transcriptional machinery and thus affect transcription. Modifications on core histones and their associated covalent bonds are known as the “histone code” (Jenuwein and Allis, 2001). Changes in the histone code may facilitate fine tuning of gene expression in response to

developmental programs or changes in environmental signals. Disruption of histone deacetylases results in growth and developmental abnormalities and aging in yeast cells (Imai et al., 2000). These developmental changes are associated with down- or up-regulation of several hundred genes (Bernstein et al., 2000; Robyr et al., 2002), suggesting that histone deacetylases are key regulators of eukaryotic development. Histone acetylation and deacetylation may also play a role in gene expression and development in animals. For examples, mouse histone deacetylase 1 (HD1) is a growth factor-inducible gene (Bartl et al., 1997). Histone hyperacetylation plays a role in establishing stable states of differential gene activity during gastrulation in *Xenopus* (Almouzni et al., 1994).

The *Arabidopsis* genome contains 18 putative HDs (*HDAs* or *HDACs*) and 12 putative histone acetyltransferases (*HATs*) distributed among all five chromosomes (Arabidopsis Genome Initiative, 2000; Initiative, 2000; Pandey et al., 2002). There are four classes of histone deacetylases in plants (Lusser et al., 2001). First, the RPD3-like protein is the major histone deacetylase in yeast and mammals. Mutations in RPD3 affect ~500 genes in yeast (Bernstein et al., 2000; Robyr et al., 2002). *Arabidopsis thaliana* HD1 (AtHD1; GenBank accession no. AAB66486), also known as AtHDA19 (Pandey et al., 2002), is a putative homolog of yeast RPD3 and the major histone deacetylase (HD1) in humans and mice (Rundlett et al., 1996; Taunton et al., 1996; Bartl et al., 1997; Lusser et al., 1997). This class consists of at least two isoforms (HD1B-I and –II) in maize embryos (Rossi et al., 1998; Lechner et al., 2000) and four genes (*HDA6*, *-7*, *-9*, and *-19*) in *Arabidopsis*. One of the maize genes, *ZmRpd3* or *HD1B-II*

(Lechner et al., 2000), complements a yeast *rpd3* null mutant (Rossi et al., 1998). Second, on the basis of DNA sequences, HDA- and HOS-like HDs are related to RPD3 but have different specific activities in deacetylating histones (Vidal and Gaber, 1991; De Rubertis et al., 1996; Rundlett et al., 1996; Rundlett et al., 1998). Eight *Arabidopsis* genes in this category are predicted and some of them may be distinct from other members in this group (Pandey et al., 2002). Third, HD2 is a plant-specific histone deacetylase (Lusser et al., 1997) localized in the nucleolus. At least two isoforms exist in maize and four genes in *Arabidopsis* (Lusser et al., 1997; Dangl et al., 2001). Fourth, a NAD-dependent HD (SIR2-like protein) forms a newly discovered class of HDs. In yeast, the deacetylation by SIR2 is NAD dependent and possibly coupled to ADP ribosylation (Tanner et al., 2000). *Arabidopsis* has two SIR2-like genes (Pandey et al., 2002).

The role of histone acetylation and deacetylation in plant gene regulation is poorly understood (Verbsky and Richards, 2001; Li et al., 2002). Transgenic plants treated with propionic or butyric acid, chemical inhibitors of histone deacetylases, display increased levels of DNA methylation and epigenetic variegation (ten Lohuis et al., 1995). HC toxin, the host-selective toxin of the maize fungal pathogen *Cochliobolus carbonum*, inhibits histone deacetylases in host plants (Brosch et al., 1995). Blocking deacetylation by sodium butyrate or trichostatin A derepresses silent rRNA genes subject to nucleolar dominance (Chen and Pikaard, 1997). In a genetic screen for auxin-insensitive mutants, Murfett et al. 2001 identified mutagenized plants with enhance expression of *gusA* and *hptII* transgenes. Further analysis indicated that several of these

mutations were in *AtHDA6*, a presumed histone deacetylase, suggesting that this gene is important for transcriptional regulation of the promoters controlling these transgenes. Indeed, *AtHDA6* is required to maintain DNA methylation patterns induced by double-stranded RNA (Aufsatz et al., 2002). Overexpression of *OsHDAC1*, a putative histone deacetylase 1 gene in rice, is correlated with the induction of OsHDAC1, increased growth rate, and altered plant morphology (Jang et al., 2003). Antisense-mediated down-regulation of the *Arabidopsis* genes *AtHD1* or *AtHD2*, which putatively encode histone deacetylases, results in a variety of abnormal phenotypes in early and late stages of *Arabidopsis* development (Wu et al., 2000a; Wu et al., 2000b; Tian and Chen, 2001).

Down-regulation of histone deacetylation induces pleiotropic effects on *Arabidopsis* development (Tian and Chen, 2001), suggesting that histone deacetylation directly or indirectly affects the expression of many genes in regulatory networks (Finnegan, 2001). However, it is not known whether the abnormal phenotypes observed in the antisense *AtHD1* plants result from the disruption of single gene or related homologs in the *Tad* multi-gene family. Moreover, it is unclear whether disruption of histone deacetylation induces other epigenetic lesions and whether the induced phenotypic changes are heritable in the absence of the original *AtHD1* defect. Finally, the acetylation code is reversible and dynamic because core histones can be acetylated or deacetylated through the activities of histone acetyltransferases (HATs) or histone deacetylases (HDs, HDAs, HDACs) during growth and development (Allfrey et al., 1964), whereas the code of DNA and histone methylation is stable because no active demethylation pathway has been identified (Jenuwein and Allis, 2001). However, both

the stable and reversible codes can be stored in chromatin and propagated through meiosis. Thus, compared to the stable code, the reversible histone acetylation code may contribute differently to gene regulation and development. In this study, we show that defects induced by expressing antisense *AtHD1*, or by T-DNA insertion mutagenesis of *AtHD1*, result in similar developmental pleiotropy. Unlike the situation with *ddm1* (decrease in DNA methylation) mutants, defects in *AtHD1* do not induce cumulative epigenetic lesions after four to five generations of selfing. Furthermore, genetic analyses indicate that disruption of developmental programs, *AtHD1* expression, and histone acetylation is immediately corrected in *AtHD1/athd1-t1* heterozygous plants probably through restoration of the reversible histone acetylation code.

RESULTS

Isolation of a stable antisense *AtHD1* transgenic plant

AtHD1 is constitutively expressed throughout plant development, although a slightly higher level of expression is detected in reproductive tissues. Expressing CASH in transgenic *Arabidopsis* plants results in the reduction of tetra-acetylated H4 and a variety of developmental abnormalities (Tian and Chen, 2001). Many lines showed variable phenotypes and deceased in early developmental stages. To understand better the *AtHD1* effects, we isolated a CASH126 line that displayed consistent phenotypes in selfing progeny. CASH126 plants displayed phenotypes in the vegetative and flowering stages in the fourth generation (Figure 3.1) similar to the “pinhead” phenotype observed

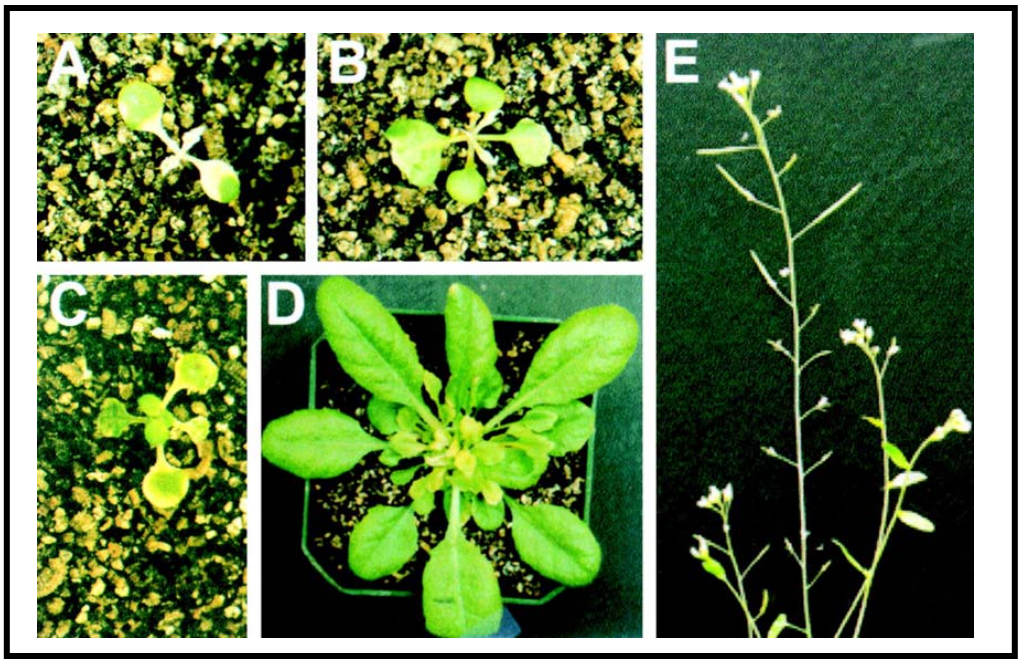


Figure 3.1. Development and inheritance of phenotype in a stable CASH 126 plant.

Only the plants in the fourth generation are shown.

(A) A 7-day-old seedling displayed a defective SAM.

(B) The defective SAM in the 10-day-old seedling developed a pair of lobed true leaves.

(C) A lateral SAM was initiated after 3 weeks.

(D) The transition from vegetative to flowering development was delayed.

(E) In the flowering stage, the plants display low fertility. About 75% of siliques were small and contained few viable seeds.

in *Argonaut* mutants (Mossman et al., 1998; Lynn et al., 1999; Morel et al., 2002). The plants had defective shoot apical meristems and grew slowly *in vitro*. After the plants were transferred to soil, cotyledonary leaves became bleached and yellow. The first pair of true leaves was very small, narrowly elongated, and lacked chlorophyll (Figures 3.1A and 3.1B). Starting with the second pair of true leaves, leaf development appeared normal except for a delay of the transition from adult vegetative to inflorescence development (Figures 3.1C and 3.1D). CASH126 plants developed additional true leaves, probably resulting from a developmental transition initiated from axillary meristems. Vegetative development could continue up to 4-5 weeks under long-day (16/8 hr of day/night) conditions. Eventually inflorescences developed from lateral meristems similar to those of wild-type plants. Although some inflorescence branches were sterile (Figure 3.1 E), seeds could be harvested and germinated and the resulting plants showed developmental abnormalities similar to those observed in plants of previous generations.

One explanation for the abnormal phenotypes in CASH126 plants would be that insertion of the transgenes into the genome disrupted a locus important for plant development, such as a homeotic gene controlling flowering. The endogenous *AtHD1* is a single-copy gene located on chromosome 4 (Arabidopsis Genome Initiative, 2000; Tian and Chen, 2001). We analyzed the copy number of transgenes in CASH126 plants using DNA blot analysis and detected two fragments for the transgenes (Figures 3.2A and 3.2B), indicating that these transgenic plants contain two copies of the *AtHD1* transgene. We further used a PCR method (Zhou et al., 1997) to identify the transgene

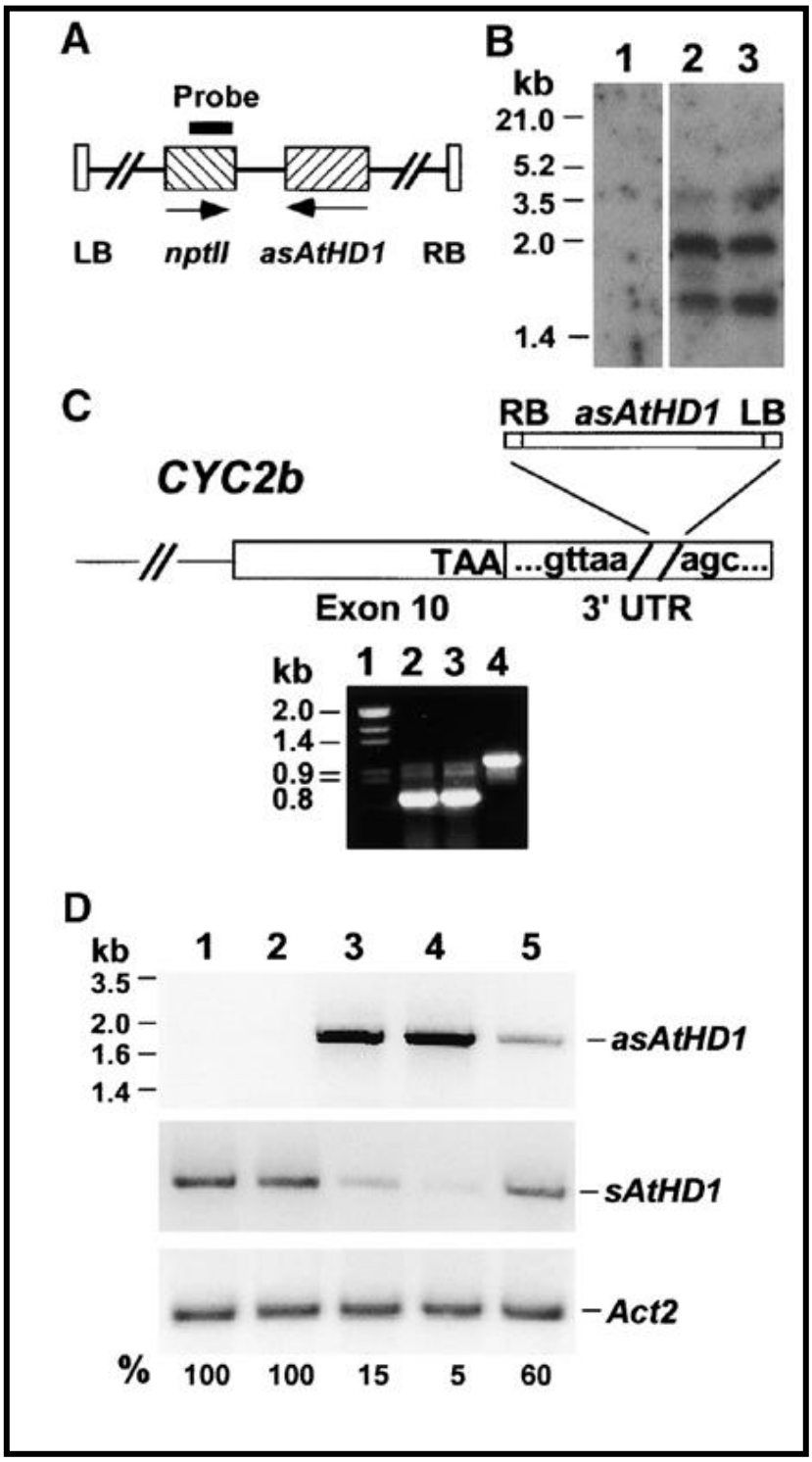
Figure 3.2. Detection of *AtHD1* transgenes in the genome and expression of the transgene and endogenous *AtHD1* in CASH126 plants.

(A) Map of the transgene construction indicating the *nptII* and antisense *AtHD1* (*asAtHD1*) genes located between the left and right T-DNA borders (LB and RB, respectively).

(B) DNA blot containing genomic DNA digested with *Bam*HI and *Eco*RI was hybridized with the *nptII* probe. Two copies of the transgene containing *nptII* and *AtHD1* were detected in CASH126 plants (lane 2 and 3), whereas no transgene was present in the control of plant (lane 1).

(C) One copy of the *asAtHD1* transgene is inserted into the 3' untranslated region (UTR) of the *CYC2b* gene, as shown in the top diagram. RT-PCR analysis indicated that *CYC2b* expression was similar in control (lane 2) and CASH126 (lane 3) plants. DNA size markers are shown in lane 1. PCR product amplified from a genomic DNA is shown in lane 4.

(D) Strand-specific RT-PCR analysis in CASH126 plants. (Top) *asAtHD1* was highly expressed in CASH126 plants (lanes 3-5) and undetectable in control plants (ecotype Columbia, lane 1, and ecotype Ws, lane 2). (Middle) RT-PCR and DNA blot analyses were performed to detect the expression levels of sense *AtHD1* (*sAtHD1*). The strand-specific RT-PCR product was resolved by electrophoresis through a 1.0% agarose gel and transferred onto a Hybond-N+ membrane (Amersham Pharmacia). The blot was hybridized with an *AtHD1* full-length cDNA probe. The relative expression levels (%) of sense *AtHD1* (*sAtHD1*) transcripts were estimated using *Act2* as an internal control.


A
Probe
LB
nptII
asAtHD1
RB
B
1 2 3
kb
21.0
5.2
3.5
2.0
1.4
C
RB asAtHD1 LB
CYC2b
TAA ...gttaa/ /agc...
Exon 10
3' UTR
kb
1 2 3 4
2.0
1.4
0.9
0.8
D
kb
1 2 3 4 5
3.5
2.0
1.6
1.4
asAtHD1
sAtHD1
Act2
%
100 100 15 5 60

insertion sites in the genome in CASH126. As expected, two fragments were amplified from DNA of transgenic plants. DNA sequencing results indicated that one of the transgene insertion sites was located ~150bp downstream of the *CYC2b* stop codon of chromosome 4. The insertion did not affect *CYC2b* RNA accumulation; we detected an approximately equal amount of *CYC2b* transcripts in the control and CASH126 plants (Figure 3.2C). The other insertion site remains unknown, as the sequenced fragment did not match *Arabidopsis* sequence in the database.

We used strand-specific RT-PCR to determine the expression levels of antisense and endogenous *AtHD1* genes in transgenic and control plants. Antisense *AtHD1* (*asAtHD1*) transcripts were abundant in each of three CASH126 plants that were derived from single-seed descent, but absent in control plants (Figure 3.2D, lanes 1 and 2). To determine the expression levels of the endogenous sense *AtHD1* (*sAtHD1*) transcripts, we performed a semiquantitative RT-PCR analysis using *Act2* (An et al., 1996) as an internal control. The *sAtHD1* and *Act2* transcripts were amplified and subjected to DNA blot analysis, and the relative expression ratios of *sAtHD1* and *Act2* transcripts were calculated in control and CASH126 plants. The results indicated that endogenous sense *AtHD1* transcripts were greatly reduced in the CASH126 plants, ranging from 5 to 60% of the level detected in the control plants. It is notable that *sAtHD1* RNA levels were inversely correlated with the amount of *asAtHD1* transcripts (Figure 3.2D: compare lanes 3-5). Overexpressing the antisense *AtHD1* gene reduced the accumulation of endogenous *AtHD1* transcripts, suggesting that the penetrance of developmental abnormalities is correlated with the level of *sAtHD1* transcript.

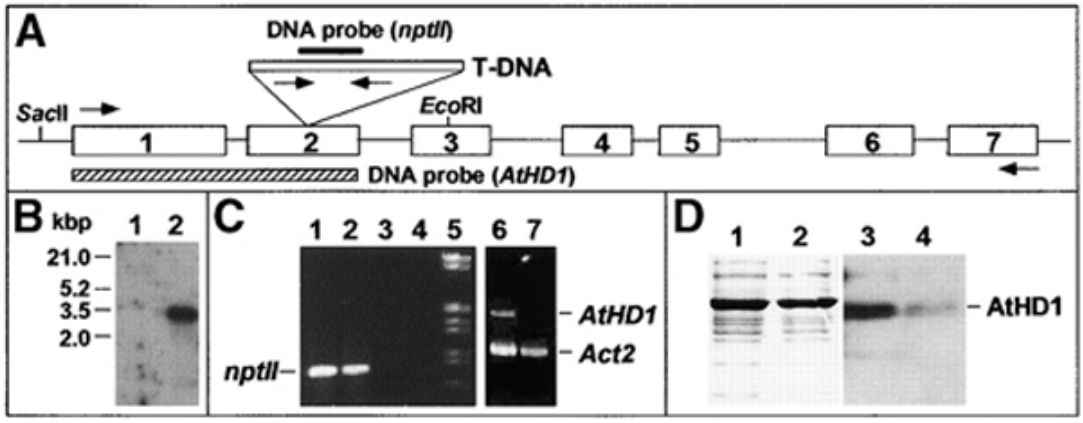


Figure 3.3. A null mutation (*athd1-t1*) is generated by T-DNA insertion into the *AtHD1* gene.

(A) *AtHD1* contains seven exons. The T-DNA is inserted into exon 2. Primers used in RT-PCR for detecting *AtHD1* expression are shown above the first exon and below the last exon. Primers used to amplify the *nptII* gene in RT-PCR reactions (C) and the *nptII* fragment used for DNA blot analysis (B) are shown below and above the T-DNA diagram, respectively. Striped box indicates the location of the *AtHD1* fragment used for DNA blot analysis in Figure 3.7.

(B) DNA blot analysis shows a single T-DNA insertion in the *athd1-t1* line.

(C) RT-PCR analysis indicates that *nptII* gene expression is detected in the *athd1-t1* plants (lanes 1 and 2), but absent in wild-type plants (ecotype Columbia, lane 3; Ws, lane 4). T-DNA insertion produces null alleles in the *AtHD1* locus. *AtHD1* expression was detected in Ws (lane 6) but absent in the *athd1-t1* line (lane 7). *Act2* was amplified as a positive internal control.

(D) AtHD1 production is reduced in the *athd1-t1* plants. Crude protein extracts (25 µg) were subjected to electrophoresis through an SDS-polyacrylamide gel and immunoblotted onto Immobilon-P (Millipore, Bedford, MA) or Hybond-ECL (Amersham) membranes. The membrane was probed with antibodies against the N terminus of AtHD1. A band cross-reacting with anti-AtHD1 antibodies in *athd1-t1* plants was reduced to only a trace amount (lane 4) compared to the band detected in the control plant (lane 3). A protein loading control is shown in an 8% SDS-PAGE stained with Coomassie Blue (lanes 1 and 2).

A T-DNA insertion in *AtHD1* results in an *AtHD1* null mutation

Phenotypic abnormalities in transgenic plants may fluctuate because of variable levels of transgene expression (Figure 3.2D). Moreover, although down-regulation of *AtHD1* in antisense transgenic plants results in a variety of developmental abnormalities, it is unclear whether these phenotypic changes result from disrupted expression of *AtHD1*. The expression of antisense *AtHD1* may also affect the expression of other genes, because there are several *AtHD1* homologs (Pandey et al., 2002). We therefore identified an *Arabidopsis* (ecotype Ws) mutant line that contains a T-DNA insertion in the *AtHD1* gene (*athd1-t1*; Figure 3.3A). DNA blot analysis indicated only one T-DNA insertion in the genome (Figure 3.3B). Using a PCR-based reverse genetic approach (Krysan et al., 1996) and DNA sequence analysis, we determined that the T-DNA was inserted into exon 2 of *AtHD1* (Figure 3.3A). This insertion caused a null mutation of *AtHD1*; no *AtHD1* expression was detected in homozygous insertion lines (lane 7, Figure 3.3C). Furthermore, Western blot analysis using antibodies against the N terminus of AtHD1 (Tian and Chen, 2001) indicated that, in the homozygous mutant line, the level of AtHD1 protein was greatly reduced (Figure 3.3D, lane 4). Only a small amount of protein (~5% that of the wild type) was detected in homozygous plants, most likely resulting from cross-reaction of the antibodies to other AtHD1 homologs. For example, AtHD1 and AtHD6 share 69 and 84% of amino-acid sequence identity and similarity in the N termini, respectively. Alternatively, the low level of AtHD1 detected could be due to residual expression of *athd1-t1*; however, a different-sized protein would be observed because the T-DNA was inserted in the coding sequence (Figure 3.3A).

Taken together, the data suggest that insertion of T-DNA into exon 2 of *AtHD1* generated an *AtHD1* null mutation.

CASH126 and *athd1-t1* plants display similar developmental abnormalities

To determine whether CASH126 and *athd1-t1* plants show similar changes in early developmental stages, we grew them side by side in a growth chamber under short-day conditions (8/16 hr of day/night). The leaves of both CASH126 and *athd1-t1* plants were slightly chlorotic and showed disrupted radial symmetry (Figures 3.4A to 3.4D), probably resulting from defective shoot apical meristems as previously described (Figure 3.1). A prominent phenotype was the somewhat left-handed "twist" of the longitudinal axis of rosette leaves in *athd1-t1* plants (Figures 3.4B and 3.4F). This twist did not occur in other parts of the plants and was different from the previously described "lefty" mutants (Thitamadee et al., 2002). This left-handed twist was not obvious in the leaves of CASH126 plants (Figure 3.4C). However, under close examination, the leaves of CASH126 plants were also twisted (Figure 3.4G), although the orientation of the distortion was not consistent.

Both wild-type and *AtHD1* disruption lines flowered at approximately the same time, suggesting that vegetative development initiated from axillary meristems had little effect on flowering time under short-day conditions. In a separate experiment we grew plants under long-day conditions (16/8 hr of day/light). As with the CASH lines, *athd1-t1* lines flowered ~2-5 days later (31.9 ± 1.1 days, n = 43) than the wild-type plants (28.1 ± 2.3 days, n =42). Although severe phenotypes in the vegetative stage were often

Figure 3.4. CASH126 and *athd1-t1* plants show similar abnormal developmental phenotypes.

CASH126 and *athd1-t1* plants were grown side by side in a growth Chamber under short-day (A-H) or long-day (I-L) conditions.

(A) Wild-type plants (Ws) 3 weeks after germination.

(B) Homozygous *athd1-t1/athd1-t1* plants 3 weeks after germination.

(C) CASH126 plants 3 weeks after germination.

(D) Wild-type plant (Col, Columbia) 3 weeks after germination.

(E) Mature inflorescence branches in *athd1-t1* and Ws.

(F) Rosette leaves of CASH126 and control plants.

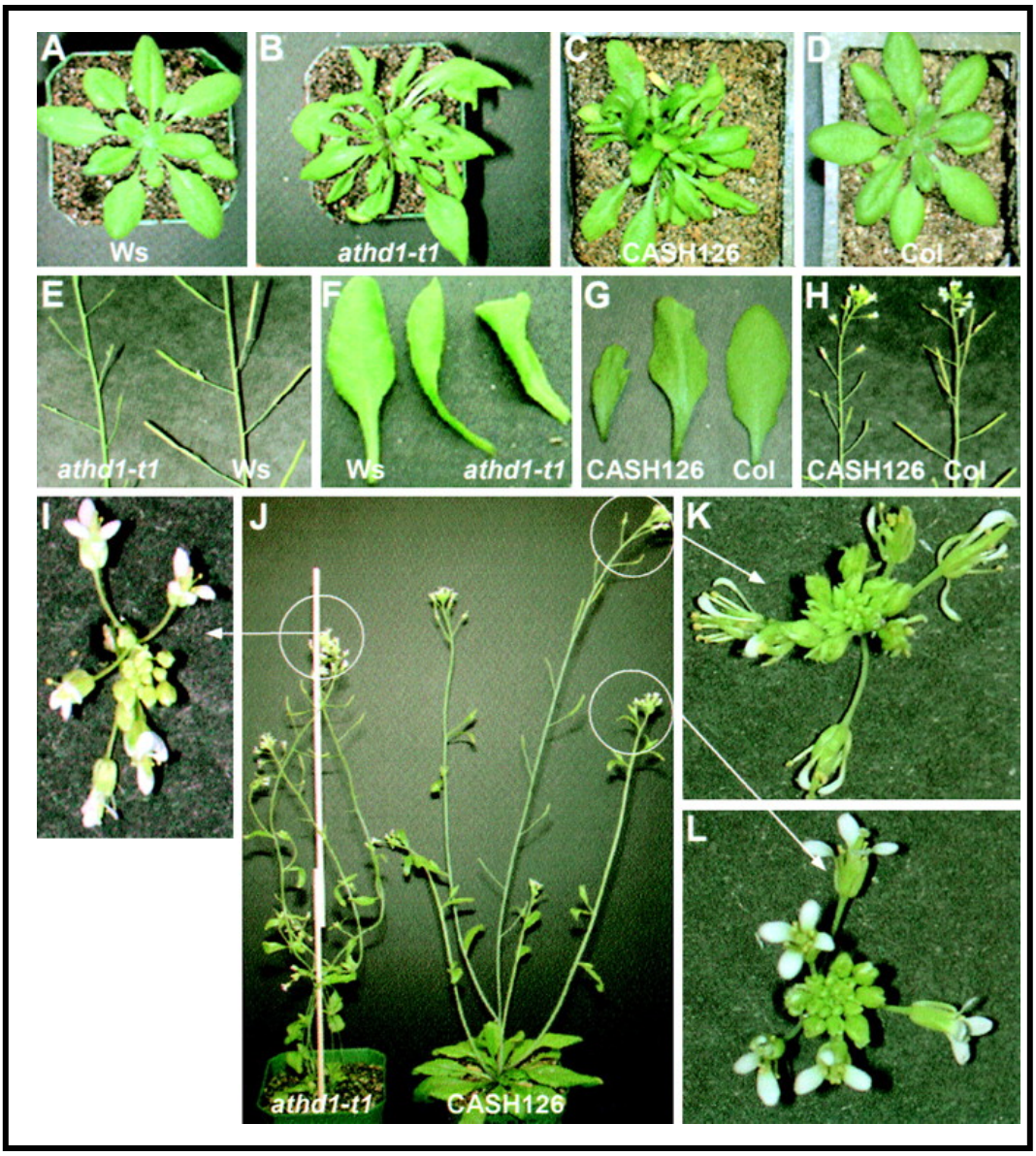
(G) Rosette leaves of Ws and athd1-t1 plants.

(H) Florescence branches of CASH126 and Columbia plants.

(I) Enlarged picture of mildly defective flowers in *athd1-t1* plants.

(J) Plant morphology of *athd1-t1* and CASH126 plants.

(K and L) Enlarged pictures of inflorescence branches showing severely defective flower organs (K) and mildly abnormal flowers (L) of CASH126 plants.


A
Ws
B
athd1-t1
C
CASH126
D
Col
E
athd1-t1
Ws
F
Ws
athd1-t1
G
CASH126
Col
H
CASH126
Col
I
J
athd1-t1
CASH126
K
L

observed under short-day conditions (Figures 3.4A to 3.4D, 3.4F, and 3.4G), similar abnormalities in the reproductive stage were observed under both long- and short-day conditions (Figures 3.4E, 3.4 H, and 3.4I to 3.4L).

CASH126 and *athd1-t1* plants developed abnormal flower structures, including reduced numbers of petals (Figures 3.4I, 3.4K, and 3.4L), split flowers (Figure 3.4K), and sterility (Figures 3.4E and 3.4H). The abnormal flower phenotypes in *athd1-t1* plants (Figure 3.4I) were not as severe as those of CASH126 plants (Figure 3.4J), but were more uniform. CASH126 lines, however, displayed a range of penetrance in developmental abnormalities, ranging from severe (Figure 3.4K) to mild (Figure 3.4L).

CASH126 and *athd1-t1* plants were partially sterile and had low seed set. To investigate the cause of sterility, we examined the flower structures of *athd1-t1* plants using light and scanning electron microscopy. Flower morphology of *athd1-t1* plants was irregular. Many flowers had missing petals and sepals (Figures 3.5B, 3.5C, and 3.5E) compared to the typical "crucifer-shaped" flowers of wild-type plants (Figures 3.5A and 3.5D). Some flowers had fewer than six stamens, and some of which were fused (Figure 3.5F). The stamens were short (Figure 3.5E). As a result, pollen would have difficulty reaching the "tall" sigma. The incompatibility between "impotent" male stamens and tall female stigmas is likely associated with sterility, because compared with wild-type stigmas that were completely covered with pollen grains (Figure 3.5G), only a few pollen grains could reach the stigmas of homozygous *athd1-t1* plants (arrows in Figure 3.5H). As a result, siliques of homozygous *athd1-t1* plants were short and contained few seeds (Figures 3.5M and 3.5O), whereas siliques of wild-type plants were

Figure 3.5. Abnormal flower development and sterility in *athd1-t1* plants.

(A) Wild-type (Ws) plants have a typical "crucifer" flower with four petals, six stamens, and a regular stigma.

(B and C) Transformation of the "crucifer" shape into an irregular form in *athd1-t1* plants resulting from fused petals (B) and missing flower parts (C).

(D) Normal flower morphology of wild-type plants revealed by scanning electron microscopy.

(E and F) Short stamens and missing petals (E) and fused stamens (F) in *athd1-t1* plants.

(G) The stigma of the wild-type flower is completely covered with pollen grains.

(H) Only a few pollen grains (arrows) are found on the stigma of *athd1-t1* plants.

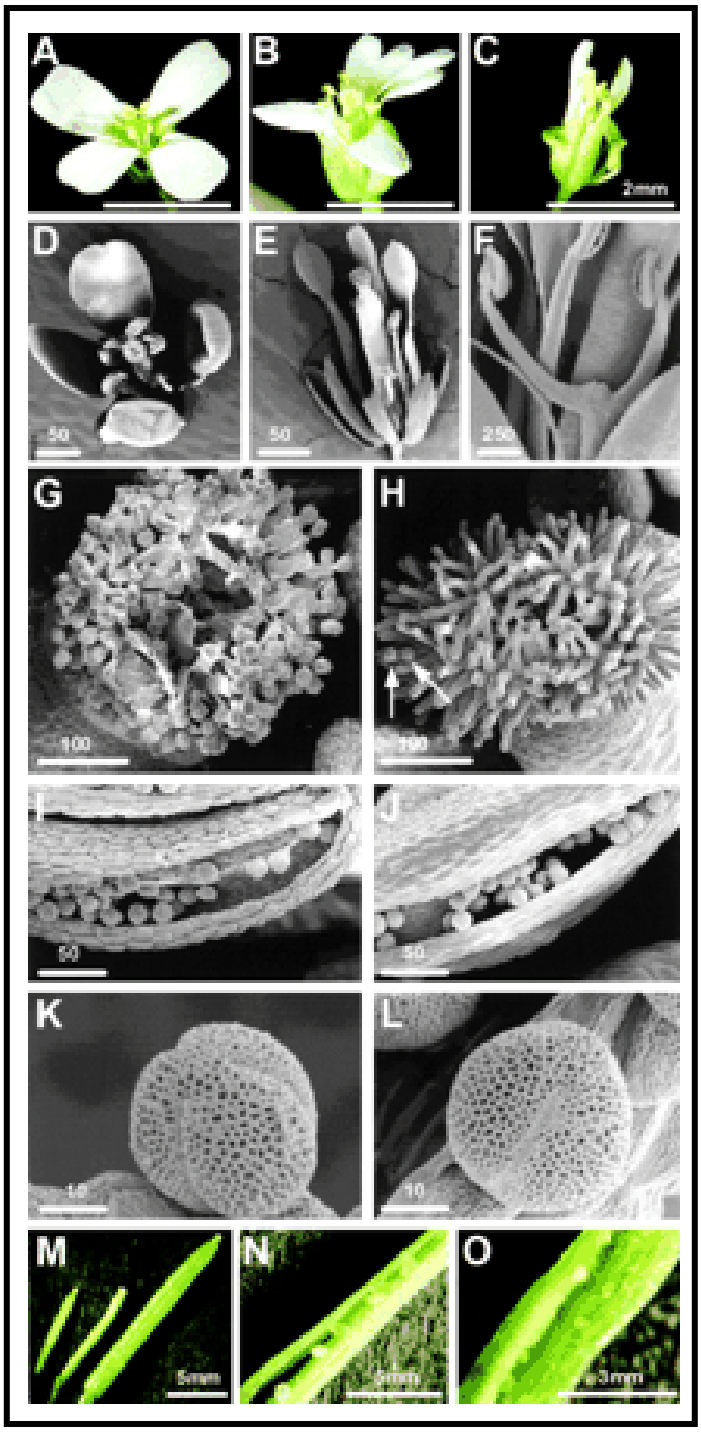
(I and J) Dehisced anthers showing normal pollen development in wild-type (I) and *athd1-t1* (J) plants.

(K and L) Pollen grains in Ws (K) and *athd1-t1* (L) anthers.

(M) Short and immature siliques (left and middle) developed in *athd1-t1* plants compared to the normal silique in the control (right).

(N) Normal seed development in Ws.

(O) Abortive seed development in *athd1-t1*.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5mm
5mm
3mm

long and contained many fully developed seeds (Figures 3.5M and 3.5N). Pollen grains of *athd1-t1* plants were apparently normal (Figures 3.5K and 3.5L). Although structural incompatibility between stigma and stamen is likely related to sterility, we do not rule out other possibilities, such as biological incompatibility between pollen and stigma interactions during pollination in *athd1-t1* plants, which may also cause sterility.

The pleiotropic phenotype resulting from *AtHD1* defects is immediately restored in heterozygous plants and displays mendelian segregation

The developmental defects of CASH and *athd1-t1/athd1-t1* plants are consistently observed in selfing generations. However, it is unknown whether the phenotypic abnormalities were dependent on the continued deficiency in *AtHD1* expression and core histone acetylation profiles. To address this, we made F1 hybrids between wild-type (Ws) and homozygous *athd1-t1/athd1-t1* plants (Figure 3.6A) and examined the phenotypes in the resulting F1 hybrids and F2 siblings. In all the F1 plants examined, most of the developmental abnormalities, including rosette leaf morphology and fertility, were reversed to those of wild-type plants, except that F1 plants still developed slightly shorter siliques. These results indicate that most of the developmental defects induced by the homozygous mutants are immediately corrected in *AtHD1/athd1-t1* heterozygotes. Indeed, the expression level of *AtHD1* in the heterozygous plants was equal to that of wild-type plants (Figure 3.6B). Furthermore, the acetylation level of histone H4 Lys12 was increased approximately three-fold in the *athd1-t1/athd1-t1* homozygous mutants compared to the wild-type plants (Figure 3.6C).

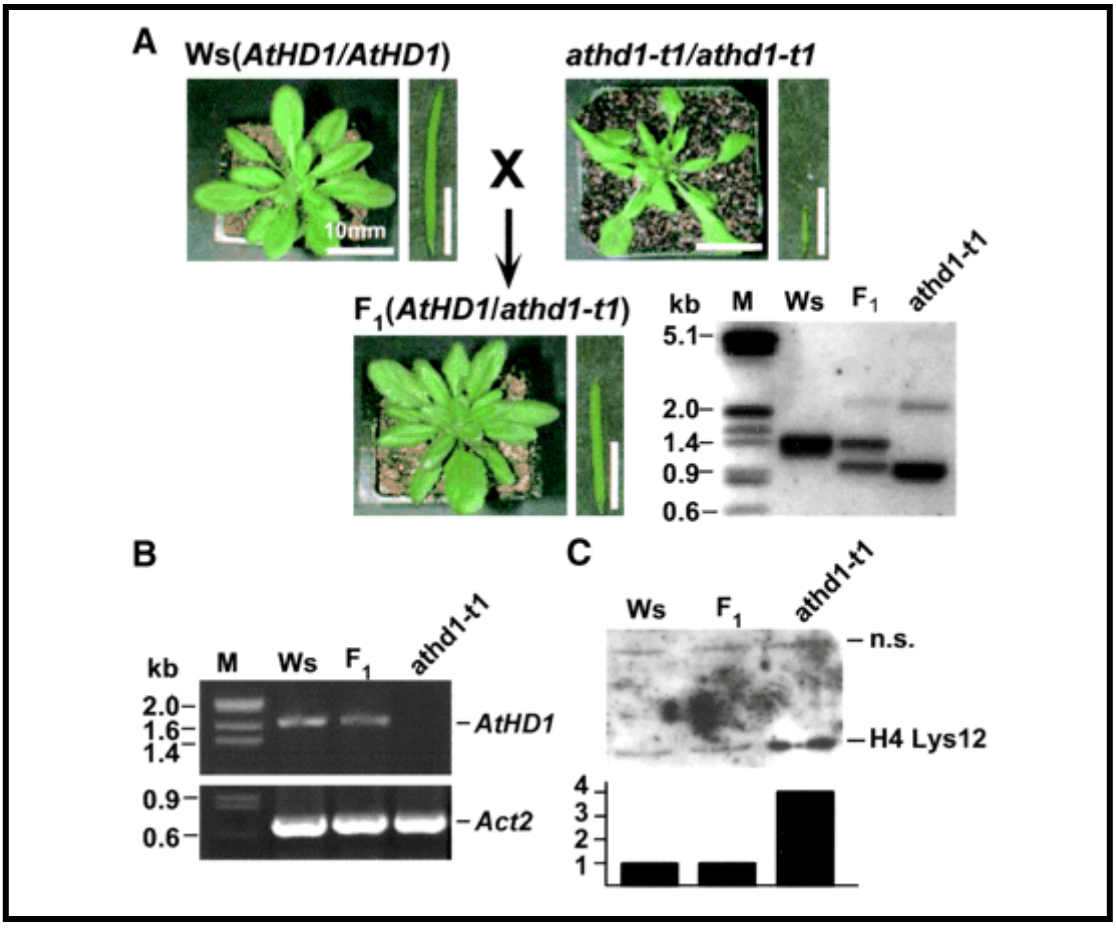


Figure 3.6. The pleiotropic phenotype of *athd1-t1* line is immediately restored in heterozygous plants.

(A) Developmental abnormalities in rosette leaves and siliques of *athd1-t1/athd1-t1* homozygous plants were restored in F1 (*AtHD1/athd1-t1*) progeny. The F1 hybrid, heterozygous for the *athd1-t1* locus, was generated by crossing a wild-type Ws plant with a homozygous athd1-t1 plant. (Right) Genomic DNA was digested with *Eco*RI and *Sac*II, subjected to electrophoresis through a 1% agarose gel, and blotted onto a Hybond N+ membrane. The blot was hybridized with a DNA fragment containing exons 1 and 2 of *AtHD1* (see Figure 3.3A). The F1 plant contained a normal *AtHD1* allele and an *athd1-t1* allele.

(B) Equal quantities of *AtHD1* transcripts were detected by RT-PCR in the F1 and Ws plants, but no *AtHD1* transcript was detected in the *athd1-t1/athd1-t1* homozygous plants. RT-PCR amplification of *Act2* was used as an internal control.

(C) Western blot indicates that histone H4 Lys12 acetylation is increased in the homozygous *athd1-t1* plants. Hybridization intensities of a nonspecific (n.s.) protein detected by the anti-H4 Lys12 antibody were used as internal controls to calculate the relative ratio of H4 Lys12 acetylation accumulation, which is shown in a histogram below the Western blot.

H4 Lys12 is one of the primary deacetylation sites targeted by RPD3 in yeast (Vidal and Gaber, 1991; Rundlett et al., 1996). The H4 Lys12 level was reversed to that of the wild-type plants in the heterozygous plants, implying that acetylation at some specific sites is responsible for the changes in developmental abnormalities and gene expression in these plants. In the F2 progeny, the wild-type and abnormal phenotypes segregated 3:1, cosegregating with the level of *AtHD1* expression in these plants. These data indicate that the developmental abnormalities are dependent on disruption of *AtHD1* expression and of some specific acetylation sites (e.g., H4 Lys12) in the *athd1-t1* lines.

Does *athd1-t1* induce additional epigenetic changes?

The *ddm1* mutant induces epigenetic lesions in addition to those observed in the original *ddm1* mutant (Stokes and Richards, 2002). To determine whether *athd1-t1* acts as an epigenetic modulator, we examined phenotypic changes in the plants derived from single-seed descent of one *athd1-t1/athd-t1* homozygous plant. Consistent with the results in CASH plants, within four generations of selfing we did not observe abnormal phenotypes in these plants in addition to developmental abnormalities observed in the original homozygous plants (Figure 3.7). Phenotypic abnormalities were less severe under long-day conditions than under short-day conditions, suggesting that the penetrance of phenotypes is dependent on day length. *AtHD1* expression was not detected in the plants after four generations of selfing. These data, together with the immediate restoration of normal development in *AtHD1/athd1-t1* heterozygous plants,

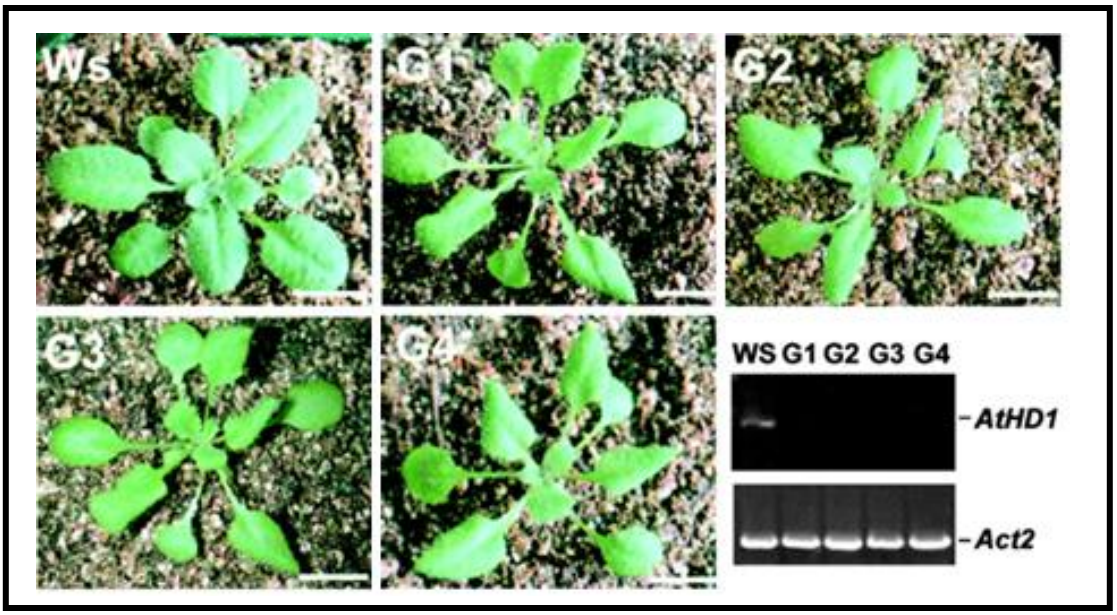


Figure 3.7. *athd1-t1/athd1-t1* plants showed constant developmental abnormalities during four generations (G1-G4) of selfing.

The plants were grown side by side in the same growth chamber. No AtHD1 expression was detected by RT-PCR in the homozygous *athd1-t1/athd1-t1* plants obtained in four generations of selfing.

suggest that developmental changes induced by disruption of *AtHD1* expression are reversible and dependent on *AtHD1* expression and histone deacetylation.

DISCUSSION

Heritable changes of developmental abnormalities induced by *AtHD1* disruption

Blocking histone deacetylation induces a wide range of developmental changes. The abnormal development includes defective shoot apical meristems (SAM), irregular trichomes and cellular patterns, late flowering, abnormal inflorescences and flowers, and aborted seeds. These developmental abnormalities are stable in the presence of *AtHD1* disruption after four to five generations of selfing. Moreover, a T-DNA insertion into the *AtHD1* gene (*athd1-t1*) results in a mutant line (Ws ecotype) that shows developmental abnormalities similar to those of CASH126 plants (Columbia ecotype), confirming that AtHD1 plays an important role in reprogramming developmental processes. The affected plants develop through initiating axillary meristems and additional changes that ensure the completion of a life cycle, although they have to overcome structural and developmental incompatibilities resulting from irregularly orchestrated patterns and tempos of organogenesis. Histone deacetylation and the resulting effects on gene regulation may contribute to the fundamental and dynamic process of developmental plasticity in plants (Walbot, 1996; Meyerowitz, 1997). Disruption of AtHDs may directly affect expression of genes such as *superman* (Tian and Chen, 2001) in specific developmental stages. Alternatively, the disruption may induce a series of changes in regulatory networks. Complex phenotypes such as

defective apical shoot meristems and abnormal flowers could be some of these pleiotropic effects. It will be interesting to identify target genes whose expression is affected in the *athd1-t1* and antisense *AtHD1* transgenic lines.

Developmental abnormalities induced by disruption of histone deacetylation are different from those induced by DNA methylation defects (Vongs et al., 1993; Finnegan et al., 1996; Ronemus et al., 1996; Gendrel et al., 2002; Stokes and Richards, 2002). First, the abnormal development in the *athd1-t1* homozygous plants can be immediately corrected in the heterozygous state. Second, no additional visual abnormalities accumulate in the selfing progeny of *athd1-t1/athd1-t1* homozygous plants. The data suggest that the dynamic and reversible process of acetylation and deacetylation provides a spatial and temporal code for gene activation and silencing. Consistent with this hypothesis, histone deacetylation may affect the expression of genes in response to environmental (*e.g.*, light, day length) and developmental (*e.g.*, homeotic genes) changes. Indeed, both antisense *AtHD1* transgenic and *athd1-t1* homozygous lines show variable flowering time and severity of developmental abnormalities under short- and long-day conditions.

AtHD1 is a member of a multi-gene family that may diverge in functions. For examples, AtHDA6, a RPD3-like homolog, is involved in the release of transgene silencing (Murfett et al., 2001) and is associated with the maintenance of DNA methylation patterns of transgenes (Aufsatz et al., 2002). However, recessive mutations in *AtHDA6* do not induce visible abnormal phenotypes (Murfett et al., 2001). Antisense *AtHD2* lines (Wu et al., 2000a; Wu et al., 2000b) show less severe developmental

abnormalities than antisense *AtHD1* lines do (Tian and Chen, 2001). Significantly, although histone deacetylases are encoded by a multi-gene family and share sequence homology (Pandey et al., 2002), other AtHD homologs cannot compensate for the loss of AtHD1 activity in the *athd1-t1* lines, suggesting that *AtHD1* is a key member of the histone deacetylase gene family. Unlike the tetra-acetylated histone H4 induced by overexpressing antisense *AtHD1* (Tian and Chen, 2001), hyper-acetylation of core histones in the *athd1-t1* line is restricted to a specific set of lysine residues including H4 Lys12. These data suggest that specific patterns of histone acetylation may be established by AtHD1, consequently controlling the expression of a subset of genes during development.

The role of a histone code in genetic and epigenetic regulation

Chromatin-based gene regulation in eukaryotes is controlled by a chromatin code (Jenuwein and Allis, 2001) that can be further classified as stable or reversible. The stable code includes DNA and histone methylation (Richards and Elgin, 2002). Once the stable code is established, it is difficult to alter unless through a reset of developmental programming during meiosis, because no active demethylation pathway has been identified (Jenuwein and Allis, 2001; Li et al., 2002; Richards and Elgin, 2002). As a result, changing a stable code results in epigenetic inheritance or variation (Stokes et al., 2002; Stokes and Richards, 2002). Moreover, alteration in the stable code may serve as an epigenetic "modulator" that induces changes at other loci, independent of the original chromatin conformation. Indeed, in the *ddm1* genetic background, epigenetic

lesions are induced in a genomic region containing multiple repeats associated with disease resistance genes, presumably because they are vulnerable in response to changes in DNA methylation (Stokes and Richards, 2002). Moreover, these epialleles may be affected not only by demethylation, but also by allelic interactions among loci or within a locus (Stokes et al., 2002), a phenomenon similar to meiotic transvection (Aramayo and Metzenberg, 1996) or paramutation (Hollick et al., 1997). Methylation-associated epialleles have also been reported in mutants with defective flower structure (e.g., SUPERMAN) or a delay in flowering time (e.g., FWA), which may result from aberrant DNA methylation patterns (Jacobsen and Meyerowitz, 1997; Kakutani, 1997; Soppe et al., 2000).

The reversible code (e.g., histone acetylation and deacetylation) is heritable (Jenuwein and Allis, 2001) but the action of the code is dependent on the respective biochemical activities that set the code. The code is dynamic and reversible during any developmental stage and is independent of meiosis. Although the connection between a specific acetylation code and gene regulation remains to be elucidated, the heritable and reversible nature of developmental abnormalities observed in *AtHD1* disruption lines is likely associated with changes in histone acetylation (e.g., H4 Lys12) and methylation. It is conceivable that under normal conditions, genes, including homeotic genes (Tian and Chen, 2001) responsible for plant development, may be controlled by the reversible code of acetylation and deacetylation. However, the acetylation code is reversible and dependent on histone deacetylases or other factors in the chromatin control. As soon as the proper code is restored, controls of regulatory networks and developmental programs

return to normal, independent of reprogramming through meiosis. The concept of a stable or reversible code is also supported by the results obtained from a recent study indicating that the loss of histone H4 Lys16 acetylation in *ddm1* homozygous plants is compensated in DDM1/ddm1 heterozygous plants, whereas DNA and histone H3 Lys9 methylation remain unchanged (Soppe et al., 2002).

The relationship between reversible and stable codes is largely unknown. Acetylation and deacetylation may act on active or inactive chromatin as a competitor for histone methylation sites (Jenuwein and Allis, 2001). For example, histone acetyltransferases and methyltransferases may compete for histone H3 Lys9 to establish an active or inactive form of chromatin (Jenuwein and Allis, 2001; Litt et al., 2001). Histone deacetylation and DNA methylation may also be interdependent (Selker, 1998; Soppe et al., 2002). Moreover, a putative histone deacetylase (AtHDA6) is needed to enhance DNA methylation induced by double-stranded RNA (Aufsatz et al., 2002). Finally, some DNA methyltransferase (e.g., CMT) or histone deacetylases (e.g., HD2) were identified only in plants (Lusser et al., 1997; Henikoff and Comai, 1998; Lindroth et al., 2001), suggesting that plants may control gene regulation via both general and unique chromatin pathways. It will be interesting to test how the reversible code of histone acetylation controlled by AtHD1 interacts with the biochemically stable code of DNA and histone methylation and affects the plastic nature of plant development.

METHODS

Plant materials

Constitutive antisense histone deacetylase (CASH) transgenic plants were described previously (Tian and Chen, 2001). All plants were grown in vermiculite mixed with 10% soil in a growth chamber with growth conditions of 22º/18º (day and night) and 16 hr of illumination per day, except as noted otherwise. Seeds from CASH plants were germinated in Murashige/Skoog medium (Sigma, St. Louis) in the presence of 15-50 μg/ml of kanamycin. After 2 weeks the plants were transferred to soil and grown in a growth chamber. The T-DNA insertion line was grown without kanamycin selection except as noted otherwise. All photographs, except those taken with the imaging system in a scanning electron microscope, were taken using a Nikon N-900 digital camera or the CCD system of a Nikon SMZ-100 fluorescence microscope.

Scanning electron microscopy

Scanning electron microscopy was performed using a modified protocol (Murai et al., 2002) with a Hitachi S570 scanning electron microscope (Tokyo) at an accelerating voltage of 5-15 kV. Tissue samples were fixed in a solution containing 3% (v/v) formaldehyde/glutaraldehyde and 0.1M sodium cacodylate buffer (pH7.4) at room temperature for 13 hr, washed three times with 0.2M sodium cacodylate buffer (pH7.4), dehydrated through an ethanol series, critical point dried with CO_2, and sputter-coated with gold before viewing by scanning electron microscopy.

Nucleic acid preparation and analysis

RNA and DNA were isolated from at least five leaves of each plant at the same developmental stages as previously described (Chen and Pikaard, 1997). For DNA and RNA blot analyses, hybridization was performed following the method of CHURCH and GILBERT (1984). The DNA and RNA blots were washed in 2 x SSC and 0.2% SDS at 65ºC for 30-60 min and hybridization signals were detected using a digital imaging system or exposure to X-ray film or to a PhosphorImager.

PCR-based reverse genetic approach to identify T-DNA insertions in the *AtHD1* gene

We used a PCR-based approach similar to that described by Krysan et al. 1996 to identify *Arabidopsis* (ecotype Ws) mutants containing a T-DNA insertion in or near the *AtHD1* gene. Pooled samples of DNA from 1000, 100, and 20 plants from the Feldmann T-DNA insertion library (Feldmann and Marks, 1987; Forsthoefel et al. 1992) were successively assayed for insertions, followed by assay of individual plants from the pool of 20 mutant plants. The zygosity of a mutant allele was determined using forward and reverse primers specific to the *AtHD1* gene and one primer specific to the *AtHD1* gene in combination with either a T-DNA left- or right-border primer. *AtHD1* primer sequences were 5'- GCA CTA GTG GCG CGC CAT GGA TAC TGC GGC AA -3' and 5'-GCA GAT CTA TTT AAA TCG CCT GCT CCG CCC CAC C-3'. T-DNA primer sequences were left border, 5'-GAT GCA CTC GAA ATC AGC CAA TTT TAG AC-3'; right border, 5'-TCC TTC AAT CGT TGC GGT TCT GTC AGT TC-3'. PCR was carried

out using 0.5 unit ExTaq (Takara, Berkeley, CA) DNA polymerase with a robocycler (Stratagene, La Jolla, CA) using one cycle of 95º for 5 min, 30-36 cycles of 94º for 40-60sec, 56º for 1 min, and 72º for 3 min, plus an extension cycle of 72º for 10 min followed by 4º on hold. We used 0.24 µM of each primer and 0.2 mM of each dNTP and either 100ng DNA (for screening superpools of 1000 plants) or 20ng DNA (for screening pools of 100, 20, or individual plants) in a 50-µl final reaction volume.

T-DNA junction sequence identification in antisense transgenic lines

T-DNA junction sequences in the CASH lines were identified using a modification of a procedure previously described (Zhou et al., 1997). Briefly, 1 µg genomic DNA and 1 µg pBluescript plasmid DNA were separately digested at 37ºC overnight in a 40-µl reaction containing 10 units of *Pst*I. The digested genomic DNA solution was extracted with phenol/chloroform/isoamylalcohol (25:24:1; Fisher) and precipitated using 2 volumes of ethanol and 1/10 vol of 3M sodium acetate (pH 5.2). The linear form of pBluescript plasmid was purified using a Gelpure kit (GeneMate; ISC Bioexpress, Kaysville, UT). For DNA ligation, 250 ng of digested genomic DNA and 180 ng of digested pBluescript plasmid DNA were mixed in a 20-µl solution containing 3 units of T4 DNA ligase (Promega, Madison, WI) and incubated at 16º overnight.

Two consecutive PCR reactions were performed using three different primers. T3 primer (5'-AAT TAA CCC TCA CTA AAG GC-3') was derived from an endogenous sequence of the pBluescript plasmid. TR2 (5'-GAT GGG AGT CAG ATT GTC GTT TC-3') and TR3 (5'-GTC GTT TCC CGC CTT CAG TTT A-3') were two

nested primers derived from the T-DNA sequence close to the right border. The first PCR reaction was performed using TR2 and T3 primers and 5 µl of ligation solution as template. After purification using a QIAquick PCR purification kit (QIAGEN, Chatsworth, CA), an aliquot of 2 µl PCR product from the first reaction was added to the second PCR reaction containing TR3 and T3 primers. Both PCR reactions were performed for 35 cycles with a program of 30 sec at 94º for denaturation, 30 sec at 52º for annealing, and 3 min at 72º for extension, followed by a final extension at 72º for 8 min. The products of the second PCR amplification were subjected to electrophoresis through a 1.0% agarose gel. The band containing the DNA fragment of interest was excised from the gel and the DNA was purified using a DNA purification kit (GeneMate). The purified PCR product was cloned into the plasmid pGEM T-easy (Promega) and sequenced using an ABI Prism Big Dye terminator cycle sequencing reaction kit (PE Applied Biosystem). The small fragment that appeared in one-step reverse transcriptase (RT)-PCR was cloned into pGEM T-easy (Promega). The plasmid DNA was isolated using a QIAprep spin miniprep kit (QIAGEN) and sequenced.

RT-PCR

RT-PCR was carried out using 500ng of total RNA prepared from leaf tissues. SuperScript one-step RT-PCR was performed using the Platinum *Taq* system (Invitrogen, San Diego) according to the manufacturer's instructions. The primers used for detecting *AtHD1* transcripts were AtHD1-R, 5'-GCT TAC AAC AAC AAC AAC TCC AGA AAC TT-3' and AtHD1-F, 5'-AGA AAG CCA GAG AGA GAG AGA GAG ACT AC -

3'. For detecting *CYC2b* transcripts, we used the following primers: Cyc2b-F, 5'-TCG GTG TAG AGA TGA AGA GAC AGA-3' and Cyc2b-R, 5'-GCA ACT AAA CCA ACA AGC TGA AGC-3'.

Strand-specific first-strand cDNAs were synthesized using 500 ng of total RNA and Omniscript reverse transcriptase (QIAGEN). AtHD1-R and AtHD1-F primers were used to synthesize sense and antisense strands of *AtHD1*, respectively. RT was performed at 37º for 60 min and the enzyme was then inactivated by incubation at 93º for 5 min. For antisense *AtHD1* detection, following an initial denaturation step, 35 cycles of PCR were performed using the program of 94º for 30 sec, 57º for 30 sec, and 72º for 1.5 min with a final extension of 10 min at 72º. A 5-ul aliquot of PCR products was resolved by electrophoresis through a 1% agarose gel and subjected to DNA blot analysis. For sense *AtHD1* analysis, PCR was performed using the same conditions as described above, except that 20 cycles were used. Hybridization was performed using a full-length *AtHD1* cDNA as a probe. The actin gene *Act2* (An et al., 1996) was used as an internal control for quantification. Relative intensities of individual DNA fragments were measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis

Western blot analysis was carried out according to Tian and Chen, 2001. In brief, crude protein and histone extracts were prepared and subjected to electrophoresis through 8 and 15% SDS polyacrylamide gels. Immunoblots were prepared and probed with antisera against the N-terminal portion of AtHD1 (Tian and Chen, 2001) or with a

site-specific antibody against histone H4 Lys12 (Upstate Biotechnology, Lake Placid, NY) and developed by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK).

CHAPTER IV

TRANSCRIPTOME CHANGES IN *ARABIDOPSIS THALIANA* HISTONE DEACETYLASE 1 MUTANT

OVERVIEW

Histone deacetylases (HDACs, HDAs or HDs) are involved in modulating chromatin structure and thus affect gene transcription. AtHD1 is a putative homolog of yeast RPD3 histone deacetylase in *Arabidopsis*, which is extensively expressed in various tissues. In previous studies, a T-DNA insertion line in *AtHD1* gene (*athd1-t1*) displayed various pleiotropic developmental abnormalities, indicating that AtHD1 plays an important role in plant development. In this study, the biological roles of AtHD1 were further investigated at molecular level. It was determined by microarrary analyses that 6.7% (1,753) of the genes were differentially expressed in leaves between the *athd1-t1* mutant and wild type, whereas 4.8% (1,263) of the genes were affected in flower buds of the mutant. These differentially expressed genes represented a wide range of biological functions and were randomly distributed in the five chromosomes of *Arabidopsis*. Some of the activated genes were directly associated with accumulation of hyperacetylated histones caused by inhibition of *AtHD1* expression. Especially, the acetylation levels of H4 tetra lysines and H3 lysine 9 were dramatically increased in the promoter region of the candidate genes. From this research, we conclude that AtHD1 is a global regulator in *Arabidopsis* gene regulation and development, providing both

positive and negative regulation of gene transcription through direct effects on target genes or indirect effects on down-stream genes.

INTRODUCTION

In eukaryotes, DNA is tightly packaged into chromatin, a highly organized protein-DNA complex. It is now believed that chromatin is not just a physical structure that contains genetic information, but it also plays a pivotal role in regulating gene expression by structural alteration (Narlikar et al., 2002). Reversible acetylation and deacetylation of lysine residues in the conserved tails of histones account for one of the fundamental mechanisms for modulating chromatin states and thereby manipulating gene transcription (Jenuwein and Allis, 2001; Berger, 2002). Hyperacetylation of core histones helps to relax chromatin structure thus to promote transcription, whereas hypoacetylation of core histones turns off the "open" chromatin states, leading to gene repression. Histone acetyltransferases (HATs) catalyze acetylation of core histones, whereas histone deacetylases (HDACs, HDAs, HDs) are responsible for removing acetyl groups from core histones. Therefore, the role of histone acetylation and deacetylation in gene regulation can be elucidated through studying the functions of histone acetyltransferases and histone deacetylases.

Yeast RPD3 protein was first identified as a positive and negative transcriptional regulator for a subset of yeast genes (Vidal and Gaber, 1991). The mammalian homolog of RPD3 protein, HDAC1, was shown to possess histone deacetylase activity (Taunton et al., 1996), providing the direct evidence that histone deacetylation is an important

factor in gene regulation. A number of histone deacetylases have been identified in various species and can be further classified into four different groups; they are RPD3-like proteins, HDA1-like proteins, SIR2 homologs, and plant-specific HD2-like proteins (Kolle et al., 1999; Pandey et al., 2002). Different classes of histone deacetylases might have evolved specialized functions (Khochbin et al., 2001).

Histone deacetylases have been extensively studied in yeast and mammalian systems. A number of repressors, such as Sin3 (Heinzel et al., 1997), pRB (Brehm et al., 1998; Luo et al., 1998), YY1 (Yang et al., 1996), and NcoR (Alland et al., 1997), are associated with HDACs, suggesting that the enzymatic modulation of histone acetylation/deacetylation is an integral component of transcription regulation machinery (Pazin and Kadonaga, 1997). The presence of different HDACs in association with different repressors indicates that HDACs may contribute to diverse biological activities. Indeed, genome-wide studies on histone deacetylases have demonstrated that a few hundred genes are either up- or down-regulated by *RPD3* deletion in yeast (Bernstein et al., 2000). By combining chromatin immunoprecipitation (ChIP) analysis and intergenic DNA microarray, it was shown that histone H4-K12 and H4-K5 acetylation was enriched for chromatin fragments associated with activated genes in *RPD3* deletion mutant (Robyr et al., 2002), suggesting that RPD3 is a global transcriptional repressor.

Little is known about the role of histone acetylation and deacetylation in plant gene regulation and development. Previous studies on plant histone modifications were mainly performed by reverse genetic approaches. Down-regulation of *AtHD1* or *AtHD2* expression by antisense technology or by T-DNA insertion resulted in various

developmental abnormalities (Wu et al., 2000a; Wu et al., 2000b; Tian and Chen, 2001; Tian et al., 2003), and overexpression of OsHDAC1, a rice RPD3 homolog, correlated with increase in HDAC expression and dramatic alterations in phenotype. However, the molecular mechanisms by which HDACs mediate gene regulation in plants remain unknown.

My previous studies demonstrated that developmental abnormalities and ectopic expression of *SUPERMAN* by down-regulation of *AtHD1* expression correlated with overall accumulation of tetra-acetylated histone H4 or acetylated H4 at lysine 12 (Tian and Chen, 2001; Tian et al., 2003). F_1 hybrids derived from the cross between the wild-type and the *athd1-t1/athd1-t1* mutant showed normal phenotypes (Tian et al., 2003). The data suggest that AtHD1 plays an important role in plant growth and development.

AtHD1 is closely related to the yeast RPD3 protein. By using DNA and RNA blot analysis, it was determined that *AtHD1* is a single-copy gene expressed in various tissues in *Arabidopsis*. The T-DNA insertion line for *AtHD1* (*athd1-t1*) has been characterized (Tian et al., 2003). In this study, I designed experiments to find out the biological substrates of AtHD1 in the *Arabidopsis* genome, which helps us understand the underlying mechanism for developmental defects in the *athd1-t1* mutant. High-density oligo-gene microarrays, each containing all 26,090 annotated genes, were used in the experiments. Transcription profiles were analyzed in both leaves and flower buds of the *athd1-t1* mutant by microarray analysis. Approximately 6.7% (1,753) of the genes were either up- or down-regulated in leaves of the *athd1-t1* mutant and 4.8% (1,263) of the genes were affected in flower buds. The affected genes were in a wide

range of functional categories and distributed randomly across the five chromosomes of *Arabidopsis*. Some candidate genes encode transcription factors and are predicted to be involved in various pathways of plant development. For instance, NAM (no apical meristems protein) was down-regulated in leaves of the *athd1-t1* mutant, which may contribute to the phenotype of defective shoot apical meristem described previously (Tian et al., 2003). However, an equal number of candidate genes were up- and down-regulated, which is reminiscent of the results observed in the yeast *RPD3* deletion mutants (Bernstein et al., 2000). The data suggest that AtHD1 may positively and negatively regulate transcription. Furthermore, some of the candidate genes were associated with accumulation of hyperacetylated histones in chromatin immunoprecipitation (ChIP) assays, suggesting that these genes are the direct targets of the AtHD1 defect. The differentially expressed genes showing no changes in acetylation states may result from the downstream regulation of the AtHD1-targeted genes, such as transcription factors. Futhermore, the hyperacetylated histones were accumulated in the promoter region, suggesting that AtHD1 is recruited to specific genomic loci to control the acetylation states of local chromatin structure. Our results demonstrate the important roles of AtHD1 in plant gene regulation and development.

RESULTS

AtHD1 is constitutively expressed in *Arabidopsis thaliana*

A total of 16 putative histone deacetylases are encoded in the *Arabidopsis* genome, ten of which belong to RPD3/HDA1 superfamily (Pandey et al., 2002). The

proteins in the RPD3/HDA1 superfamily can be subdivided into three classes. AtHD1 (AtHDA19, At4g38130) is a member of class I RPD3/HDA1-like HDACs that include other putative members, AtHDA6 (At5g63110), AtHDA7 (At5g35600), and AtHDA9 (At3g44680) (Pandey et al., 2002). These four members constitute the group of proteins most closely related to yeast RPD3 protein in *Arabidopsis* genome. The protein sequences of AtHD1, AtHDA6, AtHDA7 and AtHDA9 were aligned with yeast RPD3 (GenBank accession number P32561), maize RPD3 homolog (ZmRPD3, GenBank accession number P56521) (Rossi et al., 1998), and the human HDAC1 (GenBank accession number NP_004955) (Taunton et al., 1996). As shown in Figure 4.1A, AtHD1 and AtHDA6 are the most conserved RPD3-like proteins in *Arabidopsis*. The residues that are essential for histone deacetylase activities (Hassig et al., 1998) were conserved (Figure 4.1A).

AtHD1 is a single-copy gene in two *Arabidopsis* ecotypes, Columbia and Lansberg (Tian and Chen, 2001). In this study, the *athd1-t1* mutant was in the Ws background. Therefore, a DNA blot was performed to determine the copy numbers of *AtHD1* in Ws. Genomic DNA from both Ws and Columbia was digested with four different restriction enzymes (*Eco*RI, *Eco*RV, *Bam*HI and *Dra*I), and then blotted onto Hybond N+ membrane (Amersham). Using the 3' region of the cDNA fragment as a probe as previously described (Tian and Chen, 2001), only a single fragment was detected in each genome (Figure 4.1B), suggesting that *AtHD1* is a single-copy gene in Ws ecotype. A restriction fragment length polymorphism (RFLP) in *AtHD1* was detected between Columbia and Lansberg (Tian and Chen, 2001), whereas no

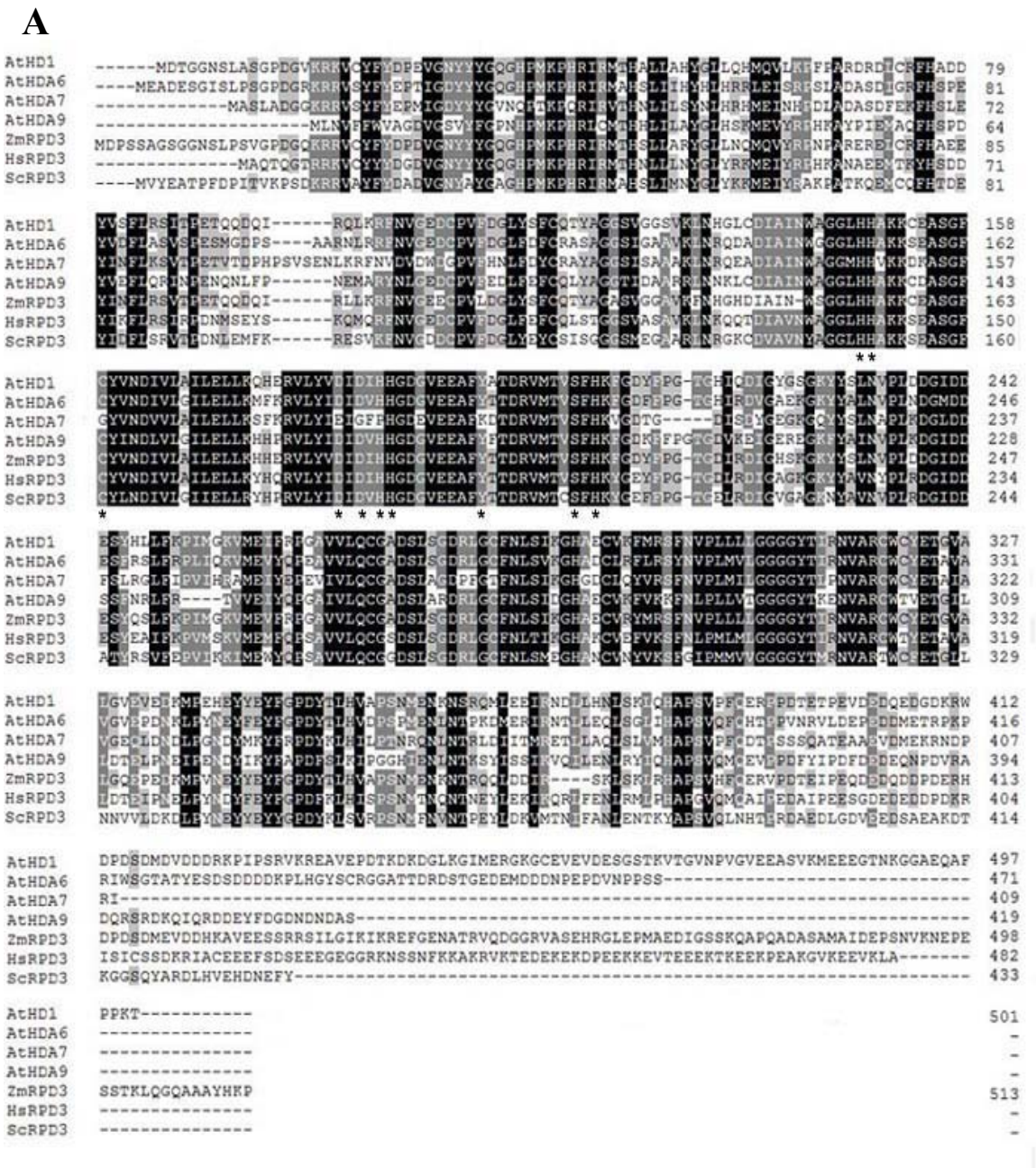


Figure 4.1. AtHD1 is a putative RPD3-like histone deacetylase in *Arabidopsis*.

(A) The amino acid sequences of AtHD1 (AtHDA19, At4g38130), AtHDA6 (At5g63110), AtHDA7 (At5g35600), and AtHDA9 (At3g44680) are aligned with amino acid sequences of maize RPD3 homolog (ZmRPD3, GenBank accession number P56521), human HDAC1 (GenBank accession number NP_004955) and yeast RPD3 (GenBank accession number P32561) using ClustalW 1.82 software (http://www.ebi.ac.uk/clustalw/). The number of amino acids is indicated at the left-hand side. Identical and similar amino acids are shaded in black and grey, respectively (GeneDoc 2.6.002, http://www.psc.edu/biomed/genedoc). The amino acids marked by asterisks are the residues with predicted functions in histone deacetylase activity.

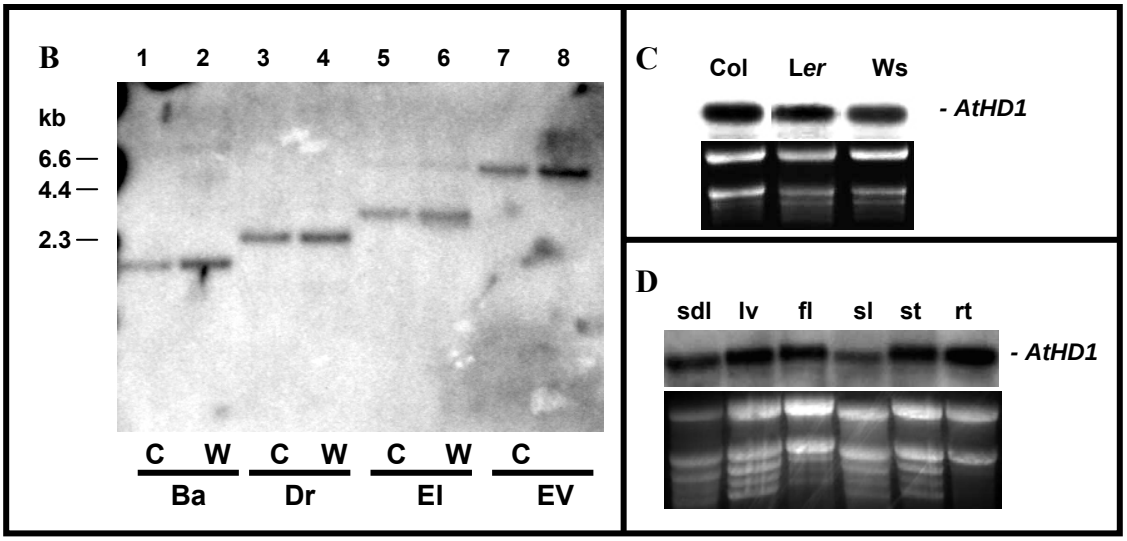


Figure 4.1. Continued

(B) Genomic DNA hybridization analyses of *AtHD1* in two ecotypes, Columbia (C) and Ws (W). The probe used for hybridization is derived from 3' region of *AtHD1* cDNA (Tian and Chen, 2001). Ba, *Bam*HI; Dr, *Dra*I; EI, *Eco*RI; EV, *Eco*RV.

(C) RNA blot analysis of *AtHD1* transcripts in three different ecotypes of *Arabidopsis*. About 10 μg of total RNA from leaves was electrophoresized in 1.5% MOPS (3-(N-Morpholino)-propanesulfonic acid) gel, transferred onto Hybond-N+ membrane (Amersham), and hybridized with ^{32}P- labeled full-length *AtHD1* cDNA fragment. Col: Columbia; L*er* - Lansberg *erecta*; Ws - Wassilewskija.

(D) *AtHD1* is expressed in a variety of tissues. Total RNA samples were isolated from seedlings (sdl), leaves (lv), flower buds (fl), siliques (sl), stems (st), and roots (rt), as described in the methods. About 10 μg of total RNA from each sample was electrophoresized in a 1.5% MOPS gel and blotted. The blot was hybridized with the same probe as described in (C). The RNA gel was stained with Ethidium bromide (EtBr) before blotting to show the amount of RNAs loaded in each lane (C and D).

difference between Columbia and Ws.

RNA blot analysis was performed to determine *AtHD1* expression patterns in *Arabidopsis*. *AtHD1* was expressed in all three ecotypes, Columbia, L*er* and Ws (Figure 4.1C). Moreover, *AtHD1* was expressed in all tissues tested, including seedlings, leaves, flower buds, siliques, stems, and roots (Figure 4.1D). *AtHD1* was highly expressed in flower buds, but expressed in a much lower expression level in siliques, suggesting a different role of *AtHD1* in seed production. The closest relative of *AtHD1* is *AtHDA6*. Transcripts of *AtHDA6* were detected in seedlings, leaves and flower buds at much lower expression levels, and barely detectable in siliques (http://chromdb.biosci.arizona.edu/cgi-bin/v3/rnaexp.cgi?id=HDA000006). The other members in the class I subgroup of RPD3/HDA1 superfamily, were either not expressed (AtHDA7, http://chromdb.biosci.arizona.edu/cgi-bin/v3/query.cgi?id=HDA000007), or were detected at an extremely low level (AtHDA9, http://chromdb.biosci.arizona.edu/cgi-bin/v3/rnaexp.cgi?id=HDA000009). Since *AtHD1* is the only RPD3-like gene expressed in various tissues in *Arabidopsis*, it may have a role in global regulation of plant gene expression.

Gene expression profiles in the *athd1-t1* mutant

Genetic studies indicate that AtHD1 plays an essential role in gene regulation during different developmental stages in *Arabidopsis* (Wu et al., 2000a; Tian and Chen, 2001; Tian et al., 2003). Down-regulation of *AtHD1* expression by antisense (Tian and Chen, 2001) or complete knockout of *AtHD1* expression by T-DNA insertion (Tian et al.,

2003) induces various phenotypic abnormalities. *SUPERMAN* is associated with male-flower organ identity in *Arabidopsis*. It is only expressed in flower buds (Bowman et al., 1992). In CASH (Constitutive Antisense Histone Deacetylase 1) plants, ectopic expression of *SUPERMAN* was detected in leaves (Tian and Chen, 2001), suggesting that like the RPD3 proteins identified in other species, AtHD1 is a putative global transcriptional regulator (Kadosh and Struhl, 1998b). The ectopic expression of certain genes in CASH plants or the *athd1-t1* mutant might account for some abnormal phenotypes. In order to identify the candidate genes in the *AtHD1* mutant, two sets of microarray analysis were carried out to identify genes whose expression was affected by *athd1-t1* mutation in leaves and flower buds, respectively.

High-density oligo-gene microarrays, each containing all 26,090 annotated genes in the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000), were used in the experiments. As previously described (Lee et al., 2004), 70-mer oligonucleotides (oligos) were designed from the 3' end sequences of annotated genes to ensure the specific hybridization for each individual gene in the genome (Lee et al., 2004). Fluorescent cDNA probes were synthesized from total RNA isolated from leaves or flower buds of wild-type and mutant plants. Four dye-swap hybridizations were used in each experiment, resulting in eight replications. The experiments were performed in both leaves (Table 4.1A, Figure 4.2A) and flower buds of the *athd1-t1* mutant (Table 4.1B, Figure 4.2B). The hybridization signals were captured by a GenePix 4000B scanner (Axon, Foter City, CA), quantified with Genepix Pro4.1 software (Axon, Foter City, CA) and exported as a color image (Figure 4.2C). Reversible color changes in each dye-swap

Table 4.1A. Microarray experimental design for analysis of differentially expressed genes between leaves of Ws and the *athd1-t1* mutant

Experiments	Cy3	Cy5	Slide pattern	Leaf RNA
1	Ws	*athd1-t1*	1	A
2	*athd1-t1*	Ws	1	A
3	Ws	*athd1-t1*	2	A
4	*athd1-t1*	Ws	2	A
5	Ws	*athd1-t1*	1	B
6	*athd1-t1*	Ws	1	B
7	Ws	*athd1-t1*	2	B
8	*athd1-t1*	Ws	2	B

Table 4.1B. Microarray experimental design for analysis of differentially expressed genes between flower buds of Ws and the *athd1-t1* mutant

Experiments	Cy3	Cy5	Slide pattern	Flower RNA
1	Ws	*athd1-t1*	1	A
2	*athd1-t1*	Ws	1	A
3	Ws	*athd1-t1*	2	A
4	*athd1-t1*	Ws	2	A
5	Ws	*athd1-t1*	1	B
6	*athd1-t1*	Ws	1	B
7	Ws	*athd1-t1*	2	B
8	*athd1-t1*	Ws	2	B

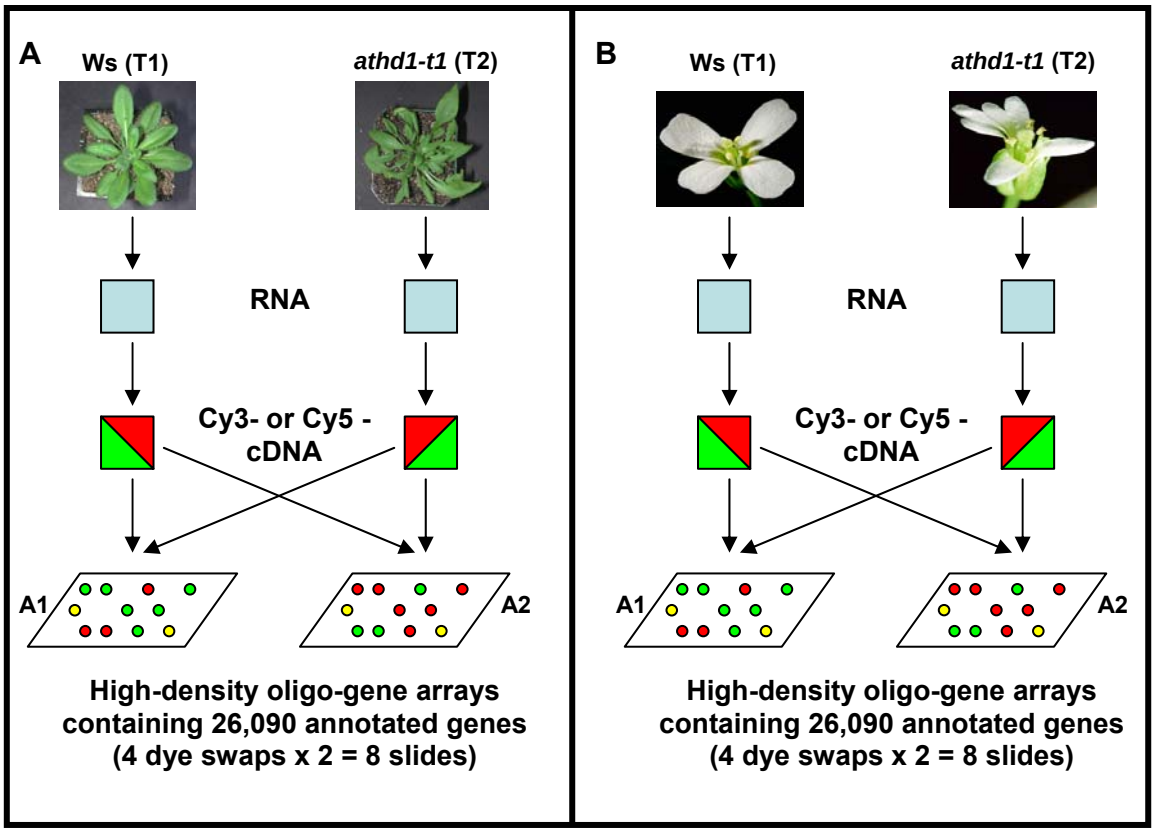


Figure 4.2. Dye-swap experiments of microarray analysis.

(A) and (B) Dye-swap scheme.

(A) Total RNA was isolated from leaves of Ws and the *athd1-t1* mutant at the pre-bolting stage. The cDNA was prepared from mRNA by reverse transcription and divided into two aliquots with an equal amount. Each aliquot was labeled with Cy3- or Cy5-fluorescent dye. The labeled cDNA probes from Ws and the *athd1-t1* mutant were mixed reciprocally and hybridized to two microarray slides. This dye-swap experiment was repeated four times, resulting in a total of 8 slide replications in each experiment.

(B) Total RNA was isolated from flower buds of Ws and the *athd1-t1* mutant. The same dye-swap experiments were performed as described in (A).

(C) Comparison of hybridization signals between two slides within a dye-swap experiment. The cDNA probe prepared from Ws leaves was labeled with Cy5, and the cDNA probe prepared from *athd1-t1* leaves was labeled with Cy3. The two probes were hybridized to an oligo-gene microarray slide containing 26,090 70-mer oligos. A color image was generated (left). Subarray 31 is enlarged as shown in the upper right side. The same block from another slide hybridized with reciprocal mix of the Cy3- and Cy5-labeled probes is shown in the bottom. A subset of five genes showing opposite color hybridization signals in the dye-swap experiment is indicated by arrows.

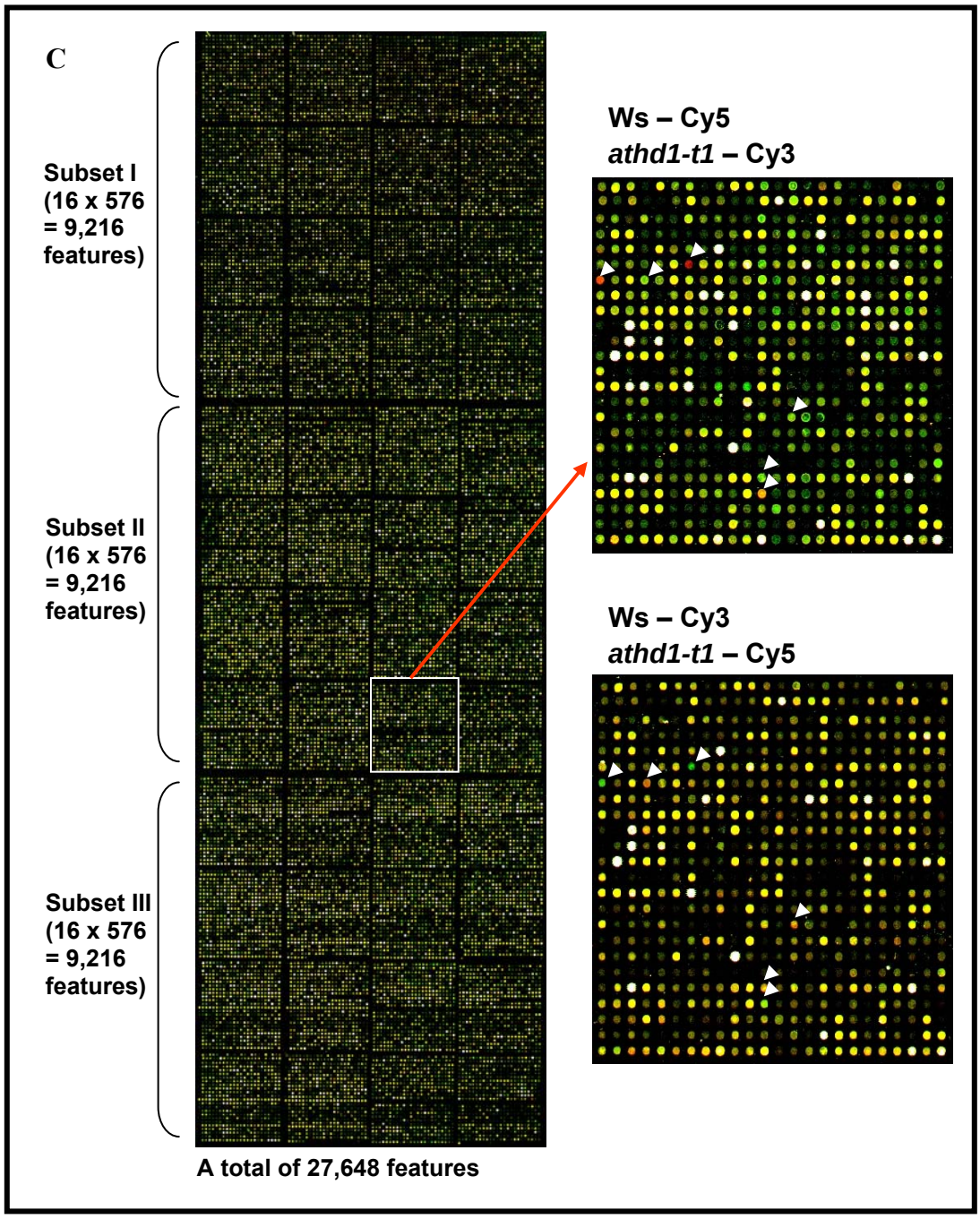


Figure 4.2. Continued

indicate that the reproducibility of the microarray analysis. The data were quantified by Genepix Pro4.1 and processed using natural logarithm (ln) transformation and subjected to statistical analysis using a linear model (Lee et al., 2004). The null hypothesis in this analysis is that there is no differential gene expression between Ws and the *athd1-t1* mutant. Thereby, the genes with significant p-values identified by the standard statistical t-test are either up- or down-regulated in the mutants. In this experiment, both common-variance and per-gene variance were used to perform t-tests. The genes detected by a common variance often had relatively large-fold changes (Figures 4.3A and 4.3C); however, many more genes were detected by per-gene variances including many genes with small-fold changes (Figures 4.3B and 4.3D). The t-tests using per-gene variances are biologically relevant, because the performance of each individual gene is biologically different among replicated experiments. However, the small fold-changes are difficult to be verified by RT-PCR or quantitative RT-PCR analysis. Therefore, an arbitrary fold-cut at ± 1.25 was performed on the genes identified by per-gene variances for further analysis (Table 4.2). Based on these criteria, approximately 6.7% (1,753) of the genes were detected to be differentially expressed in leaves of Ws and the *athd1-t1* mutant, and 4.8% (1,263) of the genes were affected in flower buds of the *athd1-t1* mutant (Table 4.2). To verify the microarray data, RT-PCR analyses or RNA blot analysis were performed on differentially expressed genes identified by statistical analysis (Tables 4.3A and 4.3B). The selected genes included those encoding transcription factors and homeotic proteins important to plant development. For instance, the expression of *NAM* (no apical meristem protein) was repressed in the

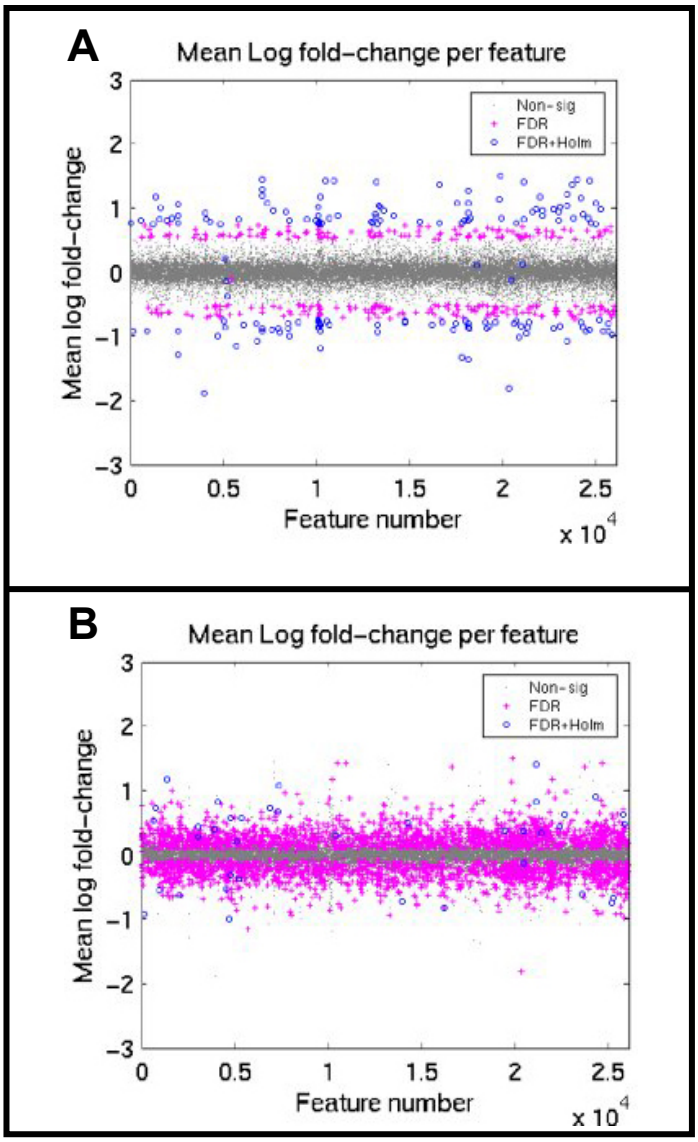


Figure 4.3. Scatter plots of mean ln fold-changes [ln(*athd1-t1*/Ws)] of genes in microarray analyses.

Both Holm's and the false discovery rate (FDR) were applied to control multiple testing errors using a significance level ($\alpha = 0.05$). Holm's is a more conserved method than FDR. All the significant genes detected by Holm's can be detected by FDR. In this study, we used significant genes detected by FDR method for analysis. When the mean ln fold-change is significantly greater than 0, the gene was up-regulated in the *athd-t1* mutants. When the mean log fold-change is significantly less than 0, the gene was down-regulated.

(A) and (B) The differentially expressed genes between Ws and *athd1-t1* in leaves were detected using a common variance and a per-gene variance, respectively.

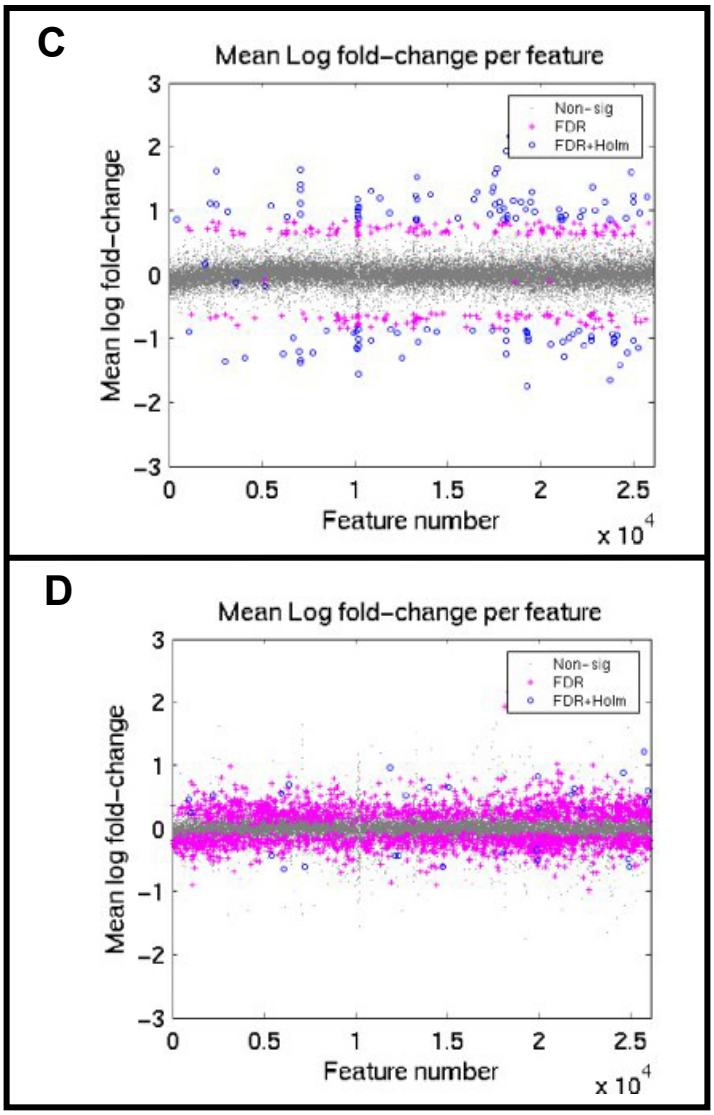


Figure 4.3. Continued

(C) and (D) The differentially expressed genes between Ws and *athd1-t1* in flower buds were detected using a common variance and a per-gene variance, respectively.

Table 4.2. The number of significant genes detected using a common variance and/or a per-gene variance

Experiments	Common variance	Per-gene variance	Common	Per-gene variance (± 1.25)
Leaf (Ws vs. *athd1-t1*)	477	2789	260	1753
Flower (Ws vs. *athd1-t1*)	359	2010	87	1263

To control multiple testing errors, FDR was employed at a significance level ($\alpha = 0.05$).
Common: common sets of significant genes detected by both common variance and per-gene variance; the last column indicates the number of differentially expressed genes detected using an arbitrary fold-change cut at ± 1.25.

Table 4.3A. Genes for verification of microarray analysis in leaves between Ws and the *athd1-t1* mutant

No.	Locus	Description	Fold Change
1	*At1g02920	glutathione transferase	0.3
2	At4g06746	AP2 domain containing protein	0.3
3	At2g26560	putative patatin	0.4
4	At2g21650	myb family transcription factor	0.4
5	At5g56870	glycosyl hydrolase family 35	0.4
6	At3g29035	NAM (no apical meristem protein)	0.4
7	At4g19170	neoxanthin cleavage enzyme (NC1)	0.4
8	At5g62920	response regulator 6 (ARR6)	0.4
9	At5g10760	nucleoid DNA-binding protein cnd41-like protein	0.4
10	At1g15125	similar to methyltransferase-related	0.5
11	At1g55860	ubiquitin-protein ligase 1 (UPL1)	2.3
12	At3g24270	hypothetical protein	2.4
13	At3g22120	cell wall-plasma membrane linker protein homolog	2.5
14	At5g07860	hydroxycinnamoyl benzoyltransferase-related	2.7
15	At2g15090	putative fatty acid elongase	2.8
16	*At2g36910	ABC transporter; multidrug resistance P-glycoprotein (pgp1)	3.0
17	At1g24020	Bet v I allergen family	3.1
18	*At2g28190	copper/zinc superoxide dismutase (CSD2)	6.3
19	*At3g23130	superman	n.d.

* These genes are also included in ChIP assays; n.d.: not determined

Table 4.3B. Genes for verification of microarray analysis in flower buds between Ws and the *athd1-t1* mutant

No.	locus	Description	Fold Change
1	*At2g22980	putative serine carboxypeptidase I	0.3
2	At1g56300	putative DnaJ protein	0.3
3	At4g27440	protochlorophyllide reductase precursor	0.4
4	At3g45640	mitogen-activated protein kinase (MAPK), putative (MPK3)	0.4
5	At1g68050	F-box protein	0.4
6	At5g45890	senescence-specific cysteine protease SAG12	0.4
7	At3g26740	Light-regulated protein - related	0.5
8	At2g38470	WRKY family transcription factor	0.5
9	At5g66070	C3HC4-type zinc finger protein family	0.5
10	At1g62360	homeobox protein –related	1.4
11	At5g32484	similar to transposon related protein	1.6
12	*At2g02850	putative basic blue protein (plantacyanin)	1.7
13	At1g74020	strictosidine synthase family	1.8
14	At1g12560	putative expansin	1.9
15	*At4g34590	bZIP family transcription factor	2.1
16	At3g62460	putative protein	2.2
17	*At1g01060	putative DNA binding protein	2.4

* These genes are also included in ChIP assays.

leaves of mutants, which may explain the phenotype of defective shoot apical meristem described previously (Tian et al., 2003). In petunia, loss-of-function *nam* mutants fail to develop shoot apical meristems (SAM) during embryogenesis, and *nam* seedlings do not develop shoots and leaves (Souer et al., 1996). The *Arabidopsis* NAM homolog has been identified as a transcription factor that contains a conserved NAC domain (Duval et al., 2002). The expression pattern and expression level of every gene selected based on microarray analysis were in good agreement with RT-PCR results (Figure 4.4A and 4.4B), indicating the reliability of microarray analysis. Interestingly, when total RNA from flower buds was included in the RNA blot analysis, two differentially expressed genes in leaves of Ws and *athd1-t1* were shown to be either up- or down-regulated by the *athd1-t1* mutation. One gene displayed ectopic expression in the leaves whereas the gene was normally expressed in flowers (Figure 4.4A). The other gene showed a high level of expression in leaves of the wild type but silenced in the *athd1-t1* mutant (Figure 4.4A). When compared with tissue-specific genes, 85 leaf-specific genes were ectopically expressed in flower buds of the *athd1-t1* mutant and 360 flower-specific genes were reactivated in leaves (data not shown). These data suggest that *AtHD1* play an important role in tissue-specific gene expression.

Of the differentially expressed genes detected, 871 (or 49.7%) genes were up-regulated and 882 (or 50.3%) genes were down-regulated in the leaves of the *athd1-t1* mutant (Figures 4.5A and 4.5B), whereas 560 (or 44.3%) genes were up-regulated and 703 (55.7%) genes were repressed in the flower buds (Figures 4.5A and 4.5B). However, there is little overlap between up-regulated genes in leaves and flower buds or between

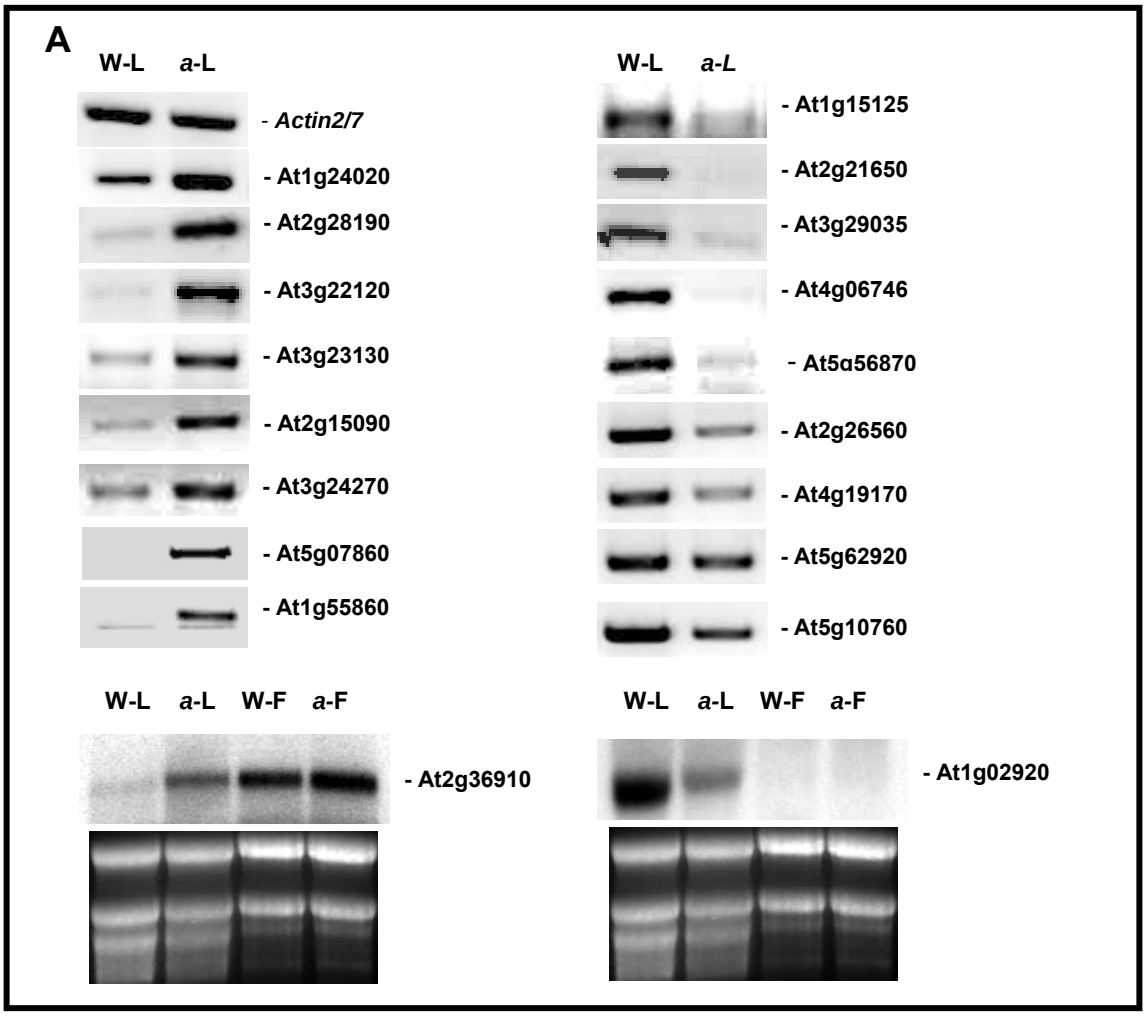


Figure 4.4. Verification of microarray analysis.

(A) Verification of differentially expressed genes in leaves between Ws and the *athd1-t1* mutant. The gene expression changes were detected by RT-PCR or RNA blot analysis. In the RNA blot analysis, the RNA samples from both leaves and flower buds were included. The *Actin2/7* gene was used as a control in RT-PCR analysis. W-L: Ws leaves; a-L: *athd1-t1* leaves; W-F: Ws flower buds; a-F: *athd1-t1* flower buds.

(B) Verification of differentially expressed genes in flower buds between Ws and the *athd1-t1* mutant. The gene expression changes were analyzed by RT-PCR. Amplification of *Actin2/7* gene was used as a control. W-F: Ws flower buds; a-F: *athd1-t1* flower buds.

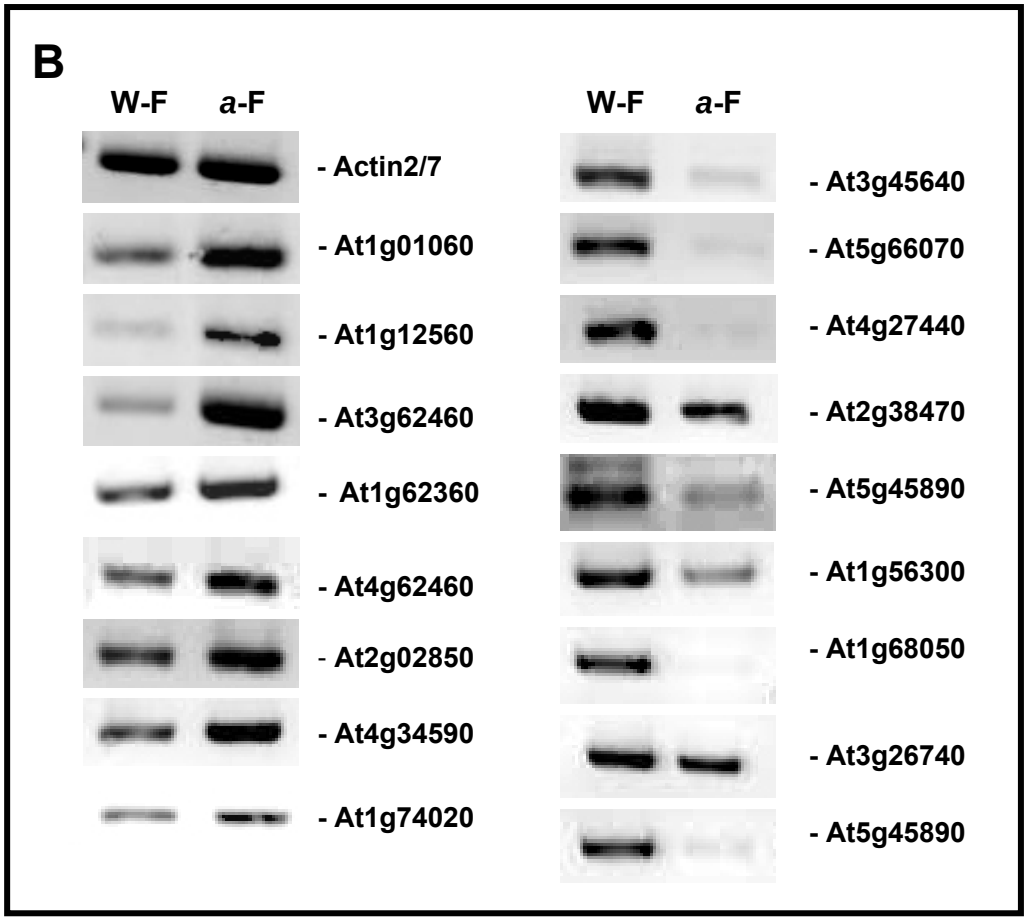


Figure 4.4. Continued

down-regulated genes in these two tissues (Figures 4.5A and 4.5B), indicating that different sets of genes were affected by *AtHD1* at vegetative and reproductive developmental stages. Approximately equal number of genes was up- or down-regulated in the *athd1-t1* mutant, suggesting that AtHD1 was not only a transcriptional repressor, but also a transcriptional activator.

About 75% of the genes exhibiting significantly higher or lower expression levels in leaves and flower buds of the *athd1-t1* mutant could be assigned to known or putative functions (Figures 4.5D and 4.5E). The remaining genes belong to unclassified. Genes whose expression was increased in leaves and flower buds of the *athd1-t1* mutant (Figure 4.5D) represent a wide range of biological functions, including those involved in cellular metabolism (12.8% and 10.7% in leaves and flower buds, respectively), cell growth, division and DNA synthesis (10.5% and 8.4%), protein destination (9.3% and 9.5%), protein synthesis (8.2% and 4.1%), cell defense and aging (7% and 8%), transcription regulation (6.7% and 8.2%), cellular biogenesis (5.9%% and 5.7%), signal transduction (5.8% and 6.4%), energy (4.8% and 4.5%), transport facilitation (4.6% and 5.5%), intracellular transport (3.6% and 3.7%). The remaining 2% are the genes encoding putative transposons and proteins involved in ionic homeostasis and plant hormonal regulation. Genes exhibiting significantly lower expression levels in leaves and flower buds of the *athd1-t1* mutant than in wild-type plants also represent a wide range of biological functions (Figure 4.5E). Although different sets of genes were affected in the leaves and flower buds of the *athd1-t1* mutant (Figures 4.5A and 4.5B), the percentage of differentially expressed genes in each functional category was

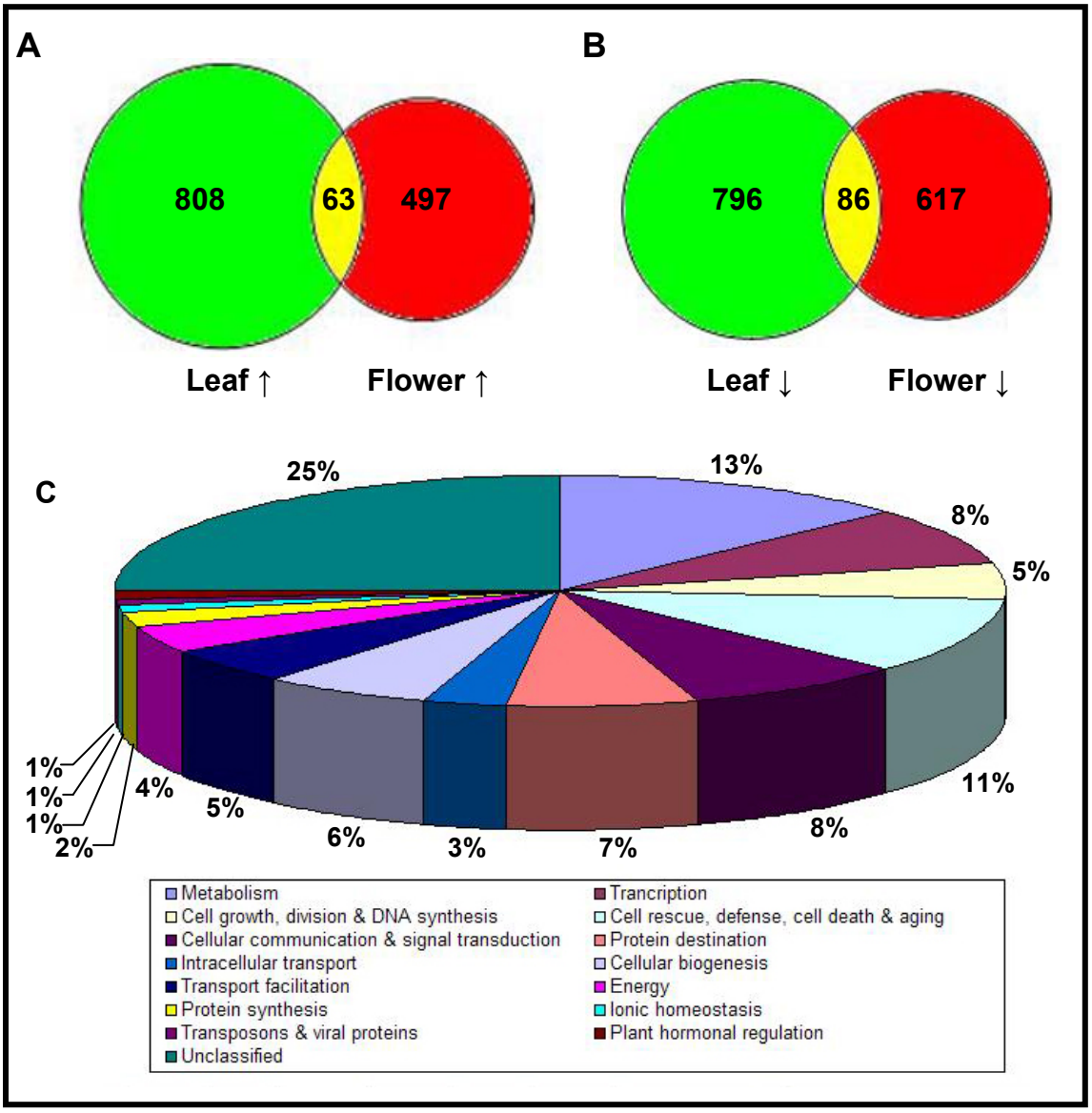


Figure 4.5. Comparative analysis of down- and up-regulated genes in the *athd1-t1* mutant.

(A) and **(B)** Venn diagrams of up-regulated genes (A) and down-regulated (B) genes in leaves and flower buds of the *athd1-t1* mutant.

(C) The 26,090 genes in *Arabidopsis* genome were classified into 15 functional categories by PENDANT (http://mips.gsf.de/proj/thal/db/index.html).

(D) and **(E)** Functional categories of up-regulated genes (D) and down-regulated genes (E) in leaves and flower buds of the *athd1-t1* mutant.

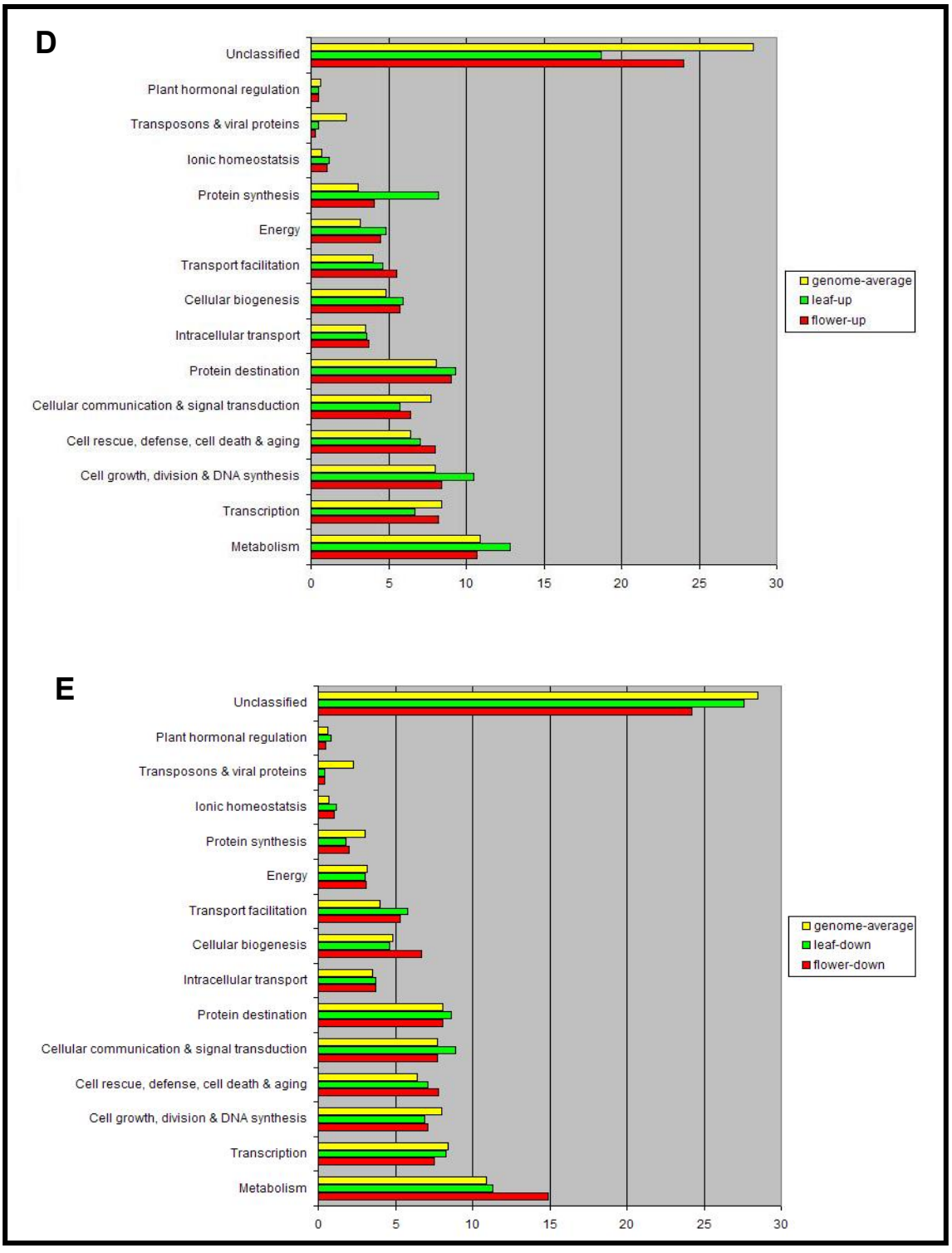


Figure 4.5. Continued

very similar between leaves and flower buds. Compared to all 26,090 genes in *Arabidopsis* genome (Figures 4.5C, 4.5D and 4.5E), the significant genes detected in each category were represented at similar percentages, indicating that AtHD1 is a global regulator involved in various biological pathways. Exceptionally, transposons were underrepresented among the differentially expressed genes detected, suggesting that AtHD1 does not affct transposons and repetitive DNA sequences (Figures 4.5D and 4.5E). In contrast, AtHDA6 has been shown to be involved in silencing of a set of retrotransposons and DNA transposons (Lippman et al., 2003). Another exception is that approximately 8% of up-regulated genes in leaves of the *athd1-t1* mutant belong to the category of protein synthesis, which is over 2 times more than the average percentage (3%) of this category in *Arabidopsis* genome (Figure 4.5D).

We further mapped the differentially expressed genes to the five chromosomes of *Arabidopsis* (Figures 4.6A and 4.6B), generating the transcription profiling in the genome of the *athd1-t1* mutant. This analysis indicates that the differentially expressed genes were randomly distributed relative to gene densities across five chromosomes (Figures 4.6A and 4.6B). There were no obvious clusters of up- or down-regulated genes.

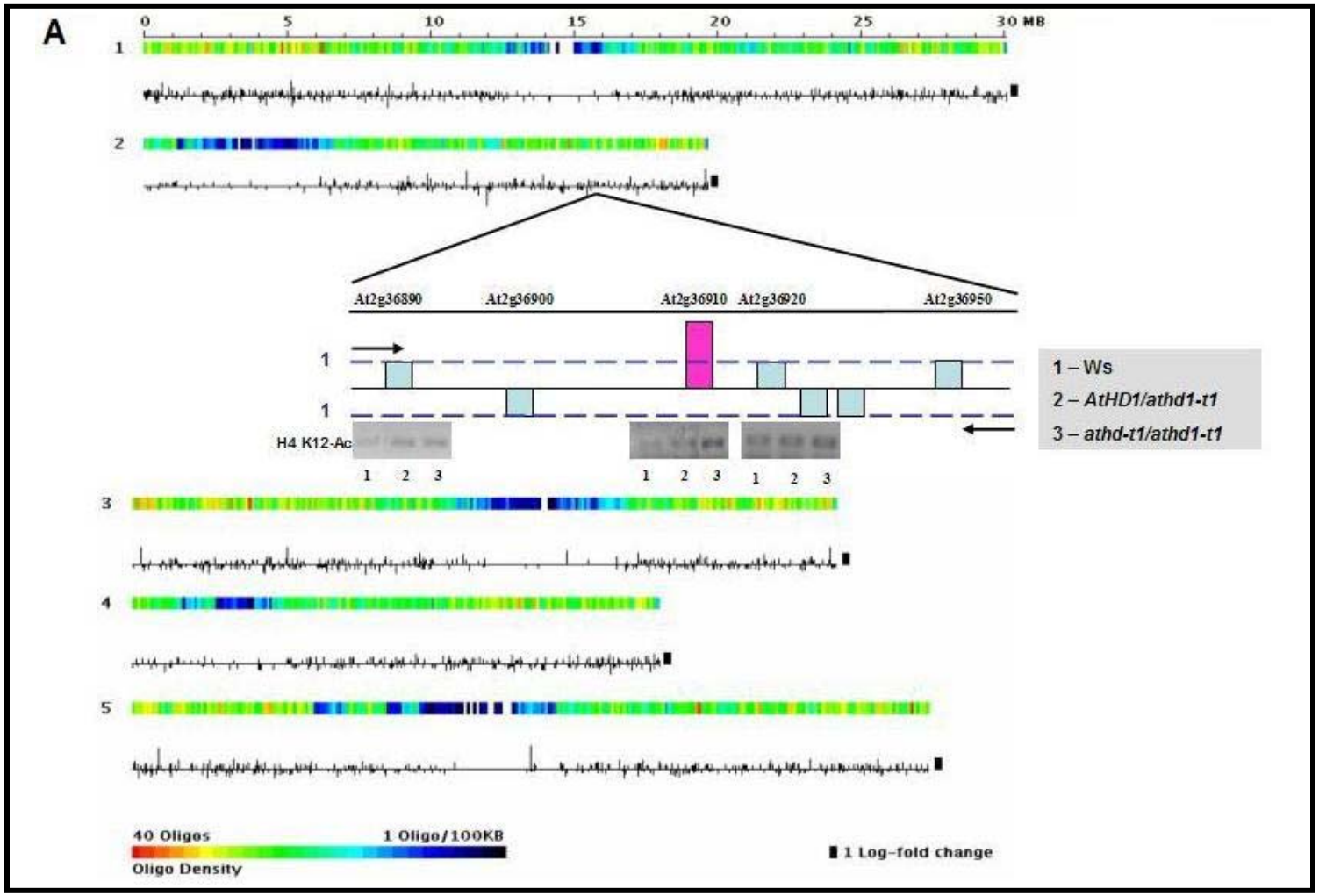
Histone acetylation/deacetylation states in the *athd1-t1* mutants

Previous data demonstrated that down-regulation of *AtHD1* induced various developmental abnormalities at different developmental stages in association with accumulation of hyperacetylated histones (Tian and Chen, 2001; Tian et al., 2003).

Figure 4.6. Chromosomal view of gene expression profiles in the *athd1-t1* mutant.

(A) Chromosomal distribution of differentially expressed genes in leaves of the *athd1-t1* mutant. The 70-mer oligos are mapped to five chromosomes using the annotated gene sequences, such that the oligo density was correspondent to the gene density in the genome. The gradients of color from red to dark blue represent the gradients of gene density from high to low level. The up- and down-regulated genes are shown above and below the chromosomes, respectively, with vertical lines proportionally representing ln fold-changes of gene expression between Ws and the *athd1-t1* mutant. A segment of chromosome 2 containing At2g36910 gene was subjected to chromatin immunoprecipitation analysis. The genes on the W and C strands of DNA are shown above and below the chromosomes. The arrows indicate the transcription directions. The genes with no changes in expression level are represented as blue rectangles, while the up-regulated genes are represented as pink rectangles. The gene At2g36910 and its two neighboring loci (At2g36890 and At2g36920) were amplified from DNA immunoprecipitated by an antibody against acetylated H4-K12.

(B) Chromosomal distribution of differentially expressed genes in flower buds of the *athd1-t1* mutant. The up- and down-regulated genes in flower buds of the *athd1-t1* mutant are mapped to five chromosomes of *Arabidopsis* as described in (A). The ChIP analysis was performed on a segment of chromosome 4 containing At4g34590 locus. The gene At4g34590 and its two neighboring loci (At4g34580 and At4g34600) were amplified from DNA immunoprecipitated by an antibody against acetylated H3-K9.


A
0
5
10
15
20
25
30 MB
1
2
At2g36890
At2g36900
At2g36910
At2g36920
At2g36950
1
1
H4 K12-Ac
1 2 3
1 2 3
1 2 3
3
4
5
1 – Ws
2 – AtHD1/athd1-t1
3 – athd-t1/athd1-t1
40 Oligos
1 Oligo/100KB
Oligo Density
1 Log-fold change

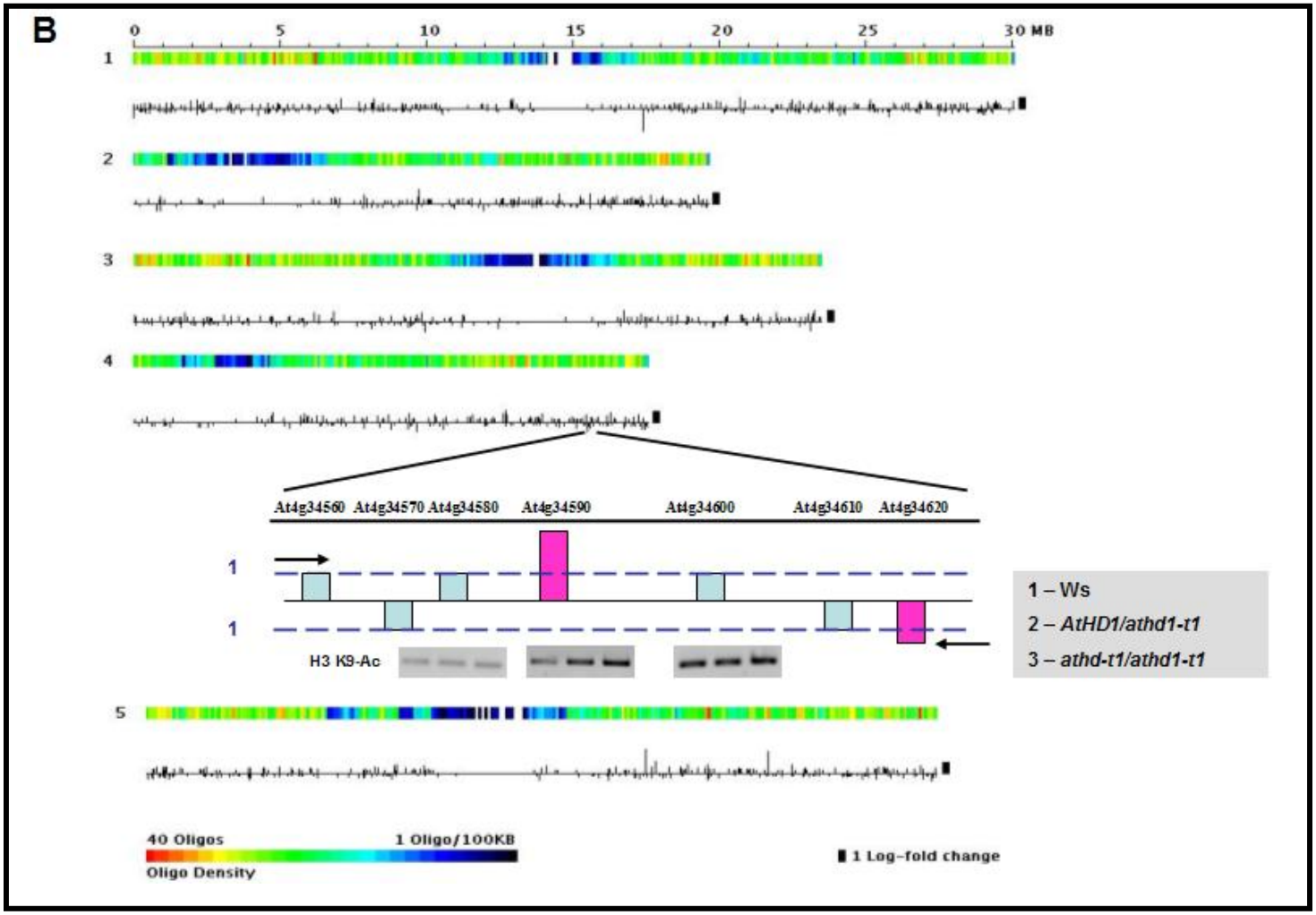


Figure 4.6. Continued

Microarray analysis detected a number of differentially expressed genes in the leaves and flower buds of the *athd1-t1* mutant. However, it is unclear whether the differential expression results from direct or indirect effects of histone acetylation and deacetylation (Finnegan 2001). If it is a direct effect, hyperacetylated histones will be accumulated in the vicinity of differentially expressed genes in the *athd1-t1* mutant. To test this, we performed chromatin immunoprecipitation (ChIP) assays to investigate whether an activated gene is associated with hyperacetylated histones. Rpd3p has been shown to deacetylate lysines K5, K8, K12 and K16 of histone H4 in yeast (Rundlett et al., 1998; Suka et al., 2001). In plants, H3 is also extensively acetylated (Waterborg et al., 1990). Therefore, ChIP assays were performed using antibodies against tetra-acetylated H4, acetylated H4-K12 and acetylated H3-K9. The antibody against dimethylated H3-K9 was also included, because acetylation and methylation of H3-K9 are mutually exclusive, resulting in gene activation or repression.

As shown in Figure 4.7A, freshly harvested leaves or flower buds were first treated with formaldehyde to cross-link proteins (including histones) to their contiguous DNA sequences. Nuclei were isolated from the treated tissues and then sonicated to fragment the chromatin material into an average length of 0.3 to 1.0 kb. Next, the sheared chromatin was incubated with specific antibodies (anti-tetra-acetylated-H4, anti-acetylated-lys12-H4, anti-acetylated-lys9-H3 or anti-dimethyl-lys9-H3) for coimmunoprecipitation of DNA that were associated with the histones. As a control, the precipitation step was also performed in the absence of antibody (mock). The immunocomplexes were heated at 65ºC from 6 hours to overnight to reverse the cross-

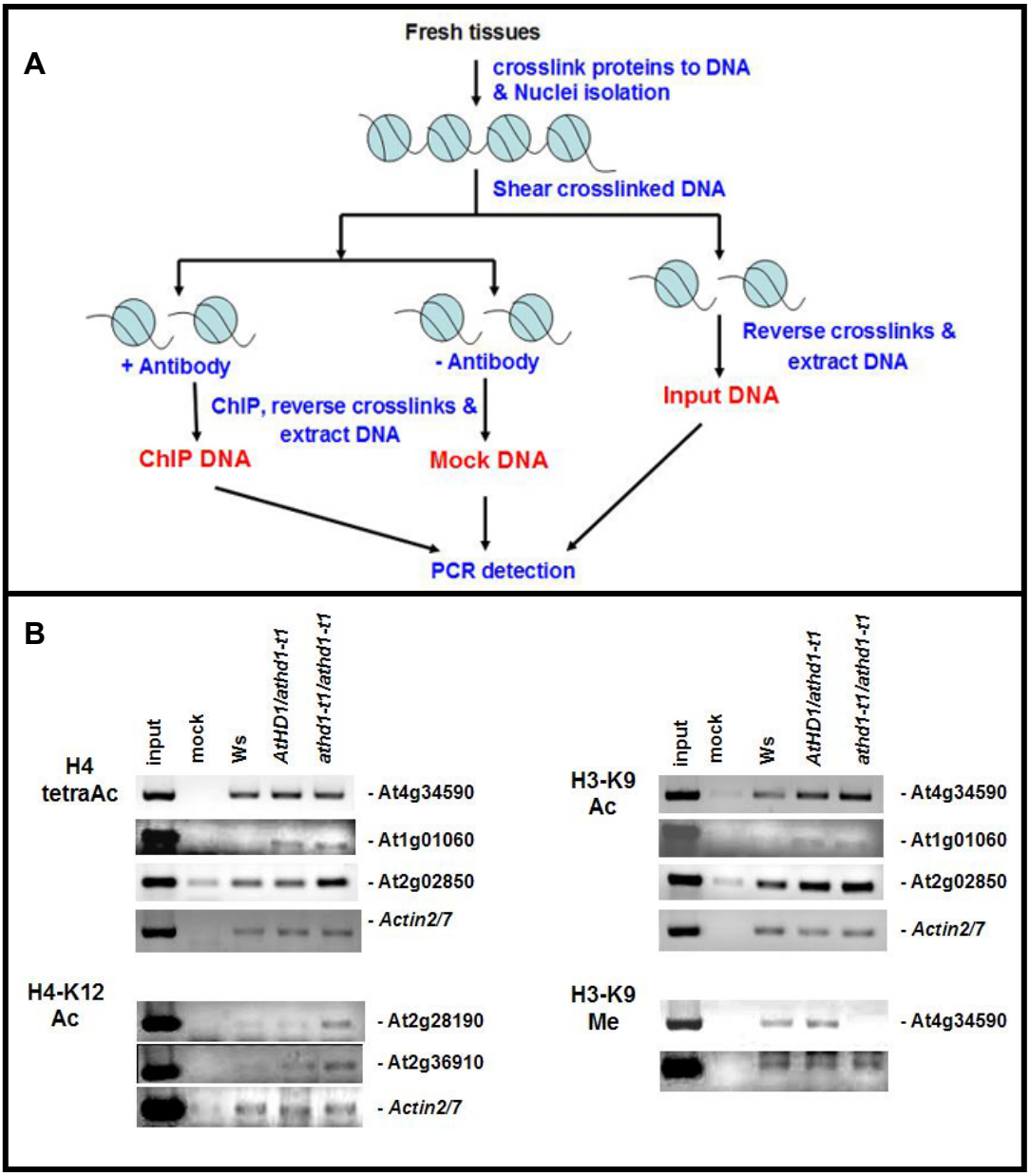


Figure 4.7. Acetylation states of individual genes in the *athd1-t1* mutant.

(A) Scheme of chromatin immunoprecipitation (ChIP) assay. Nuclei were crosslinked, sonicated, and immunoprecipitated with antibodies specific for acetylated or methylated histones. The immunoprecipitates were analyzed by semiquantitative PCR.

(B) Results of ChIP assays using specific antibodies (anti-tetraacetylated-H4, anti-acetylated-lys12-H4, anti-acetylated-lys9-H3 and anti-dimethyl-lys9-H3). The lanes labeled "Input" contain the products of PCR performed with DNA recovered from immunocomplexes before immunoprecipitation. "Mock" refers to the control, in which the chromatin was precipitated without using antibodies.

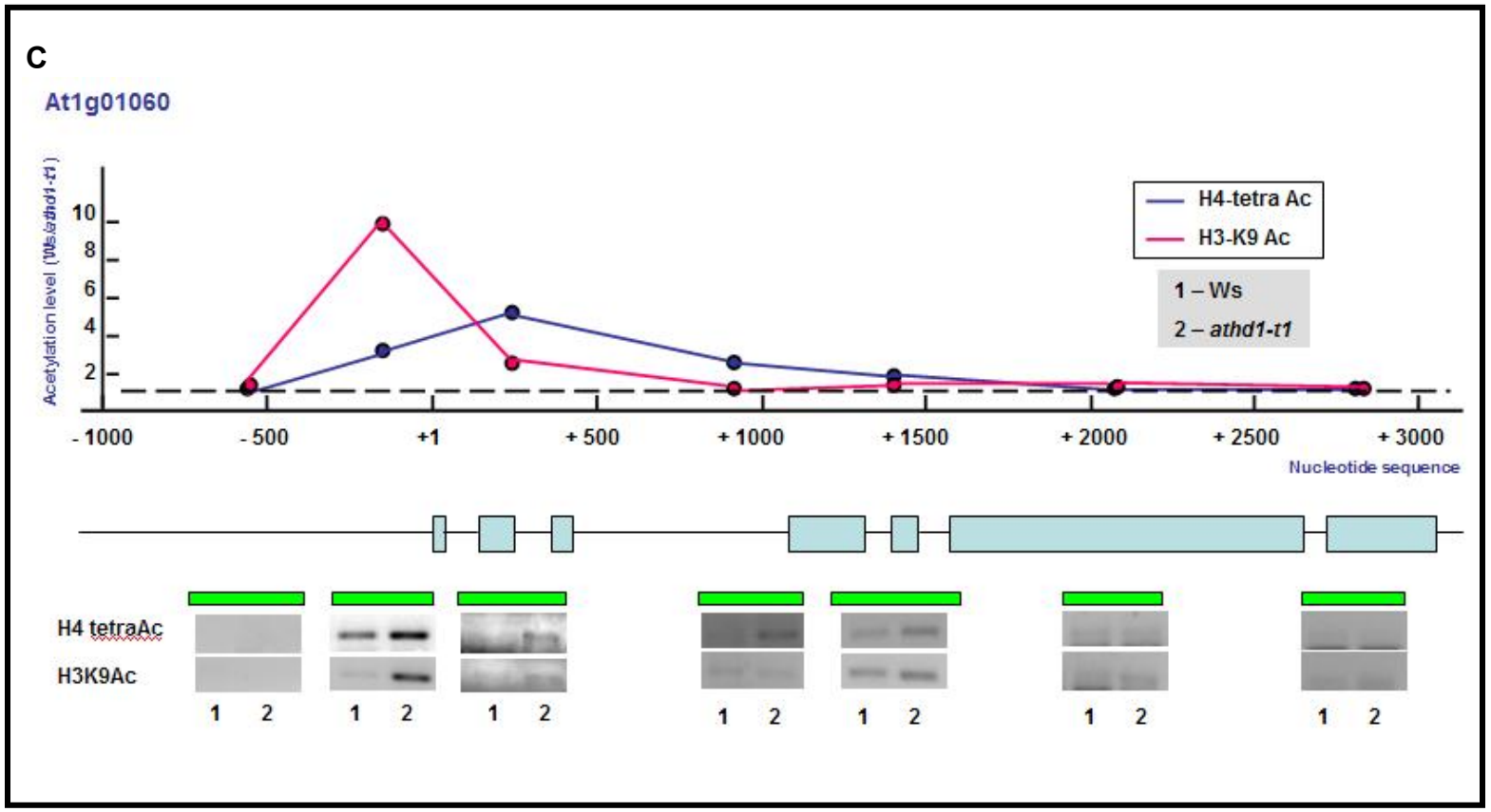


Figure 4.7. Continued

(C) and **(D)** PCR analysis of DNA immunoprecipitated by antibodies against tetra-acetylated histone H4 (blue lines) and acetylated H3-K9 (pink lines). Exons are shown as boxes and introns as lines. The predicted translation start sites are indicated as +1. The genes At1g01060 (C) and At2g02850 (D) were subjected to this analysis. The data are presented as ratios of acetylation levels in the *athd1-t1* mutant to that in the wild-type plants. Positions of the PCR fragments relative to the genomic region (green bars) are shown.

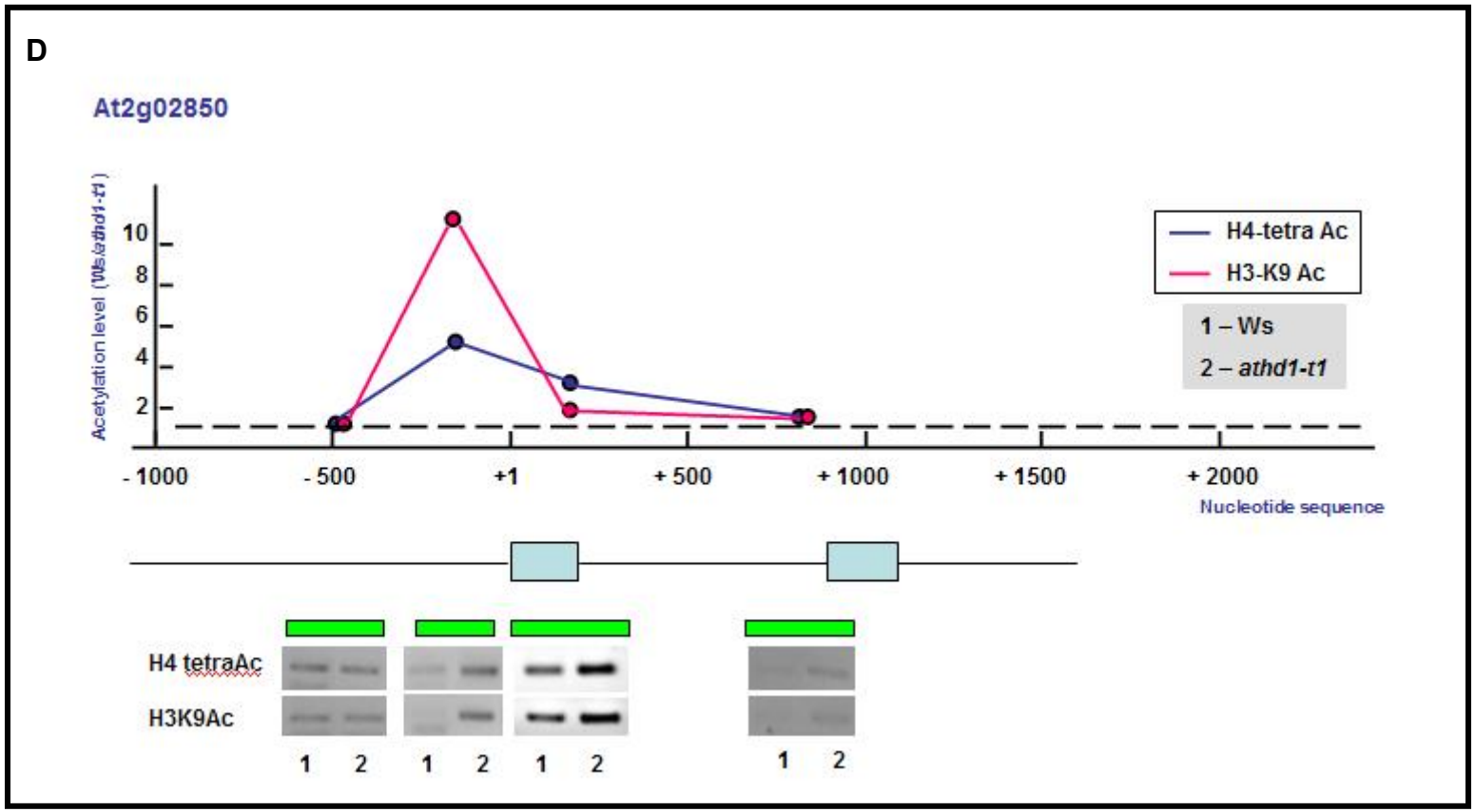


Figure 4.7. Continued

Links. DNA was extracted and analyzed by PCR.

Since the wild-type phenotypes were completely recovered in *AtHD1/athd1-t1* heterozygous plants (Tian et al., 2003), *AtHD1/athd1-t1* plants were also included in the ChIP assays to check if the acetylation states of each individual gene in the heterozygous plants were also recovered to wild-type states. The concentration of precipitated DNA from these three samples in each ChIP assay was first normalized by using a primer pair specific for *ACTIN2/7*. *ACTIN2/7* is a constitutively expressed gene (An et al., 1996), and is equally expressed in Ws and the *athd1-t1* mutant as shown by microarray and RT-PCR analyses. The genes used for ChIP assays were selected from the genes whose expression patterns have been verified by either RT-PCR or RNA blots (asterisks in Tables 4.3A and 4.3B). The primers were designed to amplify ~350 bp fragments within coding regions or promoter regions of the targeted genes.

The results are shown in Figure 4.7B and summarized in Table 4.4. There are three patterns of acetylation states associated with differentially expressed genes. In the first category, genes were activated in association with the accumulation of certain hyperacetylated histones. For instance, up-regulation of At2g36910 and At2g28190 correlated with accumulation of hyperacetylated H4-K12 (Figure 4.7B). The levels of tetra-acetylated H4 and acetylated H3-K9 associated with At2g02850 and At1g01060 gene sequences were higher in the *athd1-t1* mutant than in the wild-type plants. The acetylation level of H3-K9 was increased, whereas the methylation level at the same residue was correspondently decreased within the coding region of At4g34590. This result provided the evidence to support the notion that histone H3 Lys9 is

Table 4.4. Summary of ChIP results

	MIPs accession no.	anti-H4Ac	anti-H4K12Ac	anti-H3K9Ac	anti-H3K9Me	tissue	expression
1	**At1g14970**	~	~	~	~	**L**	↑
2	**At2g36910**	~	+	~	**n.d.**	**L**	↑
3	**At2g28190**	~	+	~	~	**L**	↑
4	**At3g23130**	~	~	~	**n.d.**	**L**	↑
5	**At1g02920**	~	~	~	~	**L**	↓
6	**At4g34590**	~	**n.d.**	+	-	**F**	↑
7	**At1g01060**	+	**n.d.**	+	**n.d.**	**F**	↑
8	**At2g02850**	+	**n.d.**	+	**n.d.**	**F**	↑
9	**At2g22980**	~	**n.d.**	~	~	**F**	↓

"~": no difference in acetylation status; "+": increase level of histone acetylation; "-": decrease level of histone acetylation/methylation; n.d.: not determined, no PCR amplification or no ChIP DNA;

"L": leaves; "F": flower buds; "↑": up-regulation in the *athd1-t1* mutant; "↓": down-regulation in the *athd1-t1* mutant

the site for competitive acetylation and methylation, leading to gene activation or repression. The expression of these genes is likely directly affected by the down-regulation of *AtHD1*. Second, in genes whose expression was up-regulated in the *athd1-t1* mutant, we observed no changes in acetylation level (i.e., At1g14970 and At3g23130 (*SUPERMAN*)) (Table 4.4). Third, for genes with lower expression than that in the wild-type (i.e., At1g02920 and At2g22980), acetylation states remained the same (Table 4.4). Thus, the expression of these two groups of genes was altered without changes in acetylation states, suggesting that they were not directly affected by down-regulation of *AtHD1*. It was also notable that the acetylation level for some genes was increased in the *AtHD1/athd1-t1* heterozygous plants, although they resembled wild-type phenotypes. This result suggests that epigenetic lesion might be induced by the AtHD1 defect.

Detailed analyses of acetylation profiles were further performed on two genes, At1g01060 and At2g02850. Primers were designed to amplify individual DNA fragments along the promoter and coding regions of these two genes. We found that the acetylation level of histone H4 and histone H3-K9 was highly increased within the promoter region of the genes (Figures 4.7C and 4.7D). However, no changes were detected in the region over 500 bp upstream of the translation start sites. Hyperacetylated histones were also accumulated within the coding region proximal to the promoter, but almost reduced to the wild-type states within the last exon of the genes (Figures 4.7C and 4.7D). It is likely that AtHD1 directly regulates gene expression by preventing the start of transcriptional initiation step.

We further tested if the accumulation of hyperacetylated histones spreaded to the neighboring chromosomal regions. The genes At2g36910 and At4g34590 were up-regulated in leaves (Figures 4.4A and 4.6A) and flower buds (Figures 4.4B and 4.6B) of the *athd1-t1* mutant, respectively, whereas the expression of their neighboring genes were not affected (Figures 4.6A and 4.6B). A segment of chromosome 2 containing At2g36910 gene (Figure 4.6A) and a segment of chromosome 4 containing At4g34590 gene (Figure 4.6A) were subjected to chromatin immunoprecipitation assays. We found that the up-regulation of At2g36910 gene and At4g34590 gene were associated with increased acetylation of H4-K12 (Figure 4.6A) and H3-K9 (Figure 4.6B), respectively. However, the hyperacetylation did not expand to the chromatin domain of their adjacent genes (Figures 4.6A and 4.6B). This result suggests that AtHD1 regulates gene expression by being recruited to its specific targeted genes without affecting the acetylation level of the adjacent chromosomal domains.

DISCUSSION

Yeast RPD3 protein and its homologs have been extensively studied in many eukaryotes. In general, RPD3-like proteins are associated with repressors or reside in repressor-containing protein complexes to modulate gene transcription (Kasten et al., 1997; Kao et al., 1998; Zhang et al., 1998b). In yeast, RPD3 protein is a positive and negative transcriptional regulator, as *RPD3* deletion induces both up- and down-regulation of gene expression (Bernstein et al., 2000). Our data suggest that AtHD1

resembles RPD3 protein in yeast and behaves as a global regulator in *Arabidopsis* genome.

AtHD1 (AtHDA19) together with three other RPD3/HDA1 superfamily members, including AtHDA6, AtHDA7 and AtHDA9, are the closest relatives of yeast RPD3 in *Arabidopsis* (Pandey et al., 2002) (Figure 4.1). AtHD1 shares the highest protein sequence identity with RPD3-like proteins in other species and possesses all the potential catalytic residues that have been characterized in human histone deacetylases (Hassig et al., 1998) (Figure 4.1), indicating a structural conservation among RPD3 homologs. In contrast to the other three members, AtHD1 is ubiquitously expressed in all *Arabidopsis* tissues analyzed (Figure 4.1D), implying that it is required in various plant developmental stages. Down-regulation of AtHD1 is associated with the overall accumulation of hyperacetylated histones in the *Arabidopsis* genome (Tian and Chen, 2001; Tian et al., 2003). Furthermore, we show that hyperacetylated histones were enriched within the coding regions of derepressed genes in the *athd1-t1* mutant (Figures 4.6A, 4.6B, 4.7B, 4.7C and 4.7D). These analyses support the notion that AtHD1 possesses a histone deacetylase activity.

AtHDA6 is another *Arabidopsis* histone deacetylase that is closely related to yeast RPD3 protein (Pandey et al., 2002). Several mutations in AtHDA6 derepress the expression of transgenes, although none of the endogenous genes were affected and no obvious developmental defects were observed (Murfett et al., 2001). Moreover, AtHDA6 has been shown to be involved in homologous DNA methylation directed by the introduction of double stranded RNA (Aufsatz et al., 2002). Different from *AtHDA6*,

down-regulation of *AtHD1* induced pleiotropic developmental abnormalities, including early senescence, suppression of apical dominance, homeotic changes, heterochronic shift towards juvenility, flower defects, and male and female sterility, as well as ectopic expression of silenced genes (Wu et al., 2000a; Tian and Chen, 2001; Tian et al., 2003). This finding suggests that these two RPD3-like proteins have distinct functions. AtHD1 is the histone deacetylase that plays an important role during plant development, while AtHDA6 is more likely to participate in the plant defense mechanism. Using microarray analysis, we examined transcription profiles of leaves and flower buds in the *athd1-t1* mutant. Unlike the results obtained from AtHDA6 mutants showing that only a few genes were affected (Murfett et al., 2001), we found that 6.7% (1,753) of the genes were differentially expressed in leaves of Ws and the *athd1-t1* mutant, and 4.8% (1,263) of the genes were affected in flower buds of the mutant. The RPD3 protein and RPD3-like HDACs of yeast and mammalian systems are thought to be involved in transcription regulation, hormone signaling, the cell cycle, differentiation, and DNA repair (Bartl et al., 1997; Bernstein et al., 2000; Thiagalingam et al., 2003). The maize RPD3 homolog ZmRPD3 was also implied in the control of cell cycle progression (Rossi and Varotto, 2002), DNA replication (Lechner et al., 2000), cell division and metabolic activity (Varotto et al., 2003). Consistent with RPD3-like proteins in other species, the up- and down-regulated genes in the *athd1-t1* mutant belonged to various functional classes (Figures 4.5D and 4.5E), including cellular metabolism, cell growth, division and DNA synthesis, protein destination, protein synthesis, cell defense and aging, transcription regulation, cellular biogenesis, signal transduction, energy, transport facilitation and

intracellular transport. It suggests that AtHD1 participates in various physiological processes. It is notable that few transposons were affected by inhibition of AtHD1 expression (Figures 4.5D and 4.5E), implying that transposons are not major biological substrates of AtHD1. Recent studies have shown that AtHDA6 is involved in silencing of a subset of transposons in *Arabidopsis* (Lippman et al., 2003), providing further evidence that AtHD1 and AtHDA6 have evolved divergent functions. Other than AtHDA6, other epigenetic modulators are also involved in transposon regulation, including DNA methylation, histone H3-K9 methylation and RNA interference (RNAi) (Lippman et al., 2003). DNA methylation and histone H3-K9 methylation have been suggested to regulate gene expression in coordination with histone deacetylation in eukaryotic organisms (Chen and Pikaard, 1997; Nan et al., 1998; Czermin et al., 2001; Deplus et al., 2002). It is possible that at different genomic loci, different histone deacetylases are recruited to participate in gene regulation together with DNA and/or histone methylation.

Generally, it is believed that histone deacetylation is associated with compact chromatin structure and thereby represses gene expression. Therefore, down-regulation of histone deacetylases induces gene transcription. However, many were down-regulated by yeast *RPD3* deletion (Bernstein et al., 2000). Similarly, our microarray analysis also indicated that almost an equal number of genes was either up- or down-regulated in the *athd1-t1* mutant. Histone deacetylases may also activate gene expression, either by creating a specific “histone code” in coordination with other histone modifying enzymes (Jenuwein and Allis, 2001), or by associating with

transcriptional cofactors leading to transcription activation. Chip-ChIP analysis demonstrates that there is a significant association between sites at which *RPD3* deletion results in increased acetylation and increased transcription, but not decreased transcription (Robyr et al., 2002). This suggests that the down-regulated genes are indirect targets of RPD3 protein. It seems more likely that RPD3 is a genome-wide repressor rather than an activator. In our study, accumulation of hyperacetylated histones were only detected to be associated with activated genes rather than repressed genes (Table 4.4). This implies that gene repression is a secondary effect of down-regulation of AtHD1. It will be interesting to apply Chip-ChIP analysis to the *athd1-t1* mutant, to address this question more thoroughly.

In this study, we detected some acetylation states that might be unique to plants. First, RPD3 protein has been shown to deacetylate both histones H3 and H4, in particular deacetylate H4 K5 and K12 (Kadosh and Struhl, 1998a; Rundlett et al., 1998; Suka et al., 1998). Chip-ChIP analysis indicated that acetylation of H4 lysines K5 and K12 and to a lesser extent H3-K18 in the *rpd3*Δ strain correlates better with increased transcription than acetylation of H4-K16 (Robyr et al., 2002). In our study, ChIP assays also detected enrichment of hyperacetylated H4-K12 in the vicinity of activated genes in leaves of the *athd1-t1* mutant. However, accumulation of both tetra-acetylated histone H4 and hyperacetylated H3 K9 was detected, especially in flower buds of the mutant. Accumulation of tetra-acetylated histone H4 implies an increase in the acetylation level of all four lysine residues of histone H4, including H4 lysine 16. The difference in acetylation sites between yeast and plants suggests that RPD3 and AtHD1 might have

evolved different choices for catalyzing substrates. A definite answer awaits *in vitro* biochemical assays using purified recombinant AtHD1 or AtHD1 complex. Second, RPD3 is recruited to the promoter regions to regulate gene transcription (Robyr et al., 2002). DNA-binding protein Ume6p probably recruits RPD3 protein, through Sin3p, to a specific DNA element URS1 in the *INO1* promoter (Kadosh and Struhl, 1997). It has been revealed that some promoters in yeast are strongly acetylated in a promoter-specific manner in the *rpd3*Δ stain; however, the surrounding regions are also acetylated (Vogelauer et al., 2000). In our chromatin immunoprecipitation assays, the acetylation levels within the promoter regions were dramatically increased and smaller enrichment of acetylated histones were observed within the coding regions proximal to translation start sites in the *athd1-t1* mutant (Figures 4.7C and 4.7D), but no or little accumulation of hyperacetylated histones was detected within the region over 500-bp upstream of the translation start sites and at the end of coding regions (Figures 4.7C and 4.7D). The enrichment of hyperacetylated histones did not further spread to the surrounding chromosomal regions (Figures 4.6A and 4.6B). It suggests that deacetylation by AtHD1 involves the targeting of this enzyme to selected promoter elements by DNA-binding proteins to suppress transcription initiation step. Unlike yeast RPD3 protein, AtHD1 may not affect global deacetylation of histones in *Arabidopsis* genome.

METHODS

Plant materials

Ws, homozygous *athd1-t1/athd1-t1*, and heterozygous *AtHD1/athd1-t1* plants were generated as previously described (Tian et al., 2003). The *AtHD1/athd1-t1* plants were generated by crossing *athd1-t1/athd1-t1* plants with Ws plants, and screened by PCR. Seeds from Ws, heterozygous plants and *athd1-t1/athd1-t1* plants were sterilized and germinated in Murashige/Skoog medium (Sigma, St. Louis). After 2 weeks of growth on the medium, the plants were transferred to soil and grown in a growth chamber with growth conditions of 22ºC/18ºC (day/night) and 14 hour of illumination per day.

DNA, RNA and mRNA analysis

Rosette leaves were harvested at pre-bolting stage. Flower buds were consecutively collected over three days after the first flower bloomed. Each tissue, including leaves, flower buds, siliques, stems and roots, was collected by pooling 32 plants for RNA preparation. Seedling samples were collected 8 days after germination. For all tissues except siliques, total RNA was extracted with Trizol reagent (Invitrogen, San Diego) according to manufacturer's recommendations. Total RNA of young siliques was isolated with Plant RNA Reagent (Invitrogen, San Diego). DNA were isolated from rosette leaves as previously described (Chen and Pikaard, 1997). DNA and RNA blot analysis was conducted as described by Tian and Chen (2001).

The mRNA for microarray analysis was isolated from 500 μg of total RNA with FastTrack 2.0 mRNA isolation kit (Invitrogen, San Diego) following the manufacturer's instructions.

Microarray analysis

We worked with Qiagen/Operon company and designed a set of 70-mer oligonucleotides (oligos) from 1,000 bp of the 3' end of well-characterized genes and predicted ORFs in the fully sequenced *Arabidopsis thaliana* genome. Gene names and GenBank accession numbers of the 26,090 70-mer oligos and the related design information can be found in http://oligos.qiagen.com/arrays/oligosets_arabidopsis.php. Each slide was printed with 27,648 features, including 26,090 70-mer oligos and controls. The microarray experiments were slightly modified from that of Lee et al. (2004).

Probe labelling, slide hybridization, and data capture

cDNA probes were prepared with CyScribe^{TM} First-Strand cDNA labeling kit (Amersham Biosciences, NJ), following the manufacturer's instructions with slight modifications. By using this method, the fluorescent dyes (Cy3 or Cy5) were incorporated into the probe after first-strand cDNA synthesis. An aliquot of 500 ng of mRNA was mixed with 1 μl oligo-dT and 1 μl nonamer primer in a volume of 11 μl. The mixture was incubated at 65ºC for 5 min, cooled down slowly at room temperature for 10 min, allowing the oligo-dT and nonamer primer to anneal with mRNA template.

The pre-annealed mixture was supplemented with 4 µl of 5 X Superscript II buffer (Invitrogen, San Diego), 2 µl of DTT, 1 µl of nucleotide mix, 1 µl of amino allyl-dUTP and 1 of µl Superscript II (200 units/µl), and then incubated at 42ºC for 2 hours. The reaction was terminated by the addition of 2 µl of 2.5 N NaOH and incubated at 37ºC for 15 min, and then neutralized with 10 µl of 2M HEPES. The synthesized first-strand cDNA was purified with QIAquick purification kit (Qiagen) and lyophilized in a speed vacuum. The lyophilized product was resuspended in 15 µl of ddH_2O, and then mixed with 15 µl of freshly prepared Cy3-Dye or Cy5-Dye (resuspended in 0.1 M $NaHCO_3$). The mixture was incubated in dark at room temperature for 2 hours. The reaction was terminated by the addition of 15 µl of 4 M Hydroxylamine hydrochloride. The synthesized cDNA probe was purified again with QIAquick purification kit (Qiagen) and lyophilized in a speed vacuum.

The lyophilized Cy3- or Cy5-probe were mixed together and resuspended in 70 µl of hybridization solution (3 X SSC, 0.2% SDS, 10 mg/ml BSA). The mixture was heated for 2 minutes at 95°C, and applied directly to the array. After covering the array with a lifterslip (Erie Scientific), the slide was placed in a microarray hybridization chamber (Corning Incorporated). Hybridization was performed overnight (14~15 hrs) at 65°C with slow agitation in a hybridization incubator. After hybridization, the slides were washed in 2 X SSC, 0.2% (w/v) SDS for 4 min, then in 0.2 X SSC for 4 min, and finally in 0.05 X SSC for 4 min. Immediately after last washing, the slides were dried by centrifugation at 800 rpm for 5 minutes.

The slides were scanned in a GenePix 4000B scanner (Axon, Foter City, CA) at a resolution of 10 μm. The absorbed fluorescent signals of hybridized microarrays were quantified using Genepix Pro4.1 software (Axon, Foter City, CA). The data of Cy3 or Cy5 signal intensities were transformed by natural logarithm (ln) and subjected to the ANOVA analysis using a liner model (Lee et al., 2004).

Experimental design

The standard dye-swap design (Lee et al., 2003) was used in this experiment. A dye swap experiment involved two treatments (wild type and *athd1-t1* mutant) and two arrays (Figure 4.2A and Table 4.1). The two treatments, T1 (Ws) and T2 (*athd1-t1*) are labeled with dye1, D1 (Cy3), and dye2, D2 (Cy5), respectively, on the first array, A1. These labellings are then reversed for the second array, A2. That is, T1 (Ws) receives D2 (Cy5), and T2 (*athd1-t1*) receives D1 (Cy3).

Data analysis

The notation X_{ijkm} was used to denote the gene m (gene 1 ~ 26090) under treatment condition k (Ws or *athd1-t1* mutant) labeled with dye j (Cy3 or Cy5) on array i (array 1 to 8). After log transformation, the notation $Y_{ijkm} = \log_2 (X_{ijkm})$ was used. The data were then subjected to the analysis of variance (ANOVA) in a linear model:

$$Y_{ijkm} = \mu + A_i + D_j + T_k + G_m + AG_{im} + DG_{jm} + TG_{km} + \varepsilon_{jikm} \text{ (ANOVA 1)}$$

Where μ represents the overall mean effect, and A, D, T and G represent main effects from the array, dye, treatment, and gene, respectively. AG, DG, and TG represent array

by gene, dye by gene, and treatment by gene interactions, and εijkm denotes the random error. The model residuals were assumed to be normally distributed with a common variance s^2 (i.e., εijkm i.i.d. N (0, s^2)). However, common variance for every gene is not always biologically meaningful. Therefore, a per-gene variance for each individual gene was also calculated using a small ANOVA model:

$$Y_{ijkm} = \mu + A_i + D^j + T_k + G_m + \varepsilon_{jikm} \text{ (ANOVA 2)}$$

Where μ represents the mean signal intensity of each individual feature on the array, and A, D, T and G represent effects from the array, dye treatment, and gene on this specific feature, respectively.

Hypothesis testing

When using common variance, the presence of differential expression is represented in T + TG terms for a particular gene (ANOVA1). The following hypotheses were tested to determine whether a gene was differentially expressed between two treatments t and t' (Ws and *athd1-t1* mutants):

H_0: $T_t + TG_{tg} = T_{t'} + TG_{t'g}$

H_1: $T_t + TG_{tg} \neq T_{t'} + TG_{t'g}$

When using per-gene variance, the presence of differential expression is represented in T terms for a particular gene (ANOVA2). The following hypotheses were used instead:

H_0: $T_t = T_{t'}$

H_1: $T_t \neq T_{t'}$

A standard t-test statistical analysis was used for the multiple comparison, using either common variance (assuming every gene has the same variance) or per-gene variance (assuming every gene has a specific variance). The standard false discovery rate (FDR) was used to control multiple testing errors (Lee et al., 2003). The significance level $\alpha = 0.05$ was chosen for this study.

Verification of expression patterns

Based on t-test, the genes with significant differential expression were subjected to RT-PCR and RNA blot analyses.

RT-PCR analysis

A total of 500 ng of mRNA was mixed with 1µl of oligo-dT (Amersham) and 2 µg random nonamer (Gene Link, Cat. No. 26-4000-06) in a total volume of 17 µl, heated at 65ºC for 5 min, and cooled down at room temperature for 10 min to allow the primers and the RNA template to anneal. Reverse transcription (RT) was then performed in a total volume of 30 µl by adding 1 µl of 20 mM dNTPs, 6 µl of 5x RT buffer, 3 µl of 0.1 M DTT, and 1 µl of SuperScript II reverse transcriptase (200U/µl, invitrogen) at 42ºC for 2 hours. The synthesized cDNA was purified by QIAquick PCR purification kit (QIAGEN, Cat. No. 28104). Being measured with a spectrometer (GeneQuant *pro*), the concentration of the purified first-stranded cDNA was adjusted to 15 ng/µl with ddH_2O. An aliquot of 0.5 µl was then used for PCR reaction in the volume of 25 µl. The primers were designed according to the 3' end sequences of *A. thaliana* genes identified in

microarray analysis. The sequences of primers were listed in Tables 4.5A and 4.5B. The *ACTIN2/7* gene was amplified and served as a control (Tian et al., 2003). The PCR reactions were conducted as follows: 1 cycle of 94ºC for 2 min, 30 cycles of 94ºC for 30 sec, 52ºC for 30 sec, and 72ºC for 1 min. An aliquot of 5 µl RT-PCR products was subjected to electrophoresis on 1% agarose gels.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were slightly modified from a previously described protocol (Gendrel et al., 2002). Leaves were collected from pooled plants of wild type (Ws), *athd1-t1/AtHD1* or *athd1-t1/athd1-t1* plants at pre-bolting stage. Flower buds from the same sets of plants were collected consecutively over three days after the emergence of the first flower. Approximately 1 g of leaves or 0.5 g of flower buds was used for each chromatin immunoprecipitation.

To cross link the chromatin proteins to DNA, the fresh tissues were subjected to vacuum infiltration in 1% formaldehyde at room temperature for 10 min. To quench the crosslinking reaction, glycine was then added to a final concentration of 0.125 M, and incubated for an additional 5 min. The tissues were rinsed three times with Milli-Q water, immediately frozen in liquid nitrogen. Approximately 1 g of tissues were resuspended in 30 ml of extraction buffer 1 (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 5 mM β- mercaptoethanol, 1 mM PMSF, 1 µg/µl aprotinin, and 1 µg/µl pepstatin A), and then was filtered through two layers of Miracloth. The filtrate was centrifuged at 2000 g for 20 min at 4ºC. The pellet was then resuspended in 1 ml of extraction buffer 2 (0.25

M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1% Triton X-100, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/μl aprotinin, and 1 μg/μl pepstatin A) and transferred to a 1.5-ml eppendorf tube. The solution was centrifuged at top speed in microcentrifuge for 10 min at 4ºC. The pellet was resuspended in 300 μl of extraction buffer 3 (1.7 M sucrose, 10 mM Tris-HCl, pH 8.0, 0.15% Triton X-100, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/μl aprotinin, and 1 μg/μl pepstatin A), and then layered on top of another 300 μl of extraction buffer 3 in a new eppendorf tube. The solution was centrifuged at top speed in microcentrifuge for 1 hour at 4ºC. The resulted chromatin pellet was resuspended in 250 μl of nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1 μg/μl aprotinin, and 1μg/μl pepstatin A) and incubated on ice. Then the chromatin was solublized with a probe sonicator (Fisher, Model 60 sonic dismembrator), 5 x 10 sec pulses at half maximal power with 3-min cooling on ice between pulses. The average size of resulted DNA fragments was 0.3 to 1.0 kb. Insoluble cellular debris was removed by centrifugation at top speed in microcentrifuge for 10 min at 4ºC. An aliquot of chromatin solution was ($1/10^{th}$ of total volume) used to determine fragment size (ideally under 1kb) and served as input DNA. The remaining of chromatin solution was diluted 10-fold with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, and 167 Mm NaCl). The diluted chromatin solution was precleared by incubation with 40 μl of salmon sperm (SS) DNA/protein A agarose (Upstate Biotechnology) for 1 hour at 4ºC with rotation. After centrifugation at 1000 rpm for 2 min at 4ºC, the supernatant was transferred into a new tube. The supernatant was divided into two equal aliquots for

Table 4.5A. Primers for verification of differentially expressed genes in leaves between Ws and the *athd1-t1* mutant

No.	Locus	Primer pairs	Product size (bp)
1	*At1g02920	F: AGTTCCAGCCTTTGAAGATGGAGA R: AGAACCTTCTTAGCAGAAGGCCTA	465
2	At4g06746	F: AAGAAGGAATGGTCATGACCGAGA R: TTACAATCCATATGCCTCCGGCAA	260
3	At2g26560	F: GCCATTGGGGAAGTAACAAATGAG R: TTGGAGCTTTTGCATGAGGTGAAC	535
4	At2g21650	F: GGCTCAATGTCTTCTTATGGCTCT R: CACTGCAGCTTCATGCTTCTCATC	296
5	At5g56870	F: ATAAAGGAGTTCTTGGTCCGGTCA R: GAGATTCCGTTAGGATCACCACCT	484
6	At3g29035	F: CGAATCGTGCTACTAAAGCCGGTT R: ACGCAATCAAGATTCACTGGACCA	658
7	At4g19170	F: TGAAGATGATGGAAACAGCGTCGT R: TAACCCATGGAATCCGTACGGAAC	526
8	At5g62920	F: TGGCTGAAGTTATGCTACCGAGGA R: ACGAGGCAAAATGTTCTCGGAGGA	368
9	At5g10760	F: CTCTCACTACCGGCGCAAACAAC R: AACCATTGGGAGCAAAACCGACTC	607
10	At1g15125	F: TTTGCATGCTAGGTCACAGGAGCT R: CATGACAGCGACTGAGAAGGGCAA	479
11	At1g55860	F: ACGAAAGTCTCGCTTTTACCACCT R: TTCTGATGTTGTTTGCAGCCACAG	485
12	At3g24270	F: GTTCCTGATTCAAGTTGGTGCAAC R: TCAAGCTAGACGAGTGGATCTGTC	374
13	At3g22120	F: CACACCTAAACCTCCCACGACCA R: AGCGGGACATTTGAAGTCAGATGG	592
14	At5g07860	F: TATCTTCTTTGCAGTCGCTGACTG R: TCCAAAGCTTCCATACACTCTGGA	517
15	At2g15090	F: ATGGCAGGATTAGCCATGAAAGGA R: TTGTTCACAATCATGGAGGCGAGT	539
16	*At2g36910	F: TCGGTTATTGTGCAGAACACAGCA R: CTTCGGTCGCACATTCATGTCCA	925
17	At1g24020	F: CCCAAAGCTTTCCCTAACGACTAC R: CAGCAAAGTCCTTGATGACATGTG	332
18	*At2g28190	F: CTGGTCTCACTCCAGGGCCTCA R: TAGAGCGGCGTCAAGCCAATCAC	358
19	*At3g23130	F: ACAGCATAGAGTTGAGGAACAGCT R: AACCCAAACGGAGTTCTAGATCCA	594

* These genes are also included in ChIP assays.

Table 4.5B. Primers for verification of differentially expressed genes in flower buds between Ws and the *athd1-t1* mutant

No.	locus	Primer pairs	Product size (bp)
1	*At2g22980	F: ACGATATCAAGAGCAGCGTCGCAT R: TTACAAAGGCTGGGCACTGATCCA	311
2	At1g56300	F: CGAAGGTGGAGGATCTAACGTCAG R: CTCCTCGAGATATTGACACGTGGT	444
3	At4g27440	F: TTGGCGGGTAATGTACCACCGAAG R: CGAGCTTCTCACTGATCTCCCACA	487
4	At3g45640	F: AGAGCACCTGAGCTTCTGTTGAAC R: TGTTGGATTGAGTGCTATGGCTTC	492
5	At1g68050	F: CCGACATGGAAAGAGATCCCGACA R: TACAGATCCGAGTCTTGCCGGCTA	518
6	At5g45890	F: GGTTGCGGCTATTGAAGGAGCAA R: AGAAGCTTTCATGGCAAGACCACA	550
7	At3g26740	F: CAGGGAGCTCTGTTTATCAAACCA R: TCAGGTTCACAGAATTCTCCTCCA	401
8	At2g38470	F: GAAACAAATGGTGGGAATGGTGGT R: AAATGAGGTCTCCTCGTTTGGTTC	543
9	At5g66070	F: GAATCTTCCCTCCTCCTTTGGCAA R: AAGATGTCTTCTGCACAAGGGACA	417
10	At1g62360	F: ATGCATATCAGTCACACCACCAAC R: GGGATTTGAATTGACACTCGACAC	495
11	At5g32484	F: ACTGAAACAAAGTGGGCGCATGTG R: TGCCTTGGTGAATAGATCGGCTGA	1066
12	*At2g02850	F: CAAGGGAAGAGGCAGTGCATCATG R: GCGGTGACTGCGATTTTCATATCG	378
13	At1g74020	F: ACAGGTGATCTTTACGTCGCCGAT R: GAATTCCAGCAAATGGTCCGGTGA	652
14	At1g12560	F: CGGCGCTAAGCACGACATTGTTC R: CTTGTAAGTCTTACCGCCGCTCCA	551
15	*At4g34590	F: GTCGTCGGGAACAACTTCGTCGA R: AGAAGACGCCATGAGAGGCTGGT	451
16	At3g62460	F: CACGGCTAGTAGCGAATACGGTAG R: AGAGTATCTTCTGGTCTGGGCGAT	548
17	*At1g01060	F: TGCAGTTCCAACTCCAGCAATGAC R: GCTTCTCCTTCCAATCGAAGCCTT	786

* These genes are also included in ChIP assays.

immunoprecipitation. In one aliquot, 10 µl of the chosen antibody was added. The antibodies are anti-tetra-acetyl-histone H4 (Cat.No. 06-866), anti-acetyl-histone H4 [Lys12] (Cat.No. 07-323), anti-dimethyl-histone H3 [Lys9] (Cat.No. 07-212), and anti-acetyl-histone H3 [Lys 9] (Cat.No. 06-942), all purchased from Upstate Biotechnology. In another aliquot, no antibodies were added, which served as control reaction (mock). After incubation overnight at 4ºC with rotation, 40 µl of SS DNA/protein A agarose was added into the solution and incubation continued for another 2 hours. The histone-DNA complexes were bound with SS DNA/protein A agarose. The agarose beads were washed using 1 ml of each of the following buffers: 1 X low salt wash buffer (150 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA, and 20 mM Tris-HCl, pH 8.0), 1 X high salt wash buffer (150 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA, and 20 mM Tris-HCl, pH 8.0), 1 X LiCl wash buffer (0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0), 2 X TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immunocomplexes were then eluted from the agarose beads by incubation with 250 µl of freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$) at 65ºC for 15 min with agitation. Elution was repeated one more time and two eluates were combined. A total of 20 µl of 5 M NaCl was added to the eluates, and crosslinks were reversed by incubation at 65ºC from 6 hours to overnight. Residual protein was degraded by the addition of 20 µg proteinase K (in 10 mM EDTA and 40 mM Tris-HCl, pH 6.5) at 45ºC for 1 hour, followed by two times of phenol/chloroform/isoamyl alcohol extraction. DNA was precipitated by adding 1 µl of 20 mg/ml glycogen and 1/10th volume of 3 M NaOAC (pH 5.2) and 2X volume of 95%

ethanol at -20ºC overnight. Pellets were washed with 70% ethanol and resuspended in 50 µl of ddH_2O. Approximately 1 µl was used for PCR reaction.

ChIP PCR

Semi-quantitative PCR was used to determine the amounts of genomic DNA immunoprecipitated in the ChIP assays. Reactions were done using primer pairs designed from promoter and/or coding regions of the candidate genes. The primers for each gene were designed to amplify a fragment of ~ 350bp. The amplification of *ACTIN2/7* gene was used as a control. The sequences of primer pairs were listed in the Table 4.6. The concentration of each immunoprecipitated DNA sample was adjusted empirically such that an equal amount of *ACTIN*2/7 was amplified (Tian et al., 2003). All PCR reactions were performed in 25 µl with 1.0 µl of immunoprecipitated DNA. Following an initial denaturation step at 95ºC for 2 min, 30-35 cycles of PCR were performed using the program of 94ºC for 30 sec, 52ºC for 30 sec, and 72ºC for 1 min with a final extension of 10 min at 72ºC. A 5-µl aliquot of PCR products was resolved by electrophoresis on a 1.5% agarose gel.

Table 4.6. Primers used for ChIP assays

	MIPs accession no.	Amplified* region	Primer pairs	Product size (bp)
1	**At1g14970**	**c**	F: CGGGTGGTCGATACAGTTCCTGA R: TCGCAAGGCTACATTATTCGCCAA	369
2	**At2g36910**	**c**	F: CTCTGTTGGTGCTTTTGTCCATGG R: CGTTGTTTGTCTCTCTCCACTCCA	374
3	**At2g28190**	**c**	F: CAACACAATCCTCGCATTCTCATC R: ACGAAGCAGATTGAACACTGAGAG	359
4	**At2g28190**	**p**	F: AGCTTTGGTCACACATAAGATAGG R: GTAGGCACAAATACTTGTGTGAGA	349
6	**At3g23130**	**c**	F: ATCAAATCATGGAAAAGCTCGCTT R: GAACTCACTTTTTTCCCCTGGGAA	264
5	**At1g02920**	**c**	F: AGGAAACCAACTTGTCTCCCTTGG R: AGCAGAAGGCCTAGAAGTGATGTC	370
7	**At4g34590**	**c**	F: GTCGTCGGGAACAACTTCGTCGA R: AGAAGACGCCATGAGAGGCTGGT	451
8	**At1g01060**	**c**	F: GGAACAACGGTACATCTTCCTCTC R: CGCCACTTACCTGCTAAAAATCAG	390
9	**At1g01060**	**p**	F: GCTGAGATTGCTTCTGGCTTCTCT R: AGTTTCTCAGCAGCCAAACAGAGA	344
10	**At2g02850**	**c**	F: CCAAGGGAAGAGGCAGTGCATC R: TTGCCCATACTTAGAAGCAGCCAT	328
11	**At2g22980**	**c**	F: TGGTGCCTTTCCTTGCAACTCAAG R: CTCTGCTGTGTGTCCACTTCCCTT	372

*c: coding region; p: promoter region; F: forward; R: reverse

CHAPTER V

SUMMARY AND DISCUSSION

SUMMARY

The acetylation and deacetylation of core histones have emerged as a fundamental mechanism for epigenetic regulation. Histone acetyltransferases and deacetylases modulate chromatin structure through antagonized enzymatic activities and reversibly manipulate gene transcription in eukaryotic organisms. Despite extensive studies on the role of histone deacetylases in yeast and mammalian systems, the biological roles of histone deacetylases in plants are poorly understood.

In this study, we have taken biochemical, genomic and molecular biology approaches to study the effects of *Arabidopsis thaliana* histone deacetylase 1 (AtHD1) on plant gene regulation and development. In summary, disruption of *AtHD1* gene expression induced various pleiotropic effects on different developmental stages in *Arabidopsis*, which was associated with enrichment of tetra-acetylated H4, acetylated H4-K12 and acetylated H3-K9. These phenotypic abnormalities were reversible and dependent on *AtHD1* expression. It was determined by microarray analysis that ~ 6.7% (1,753) and ~ 4.8% (1,263) of the transcriptome were affected in the leaves and flower buds, respectively. These affected genes represented a wide range of biological functions. The expression of some tissue-specific genes important to plant development was affected. The activation for a subset of genes was directly associated with increased

acetylation levels, especially at the promoter region. However, the increased acetylation level was not extended to the neighboring chromosomal domains. Taken together, the results provide direct evidence for a reversible role of histone acetylation and deacetylation in the control of plant gene regulation and development.

DISCUSSION

Histone acetylation/deacetylation is a key regulator in plant gene regulation and development

Blocking histone deacetylation by trichostatin A derepresses one parental set of rRNA genes that is normally silenced in a *Brassica* allotetraploid (Chen and Pikaard, 1997). It provides first evidence that histone acetylation and deacetylation is involved in regulating gene expression in plants as in other eukaryotes (Pazin and Kadonaga, 1997; Struhl, 1998). Our studies on investigating the biological roles of AtHD1 further support this notion.

Disruption of *AtHD1* gene expression induces various pleiotropic effects on different developmental stages, including twisted leaves, defective shoot apical meristem (SAM), irregular trichomes, late flowering, abnormal inflorescence and flowers, and aborted seeds (Tian and Chen, 2001; Tian et al., 2003). A large number of genes are either up- or down-regulated in the *athd1-t1* mutant (Figures 4.5A and 4.5B), which may contribute to these phenotypic abnormalities. Several NAM (no apical meristem) protein homologs are repressed in the leaves of the mutant, which well explains the phenotype of defective shoot apical meristem (Tian et al., 2003). In petunia, loss-of-

function *nam* mutants fail to develop shoot apical meristems (SAM) during embryogenesis, and *nam* seedlings do not develop shoots and leaves (Souer et al., 1996).

Significantly, some tissue-specific genes important to plant development were ectopically expressed in different tissues, one of which is *SUPERMAN* (Tian and Chen, 2001), a gene that is involved in determination of floral whorl boundaries (Sakai et al., 1995). Altogether, 85 leaf-specific genes are derepressed in flower buds of the mutant, whereas 350 flower-specific genes are ectopically expressed in leaves. This fact suggests a role of histone acetylation and deacetylation in setting tissue-specific expression patterns for a set of genes.

The affected genes in the *athd1-t1* mutant represent a wide range of biological functions. It implies that histone acetylation and deacetylation participate in various cellular pathways (Figures 4.5D and 4.5E). Recently, it has been demonstrated that histone deacetylation is a key player in controlling flower timing in plants through autonomous pathway (He et al., 2003; Ausin et al., 2004). The *athd1-t1* mutant also shows slightly delayed flowering (Tian and Chen, 2001; Tian et al., 2003), suggesting that histone deacetylase may work more efficiently through coordination with other protein factors. The ATPase activity of Mi-2 has been shown to be able to increase the efficiency of histone deacetylation by NURD complexes (Tong et al., 1998; Xue et al., 1998; Guschin et al., 2000).

Studies on AtGCN5, an *Arabidopsis* histone acetyltransferase, have revealed that histone acetylation is involved in the regulation of cold stress regulated genes (Stockinger et al., 2001; Vlachonasios et al., 2003). Intriguingly, FVE, a homolog of the

mammalian retinoblastoma-associated protein contained in a histone deacetylase (HDAC) complex, is a cold stress sensor in *Arabidopsis* (Kim et al., 2004). It has been shown that plants sense cold stress signal through FVE, which in turn promotes flowering by repressing FLC, a negative regulator in flower timing (Kim et al., 2004). Another group has independently demonstrated that cold treatment (vernalization) promotes flowering involving changes in the acetylation states of histones (Sung and Amasino, 2004). Indeed, plants regulate flower timing through both vernalization pathway and autonomous pathway mediated by negatively regulating the expression of *FLC* (Simpson and Dean, 2002). The connection between cold response and flower timing mediated through histone acetylation and deacetylation may provide evolutionary fitness to plants.

Another study on AtGCN5 has shown that histone acetylation also plays a role in regulating the floral meristem activity through the WUSCHEL/AGAMOUS pathway (Bertrand et al., 2003). The T-DNA insertion mutation of *AtGCN5* gene induces disorganized flower structure in the early-arising flowers and homeotic transformation of flower organs in the late-arising flowers (Bertrand et al., 2003), which has been also observed in the CASH transgenic plants and *athd1-t1* mutant (Tian and Chen, 2001; Tian et al., 2003). Mutations in *AtHD1* and *AtGCN5* induce similar pleiotropic effects, suggesting that the temporal control of certain gene expression requires reversible histone acetylation and deacetylation. It has been proposed that more histone deacetylase activity might be needed to counteract the increased histone acetyltransferase activity at heavily transcribed genes in yeast (Kurdistani et al., 2002).

In plants, both histone acetyltransferases (HATs) and histone deacetylases (HDAs) are multigene families (Pandey et al., 2003). The biological roles of most of these genes remain unknown. Current evidence suggests that the multigene family members may function in different biological processes. The plant-specific histone deacetylase, HD2, is localized in nucleolus, and is presumably important for rDNA chromatin structure (Lusser et al., 1997). However, recent studies suggest that the functions of nucleolus are not limited to rRNA transcription, rRNA processing and ribosome assembly, nucleolus may also be involved in gene expression (Scherl et al., 2002). AtHDA6 shares high sequence homology to AtHD1, however, it has divergent functions. It has been shown to be involved in transgene repression (Murfett et al., 2001), transposon regulation (Lippman et al., 2003), and homologous DNA methylation directed by the introduction of double stranded RNA (Aufsatz et al., 2002). It is more likely that AtHDA6 plays a role in plant defense mechanism.

Together, our data suggest that histone acetylation and deacetylation regulate gene expression during development through DNA-sequence independent or epigenetic mechanisms in plants. Unlike the stable epigenetic code, such as DNA methylation and histone methylation, histone acetylation and deacetylation represent a reversible "histone code". Understanding the role of histone acetylation and deacetylation in plant gene regulation and development will help us to reveal and control the plastic nature of plant development.

FUTURE DIRECTIONS

We have learned that AtHD1 is an important regulator involved in *Arabidopsis* development and gene regulation. However, in order to elucidate the biological roles of AtHD1, there still remain many questions to address.

What protein factor or factors are associated with AtHD1?

In yeast and mammals, HDs have been shown to either reside in repressive chromatin complexes (Jones et al., 1998; Pirrotta, 1998; Fuks et al., 2000) or be associated with transcription repressors (Alland et al., 1997; Heinzel et al., 1997; Rundlett et al., 1998). Recent studies have identified two proteins FLD and FVE that might interact with histone deacetylases to regulate the initiation of flowering in *Arabidopsis* (He et al., 2003; Ausin et al., 2004). Both FLD and FVE participate in the autonomous pathway to promote flowering by repressing *FLC* expression (Simpson and Dean, 2002). FLD encodes a plant homolog of a protein found in histone deacetylase complexes in mammals (He et al., 2003), whereas FVE is a putative Rb-associated protein which has been suggested to interact with histone deacetylases in maize (Rossi et al., 2003; Ausin et al., 2004). Both *fld* and *fve* mutation induce *FLC* acetylation. As a result, the expression of *FLC* is increased and flowering is delayed (He et al., 2003; Rossi et al., 2003). This finding is reminiscent of delayed flowering observed in the CASH plants and the *athd1-t1* mutant (Tian and Chen, 2001; Tian et al., 2003). In addition to participating in regulation of flower timing, our data have also implied that AtHD1 are involved in various biological pathways (Figures 4.5D and 4.5E), which is

likely to be mediated by interaction with different protein factors. To study AtHD1 complexes, yeast two-hybrid system will be used to "fish out" interacting proteins with AtHD1. Alternatively, transgenic plants over-expressing epitope-tagged AtHD1 in the *athd1-t1* mutant background will be generated. The antibody against the specific epitope will be used to perform immunoprecipitation, so that the associated proteins will be pulled down together with epitope-tagged AtHD1. Micro-sequencing and mass spectrometry (MS) analysis will then be performed to identify the protein factors.

Which chromosomal regions are recognized by AtHD1?

Our genome-wide expression microarray analyses have determined where gene activity is altered by disruption of *AtHD1* gene expression (Figures 4.6A and 4.6B). By using chromatin immunoprecipitation assays, we have also determined that a subset of derepressed genes is associated with accumulation of hyperacetylated histones in the *athd1-t1* mutant (Figures 4.6A, 4.6B, 4.7B, 4.7C and 4.7D) and the increased acetylation level is not extended to the neighboring chromosomal domains (Figures 4.6A and 4.6B). These results suggest that AtHD1 is recruited to its specific genomic regions to directly regulate the expression of its target genes. This analysis can be further extended to study genome-wide acetylation profiles in the *athd1-t1* mutant by performing microarray analysis with DNA immunoprecipitated by acetylated histones (acetylation microarray) (Robyr et al., 2002), which will provide a functional map of AtHD1 enzymatic action and identify its target genes in the whole genome. However, there are several RPD3-like HDs in addition to AtHD1 in *Arabidopsis* genome (Pandey et al., 2002). Functional

redundancy among histone deacetylases and the absence of histone acetyltransferases in the target regions of AtHD1 may make it complicated to determine the AtHD1-binding sites based on acetylation states. The enzyme binding microarray analysis in yeast has uniquely revealed that ribosomal protein genes are the potential targets of RPD3 protein (Kurdistani et al., 2002), which was missed by the expression and acetylation microarrays (Bernstein et al., 2000; Robyr et al., 2002). Therefore, it is important to identify localization of AtHD1 in the genome so that the biological roles of AtHD1 can be further elucidated. To do this, DNA will be immunoprecipitated by AtHD1-specific antibody (Tian and Chen, 2001) from wild-type plants and the *athd1-t1* mutant, respectively. The isolated DNA will be used to synthesize probes labeled with Cy3- and Cy5-fluorescent dyes, which will be mixed and applied on microarray slides that represent all predicated genomic sequences. The genomic sequences that are associated with AtHD1 protein will be identified by statistical analysis as described in Chapter III.

REFERENCES

Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000). Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. Cell **103,** 667-678.

Agresti, A., and Bianchi, M., E. (2003). HMGB proteins and gene expression. Curr Opin Genet Dev. **13**, 170-8.

Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R.A. (1997). Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. Nature **387,** 49-55.

Allfrey, V.G., Faulkner, R., and Mirsky, A.E. (1964). Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proc Natl Acad Sci U S A **51,** 786-794.

Almouzni, G., Khochbin, S., Dimitrov, S., and Wolffe, A.P. (1994). Histone acetylation influences both gene expression and development of *Xenopus laevis*. Dev Biol **165,** 654-669.

Amedeo, P., Habu, Y., Afsar, K., Scheid, O.M., and Paszkowski, J. (2000). Disruption of the plant gene MOM releases transcriptional silencing of methylated genes. Nature **405**, 203-8.

An, Y.Q., McDowell, J.M., Huang, S., McKinney, E.C., Chambliss, S., and Meagher, R.B. (1996). Strong, constitutive expression of the *Arabidopsis* ACT2/ACT8 actin subclass in vegetative tissues. Plant J **10,** 107-121.

Anderson, A., A., and Panning, B. (2003). Epigenetic gene regulation by noncoding RNAs. Curr Opin Cell Biol. **15**, 281-9.

Antequera, F., Boyes, J., and Bird, A. (1990). High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell **62,** 503-514.

Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408,** 796-815.

Aramayo, R., and Metzenberg, R.L. (1996). Meiotic transvection in fungi. Cell **86,** 103-113.

Aravind, L., and Koonin, E. (1998). Second family of histone deacetylaese. Science **280,** 1167a.

Aufsatz, W., Mette, M.F., Van Der Winden, J., Matzke, M., and Matzke, A.J. (2002). HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA. EMBO J **21,** 6832-6841.

Ausin, I., Alonso-Blanco, C., Jarillo, J.,A., Ruiz-Garcia, L., and Martinez-Zapater, J.M. (2004). Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nat. Genetics **36**, 162-166.

Ausio, J., Dong, F., and van Holde, K.E. (1989). Use of selectively trypsinized nucleosome core particles to analyze the role of the histone "tails" in the stabilization of the nucleosome. J Mol Biol **206,** 451-463.

Baltimore, D. (2001). Our genome unveiled. Nature **409,** 814-816.

Bartl, S., Taplick, J., Lagger, G., Khier, H., Kuchler, K., and Seiser, C. (1997). Identification of mouse histone deacetylase 1 as a growth factor-inducible gene. Mol Cell Biol **17,** 5033-5043.

Baumbusch, L.O., Thorstensen, T., Krauss, V., Fischer, A., Naumann, K., Assalkhou, R., Schulz, I., Reuter, G., and Aalen, R.B. (2001). The *Arabidopsis thaliana* genome contains at least 29 active genes encoding SET domain proteins that can be assigned to four evolutionarily conserved classes. Nucleic Acids Res **29,** 4319-4333.

Bechtold, N., and Pelletier, G. (1998). In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Methods Mol Biol **82,** 259-266.

Belmont, A.S., Sedat, J.W., and Agard, D.A. (1987). A three-dimensional approach to mitotic chromosome structure: evidence for a complex hierarchical organization. J Cell Biol **105,** 77-92.

Belmont, A.S., Braunfeld, M.B., Sedat, J.W., and Agard, D.A. (1989). Large-scale chromatin structural domains within mitotic and interphase chromosomes in vivo and in vitro. Chromosoma **98,** 129-143.

Berg, A., Meza, T.J., Mahic, M., Thorstensen, T., Kristiansen, K., and Aalen, R.B. (2003). Ten members of the *Arabidopsis* gene family encoding methyl-CpG-binding domain proteins are transcriptionally active and at least one, AtMBD11, is crucial for normal development. Nucleic Acids Res **31,** 5291-5304.

Berger, S.L. (2002). Histone modifications in transcriptional regulation. Curr Opin Genet Dev **12,** 142-148.

Bernier, G. (1986). The flowering process as an example of plastic development. Symp. Soc. Exp. Biol. **40,** 257-286.

Bernstein, B.E., Tong, J.K., and Schreiber, S.L. (2000). Genomewide studies of histone deacetylase function in yeast. Proc Natl Acad Sci U S A **97,** 13708-13713.

Bernstein, B.E., Humphrey, E.L., Erlich, R.L., Schneider, R., Bouman, P., Liu, J.S., Kouzarides, T., and Schreiber, S.L. (2002). Methylation of histone H3 Lys 4 in coding regions of active genes. Proc Natl Acad Sci U S A **99,** 8695-8700.

Bertrand, C., Bergounioux, C., Domenichini, S., Delarue, M., and Zhou, D.X. (2003). *Arabidopsis* histone acetyltransferase AtGCN5 regulates the floral meristem activity through the WUSCHEL/AGAMOUS pathway. J Biol Chem **278,** 28246-51.

Bestor, T.H. (2000). The DNA methyltransferases of mammals. Hum Mol Genet **9,** 2395-2402.

Bleecker, A.B., and Patterson, S.E. (1997). Last exit: senescence, abscission, and meristem arrest in *Arabidopsis*. Plant Cell **9,** 1169-1179.

Bordoli, L., Husser, S., Luthi, U., Netsch, M., Osmani, H., and Eckner, R. (2001). Functional analysis of the p300 acetyltransferase domain: the PHD finger of p300 but not of CBP is dispensable for enzymatic activity. Nucleic Acids Res **29,** 4462-4471.

Bourc'his, D., and Bestor, T.H. (2002). Helicase homologues maintain cytosine methylation in plants and mammals. Bioessays **24,** 297-299.

Bowman, J.L., Sakai, H., Jack, T., Weigel, D., Mayer, U., and Meyerowitz, E.M. (1992). SUPERMAN, a regulator of floral homeotic genes in *Arabidopsis*. Development **114,** 599-615.

Braunstein, M., Rose, A.B., Holmes, S.G., Allis, C.D., and Broach, J.R. (1993). Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev **7,** 592-604.

Braunstein, M., Sobel, R.E., Allis, C.D., Turner, B.M., and Broach, J.R. (1996). Efficient transcriptional silencing in *Saccharomyces cerevisiae* requires a heterochromatin histone acetylation pattern. Mol Cell Biol **16,** 4349-4356.

Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J., and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature **391,** 597-601.

Brosch, G., Georgieva, E.I., Lopez-Rodas, G., Lindner, H., and Loidl, P. (1992). Specificity of *Zea mays* histone deacetylase is regulated by phosphorylation. J Biol Chem **267,** 20561-20564.

Brosch, G., Ransom, R., Lechner, T., Walton, J.D., and Loidl, P. (1995). Inhibition of maize histone deacetylases by HC toxin, the host-selective toxin of *Cochliobolus carbonum*. Plant Cell **7,** 1941-1950.

Brosch, G., Goralik-Schramel, M., and Loidl, P. (1996). Purification of histone deacetylase HD1-A of germinating maize embryos. FEBS Lett **393,** 287-291.

Cao, X., and Jacobsen, S.E. (2002a). Role of the *Arabidopsis* DRM methyltransferases in de novo DNA methylation and gene silencing. Curr Biol **12,** 1138-1144.

Cao, X., and Jacobsen, S.E. (2002b). Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. Proc Natl Acad Sci U S A **99 Suppl 4,** 16491-16498.

Carmen, A.A., Milne, L., and Grunstein, M. (2002). Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3. J Biol Chem **277,** 4778-4781.

Carrozza, M.J., Utley, R.T., Workman, J.L., and Cote, J. (2003). The diverse functions of histone acetyltransferase complexes. Trends Genet **19,** 321-329.

Cervoni, N., and Szyf, M. (2001). Demethylase activity is directed by histone acetylation. J Biol Chem **276,** 40778-40787.

Chalfie, M. (1998). Genome sequencing. The worm revealed. Nature **396,** 620-621.

Chaudhury, A.M., Ming, L., Miller, C., Craig, S., Dennis, E.S., and Peacock, W.J. (1997). Fertilization-independent seed development in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **94,** 4223-4228.

Chen, H., Lin, R.J., Schiltz, R.L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M.L., Nakatani, Y., and Evans, R.M. (1997). Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell **90,** 569-580.

Chen, Z.J., and Pikaard, C.S. (1997). Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. Genes Dev **11,** 2124-2136.

Cheung, P., Tanner, K.G., Cheung, W.L., Sassone-Corsi, P., Denu, J.M., and Allis, C.D. (2000). Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. Mol Cell **5,** 905-915.

Chua, Y.L., Brown, A.P., and Gray, J.C. (2001). Targeted histone acetylation and altered nuclease accessibility over short regions of the pea plastocyanin gene. Plant Cell **13,** 599-612.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc Natl Acad Sci U S A **81,** 1991-1995.

Ciechanover, A., Orian, A., and Schwartz, A.L. (2000). The ubiquitin-mediated proteolytic pathway: mode of action and clinical implications. J Cell Biochem **77,** 40-51.

Cioci, F., Vogelauer, M., and Camilloni, G. (2002). Acetylation and accessibility of rDNA chromatin in *Saccharomyces cerevisiae* in (Delta) top1 and (Delta) sir2 mutants. J Mol Biol **322,** 41-52.

Clarke, J.H., Tack, D., Findlay, K., Van Montagu, M., and Van Lijsebettens, M. (1999). The SERRATE locus controls the formation of the early juvenile leaves and phase length in *Arabidopsis*. Plant J **20,** 493-501.

Clayton, A.L., Rose, S., Barratt, M.J., and Mahadevan, L.C. (2000). Phosphoacetylation of histone H3 on c-fos- and c-jun-associated nucleosomes upon gene activation. EMBO J **19,** 3714-3726.

Crane-Robinson, C., Myers, F.A., Hebbes, T.R., Clayton, A.L., and Thorne, A.W. (1999). Chromatin immunoprecipitation assays in acetylation mapping of higher eukaryotes. Methods Enzymol **304,** 533-547.

Cubas, P., Vincent, C., and Coen, E. (1999). An epigenetic mutation responsible for natural variation in floral symmetry. Nature **401,** 157-161.

Czermin, B., Schotta, G., Hulsmann, B.B., Brehm, A., Becker, P.B., Reuter, G., and Imhof, A. (2001). Physical and functional association of SU(VAR)3-9 and HDAC1 in *Drosophila*. EMBO Rep **2,** 915-919.

Dangl, M., Brosch, G., Haas, H., Loidl, P., and Lusser, A. (2001). Comparative analysis of HD2 type histone deacetylases in higher plants. Planta **213,** 280-285.

Davie, J.K., and Dent, S.Y. (2002). Transcriptional control: an activating role for arginine methylation. Curr Biol **12,** R59-61.

Davie, J.R., and Murphy, L.C. (1990). Level of ubiquitinated histone H2B in chromatin is coupled to ongoing transcription. Biochemistry **29,** 4752-4757.

Davie, J.R., Lin, R., and Allis, C.D. (1991). Timing of the appearance of ubiquitinated histones in developing new macronuclei of *Tetrahymena thermophila*. Biochem Cell Biol **69,** 66-71.

Davie, J.R., and Chadee, D.N. (1998). Regulation and regulatory parameters of histone modifications. J Cell Biochem Suppl **30-31,** 203-213.

De Rubertis, F., Kadosh, D., Henchoz, S., Pauli, D., Reuter, G., Struhl, K., and Spierer, P. (1996). The histone deacetylase RPD3 counteracts genomic silencing in *Drosophila* and yeast. Nature **384,** 589-591.

Deplus, R., Brenner, C., Burgers, W.A., Putmans, P., Kouzarides, T., de Launoit, Y., and Fuks, F. (2002). Dnmt3L is a transcriptional repressor that recruits histone deacetylase. Nucleic Acids Res **30,** 3831-3838.

Dilworth, F.J., Fromental-Ramain, C., Yamamoto, K., and Chambon, P. (2000). ATP-driven chromatin remodeling activity and histone acetyltransferases act sequentially during transactivation by RAR/RXR In vitro. Mol Cell **6,** 1049-1058.

Dressel, U., Bailey, P.J., Wang, S.C., Downes, M., Evans, R.M., and Muscat, G.E. (2001). A dynamic role for HDAC7 in MEF2-mediated muscle differentiation. J Biol Chem **276,** 17007-17013.

Duval, M., Hsieh, T.F., Kim, S.Y., and Thomas, T.L. (2002). Molecular characterization of AtNAM: a member of the *Arabidopsis* NAC domain superfamily. Plant Mol Biol. **50**, 237-48.

Earnshaw, W., C., Halligan, B., Cooke, C. A., Heck, M. M., and Liu, L. F. (1985). Topoisomerase II is a structural component of mitotic chromosome scaffolds. J. Cell Biol. **100**, 1706-15.

Eden, S., and Cedar, H. (1994). Role of DNA methylation in the regulation of transcription. Curr Opin Genet Dev **4,** 255-259.

Eden, S., Hashimshony, T., Keshet, I., Cedar, H., and Thorne, A.W. (1998). DNA methylation models histone acetylation. Nature **394,** 842.

Eisen, J.A., Sweder, K.S., and Hanawalt, P.C. (1995). Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. Nucleic Acids Res **23,** 2715-2723.

Fazzio, T.G., Kooperberg, C., Goldmark, J.P., Neal, C., Basom, R., Delrow, J., and Tsukiyama, T. (2001). Widespread collaboration of Isw2 and Sin3-Rpd3 chromatin remodeling complexes in transcriptional repression. Mol Cell Biol **21,** 6450-6460.

Feldmann, K.A., and Marks, M.D. (1987). *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non-tissue culture approach. Mol. Gen. Genet. **208,** 1-9.

Finnegan, E.J., and Dennis, E.S. (1993). Isolation and identification by sequence homology of a putative cytosine methyltransferase from *Arabidopsis thaliana*. Nucleic Acids Res **21,** 2383-2388.

Finnegan, E.J., Peacock, W.J., and Dennis, E.S. (1996). Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. Proc Natl Acad Sci U S A **93,** 8449-8454.

Finnegan, E.J., and Kovac, K.A. (2000). Plant DNA methyltransferases. Plant Mol Biol **43,** 189-201.

Finnegan, E.J. (2001). Is plant gene expression regulated globally? Trends Genet **17,** 361-365.

Fischer, D.D., Cai, R., Bhatia, U., Asselbergs, F.A., Song, C., Terry, R., Trogani, N., Widmer, R., Atadja, P., and Cohen, D. (2002). Isolation and characterization of a novel class II histone deacetylase, HDAC10. J Biol Chem **277,** 6656-6666.

Fischle, W., Emiliani, S., Hendzel, M.J., Nagase, T., Nomura, N., Voelter, W., and Verdin, E. (1999). A new family of human histone deacetylases related to *Saccharomyces cerevisiae* HDA1p. J Biol Chem **274,** 11713-11720.

Fischle, W., Dequiedt, F., Fillion, M., Hendzel, M.J., Voelter, W., and Verdin, E. (2001). Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. J Biol Chem **276,** 35826-35835.

Forsthoefel, N.R., Wu, Y., Schulz, B., Bennett, M.J., and Feldmann, K.A. (1992). T-DNA insertion mutagenesis in *Arabidopsis*: prospects and perspectives. Aust J. Plant Physiol. **19**, 353-366.

Fry, C.J., and Peterson, C.L. (2001). Chromatin remodeling enzymes: who's on first? Curr Biol **11,** R185-197.

Frye, R.A. (2000). Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem Biophys Res Commun **273,** 793-798.

Fuks, F., Burgers, W.A., Brehm, A., Hughes-Davies, L., and Kouzarides, T. (2000). DNA methyltransferase Dnmt1 associates with histone deacetylase activity. Nat Genet **24,** 88-91.

Fuks, F., Burgers, W.A., Godin, N., Kasai, M., and Kouzarides, T. (2001). Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. EMBO J **20,** 2536-2544.

Gao, L., Cueto, M.A., Asselbergs, F., and Atadja, P. (2002). Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. J Biol Chem **277,** 25748-25755.

Gavin, I., Horn, P.J., and Peterson, C.L. (2001). SWI/SNF chromatin remodeling requires changes in DNA topology. Mol Cell **7,** 97-104.

Geiman, T.M., Tessarollo, L., Anver, M.R., Kopp, J.B., Ward, J.M., and Muegge, K. (2001). Lsh, a SNF2 family member, is required for normal murine development. Biochim Biophys Acta **1526,** 211-220.

Geiman, T.M., and Robertson, K.D. (2002). Chromatin remodeling, histone modifications, and DNA methylation-how does it all fit together? J Cell Biochem **87,** 117-125.

Gendrel, A.V., Lippman, Z., Yordan, C., Colot, V., and Martienssen, R.A. (2002). Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene DDM1. Science **297,** 1871-1873.

Goldknopf, I.L., and Busch, H. (1977). Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate-protein A24. Proc Natl Acad Sci U S A **74,** 864-868.

Goldknopf, I.L., and Busch, H. (1980). N-Bromosuccinimide fragments of protein A24 (uH2A): an implication that ubiquitin is the precursor of conjugation in vivo. Biochem Biophys Res Commun **96,** 1724-1731.

Goodrich, J., and Tweedie, S. (2002). Remembrance of things past: chromatin remodeling in plant development. Annu. Rev. Cell Dev. Biol. **18**, 707-46.

Grossniklaus, U., Vielle-Calzada, J.P., Hoeppner, M.A., and Gagliano, W.B. (1998). Maternal control of embryogenesis by MEDEA, a polycomb group gene in *Arabidopsis*. Science **280,** 446-450.

Grozinger, C.M., Hassig, C.A., and Schreiber, S.L. (1999). Three proteins define a class of human histone deacetylases related to yeast Hda1p. Proc Natl Acad Sci U S A **96,** 4868-4873.

Grozinger, C.M., and Schreiber, S.L. (2000). Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. Proc Natl Acad Sci U S A **97,** 7835-7840.

Grunstein, M. (1990). Histone function in transcription. Annu Rev Cell Biol **6,** 643-678.

Guardiola, A.R., and Yao, T.P. (2002). Molecular cloning and characterization of a novel histone deacetylase HDAC10. J Biol Chem **277,** 3350-3356.

Guarente, L. (2000). Sir2 links chromatin silencing, metabolism, and aging. Genes Dev **14,** 1021-1026.

Guschin, D., Wade, P.A., Kikyo, N., and Wolffe, A.P. (2000). ATP-dependent histone octamer mobilization and histone deacetylation mediated by the Mi-2 chromatin remodeling complex. Biochemistry **39,** 5238-5245.

Habu, Y., Kakutani, T., and Paszkowski, J. (2001). Epigenetic developmental mechanisms in plants: molecules and targets of plant epigenetic regulation. Curr Opin Genet Dev **11,** 215-220.

Hansen, J.C. (2002). Conformational dynamics of the chromatin fiber in solution: determinants, mechanisms, and functions. Annu Rev Biophys Biomol Struct **31,** 361-392.

Hassan, A.H., Neely, K.E., and Workman, J.L. (2001). Histone acetyltransferase complexes stabilize swi/snf binding to promoter nucleosomes. Cell **104,** 817-827.

Hassig, C.A., Tong, J.K., Fleischer, T.C., Owa, T., Grable, P.G., Ayer, D.E., and Schreiber, S.L. (1998). A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. Proc Natl Acad Sci U S A **95,** 3519-3524.

Havas, K., Flaus, A., Phelan, M., Kingston, R., Wade, P.A., Lilley, D.M., and Owen-Hughes, T. (2000). Generation of superhelical torsion by ATP-dependent chromatin remodeling activities. Cell **103,** 1133-1142.

He, Y., Michaels, S.D., and Amasino, R.M. (2003). Regulation of flowering time by histone acetylation in *Arabidopsis*. Science **302**, 1751-4.

Heinzel, T., Lavinsky, R.M., Mullen, T.M., Soderstrom, M., Laherty, C.D., Torchia, J., Yang, W.M., Brard, G., Ngo, S.D., Davie, J.R., Seto, E., Eisenman, R.N., Rose, D.W., Glass, C.K., and Rosenfeld, M.G. (1997). A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature **387,** 43-48.

Henikoff, S., and Comai, L. (1998). A DNA methyltransferase homolog with a chromodomain exists in multiple polymorphic forms in *Arabidopsis*. Genetics **149,** 307-318.

Henry, K.W., Wyce, A., Lo, W.S., Duggan, L.J., Emre, N.C., Kao, C.F., Pillus, L., Shilatifard, A., Osley, M.A., and Berger, S.L. (2003). Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. Genes Dev **17,** 2648-2663.

Hodgkin, J. (2000). A view of mount *Drosophila*. Nature **404,** 442-443.

Hollick, J.B., Dorweiler, J.E., and Chandler, V.L. (1997). Paramutation and related allelic interactions. Trends Genet **13,** 302-308.

Horn, P.J., and Peterson, C.L. (2002). Molecular biology. Chromatin higher order folding--wrapping up transcription. Science **297,** 1824-1827.

Hsu, J.Y., Sun, Z.W., Li, X., Reuben, M., Tatchell, K., Bishop, D.K., Grushcow, J.M., Brame, C.J., Caldwell, J.A., Hunt, D.F., Lin, R., Smith, M.M., and Allis, C.D. (2000). Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. Cell **102,** 279-291.

Hu, E., Chen, Z., Fredrickson, T., Zhu, Y., Kirkpatrick, R., Zhang, G.F., Johanson, K., Sung, C.M., Liu, R., and Winkler, J. (2000). Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor. J Biol Chem **275,** 15254-15264.

Huang, E.Y., Zhang, J., Miska, E.A., Guenther, M.G., Kouzarides, T., and Lazar, M.A. (2000). Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. Genes Dev **14,** 45-54.

Hughes, T.R., Marton, M.J., Jones, A.R., Roberts, C.J., Stoughton, R., Armour, C.D., Bennett, H.A., Coffey, E., Dai, H., He, Y.D., Kidd, M.J., King, A.M., Meyer, M.R., Slade, D., Lum, P.Y., Stepaniants, S.B., Shoemaker, D.D.,

Gachotte, D., Chakraburtty, K., Simon, J., Bard, M., and Friend, S.H. (2000). Functional discovery via a compendium of expression profiles. Cell **102,** 109-126.

Imai, S., Armstrong, C.M., Kaeberlein, M., and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature **403,** 795-800.

Imhof, A., and Wolffe, A.P. (1999). Purification and properties of the *Xenopus* Hat1 acetyltransferase: association with the 14-3-3 proteins in the oocyte nucleus. Biochemistry **38,** 13085-13093.

Ito, M., Koike, A., Koizumi, N., and Sano, H. (2003). Methylated DNA-binding proteins from *Arabidopsis*. Plant Physiol. **133**, 1747-54.

Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. (2002). Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature **416,** 556-560.

Jacobsen, S.E., and Meyerowitz, E.M. (1997). Hypermethylated SUPERMAN epigenetic alleles in *Arabidopsis*. Science **277,** 1100-1103.

Jang, I.C., Pahk, Y.M., Song, S.I., Kwon, H.J., Nahm, B.H., and Kim, J.K. (2003). Structure and expression of the rice class-I type histone deacetylase genes OsHDAC1-3: OsHDAC1 overexpression in transgenic plants leads to increased growth rate and altered architecture. Plant J **33,** 531-541.

Jaskelioff, M., Gavin, I.M., Peterson, C.L., and Logie, C. (2000). SWI-SNF-mediated nucleosome remodeling: role of histone octamer mobility in the persistence of the remodeled state. Mol Cell Biol **20,** 3058-3068.

Jason, L.J., Moore, S.C., Lewis, J.D., Lindsey, G., and Ausio, J. (2002). Histone ubiquitination: a tagging tail unfolds? Bioessays **24,** 166-174.

Jeddeloh, J.A., Stokes, T.L., and Richards, E.J. (1999). Maintenance of genomic methylation requires a SWI2/SNF2-like protein. Nat Genet **22,** 94-97.

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science **293,** 1074-1080.

Jones, P.L., Veenstra, G.J., Wade, P.A., Vermaak, D., Kass, S.U., Landsberger, N., Strouboulis, J., and Wolffe, A.P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet **19,** 187-191.

Joyce, J.A., Lam, W.K., Catchpoole, D.J., Jenks, P., Reik, W., Maher, E.R., and Schofield, P.N. (1997). Imprinting of IGF2 and H19: lack of reciprocity in sporadic Beckwith-Wiedemann syndrome. Hum Mol Genet **6,** 1543-1548.

Kadosh, D., and Struhl, K. (1997). Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. Cell **89,** 365-371.

Kadosh, D., and Struhl, K. (1998a). Targeted recruitment of the Sin3-Rpd3 histone deacetylase complex generates a highly localized domain of repressed chromatin in vivo. Mol Cell Biol **18,** 5121-5127.

Kadosh, D., and Struhl, K. (1998b). Histone deacetylase activity of Rpd3 is important for transcriptional repression in vivo. Genes Dev **12,** 797-805.

Kakutani, T., Jeddeloh, J.A., and Richards, E.J. (1995). Characterization of an *Arabidopsis thaliana* DNA hypomethylation mutant. Nucleic Acids Res **23,** 130-137.

Kakutani, T., Jeddeloh, J.A., Flowers, S.K., Munakata, K., and Richards, E.J. (1996). Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. Proc Natl Acad Sci U S A **93,** 12406-12411.

Kakutani, T. (1997). Genetic characterization of late-flowering traits induced by DNA hypomethylation mutation in *Arabidopsis thaliana*. Plant J **12,** 1447-1451.

Kao, H.Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C.R., Evans, R.M., and Kadesch, T. (1998). A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes Dev **12,** 2269-2277.

Kao, H.Y., Downes, M., Ordentlich, P., and Evans, R.M. (2000). Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. Genes Dev **14,** 55-66.

Kao, H.Y., Verdel, A., Tsai, C.C., Simon, C., Juguilon, H., and Khochbin, S. (2001). Mechanism for nucleocytoplasmic shuttling of histone deacetylase 7. J Biol Chem **276,** 47496-47507.

Kao, H.Y., Lee, C.H., Komarov, A., Han, C.C., and Evans, R.M. (2002). Isolation and characterization of mammalian HDAC10, a novel histone deacetylase. J Biol Chem **277,** 187-193.

Kasten, M.M., Dorland, S., and Stillman, D.J. (1997). A large protein complex containing the yeast Sin3p and Rpd3p transcriptional regulators. Mol Cell Biol **17,** 4852-4858.

Kaszas, E., and Cande, W.Z. (2000). Phosphorylation of histone H3 is correlated with changes in the maintenance of sister chromatid cohesion during meiosis in maize, rather than the condensation of the chromatin. J Cell Sci **113 (Pt 18),** 3217-3226.

Khochbin, S., Verdel, A., Lemercier, C., and Seigneurin-Berny, D. (2001). Functional significance of histone deacetylase diversity. Curr Opin Genet Dev **11,** 162-166.

Kim, H.J., Hyun, Y., Park, J.Y., Park, M.J., Park, M.K., Kim, M.D., Kim, H.J., Lee, M.H., Moon, J., and Kim, J. (2004). A genetic link between cold responses and flowering time through FVE in *Arabidopsis thaliana*. Nat Genet **36,** 167-71.

Kimura, A., Umehara, T., and Horikoshi, M. (2002). Chromosomal gradient of histone acetylation established by Sas2p and Sir2p functions as a shield against gene silencing. Nat Genet **32,** 370-377.

Kingston, R.E., and Narlikar, G.J. (1999). ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. Genes Dev **13,** 2339-2352.

Kishimoto, N., Sakai, H., Jackson, J., Jacobsen, S.E., Meyerowitz, E.M., Dennis, E.S., and Finnegan, E.J. (2001). Site specificity of the *Arabidopsis* METI DNA methyltransferase demonstrated through hypermethylation of the superman locus. Plant Mol Biol **46,** 171-183.

Klee, H.J., Hayford, M.B., Kretzmer, K.A., Barry, G.F., and Kishore, G.M. (1991). Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. Plant Cell **3,** 1187-1193.

Knoepfler, P.S., and Eisenman, R.N. (1999). Sin meets NuRD and other tails of repression. Cell **99,** 447-450.

Kolle, D., Brosch, G., Lechner, T., Pipal, A., Helliger, W., Taplick, J., and Loidl, P. (1999). Different types of maize histone deacetylases are distinguished by a highly complex substrate and site specificity. Biochemistry **38,** 6769-6773.

Kornberg, R.D. (1977). Structure of chromatin. Annu Rev Biochem **46,** 931-954.

Krebs, J.E., Kuo, M.H., Allis, C.D., and Peterson, C.L. (1999). Cell cycle-regulated histone acetylation required for expression of the yeast HO gene. Genes Dev **13,** 1412-1421.

Kress, C., Thomassin, H., and Grange, T. (2001). Local DNA demethylation in vertebrates: how could it be performed and targeted? FEBS Lett **494,** 135-140.

Kristjuhan, A., Wittschieben, B.O., Walker, J., Roberts, D., Cairns, B.R., and Svejstrup, J.Q. (2003). Spreading of Sir3 protein in cells with severe histone H3 hypoacetylation. Proc Natl Acad Sci U S A **100,** 7551-7556.

Krogan, N.J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M.A., Dean, K., Ryan, O.W., Golshani, A., Johnston, M., Greenblatt, J.F., and Shilatifard, A. (2003). The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. Mol Cell **11,** 721-729.

Krumm, A., Madisen, L., Yang, X.J., Goodman, R., Nakatani, Y., and Groudine, M. (1998). Long-distance transcriptional enhancement by the histone acetyltransferase PCAF. Proc Natl Acad Sci U S A **95,** 13501-13506.

Krysan, P.J., Young, J.C., Tax, F., and Sussman, M.R. (1996). Identification of transferred DNA insertions within *Arabidopsis* genes involved in signal transduction and ion transport. Proc Natl Acad Sci U S A **93,** 8145-8150.

Kuo, M.H., and Allis, C.D. (1998). Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays **20,** 615-626.

Kuo, M.H., Zhou, J., Jambeck, P., Churchill, M.E., and Allis, C.D. (1998). Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes in vivo. Genes Dev **12,** 627-639.

Kurdistani, S.K., Robyr, D., Tavazoie, S., and Grunstein, M. (2002). Genome-wide binding map of the histone deacetylase Rpd3 in yeast. Nat. Genetics **31,** 248-254.

Kurdistani, S.K., and Grunstein, M. (2003). Histone acetylation and deacetylation in yeast. Nat Rev Mol Cell Biol **4,** 276-284.

Kyrylenko, S., Kyrylenko, O., Suuronen, T., and Salminen, A. (2003). Differential regulation of the Sir2 histone deacetylase gene family by inhibitors of class I and II histone deacetylases. Cell Mol Life Sci **60,** 1990-1997.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature **410,** 116-120.

Landry, J., Slama, J.T., and Sternglanz, R. (2000a). Role of NAD(+) in the deacetylase activity of the SIR2-like proteins. Biochem Biophys Res Commun **278,** 685-690.

Landry, J., Sutton, A., Tafrov, S.T., Heller, R.C., Stebbins, J., Pillus, L., and Sternglanz, R. (2000b). The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc Natl Acad Sci U S A **97,** 5807-5811.

Lechner, T., Lusser, A., Pipal, A., Brosch, G., Loidl, A., Goralik-Schramel, M., Sendra, R., Wegener, S., Walton, J.D., and Loidl, P. (2000). RPD3-type histone deacetylases in maize embryos. Biochemistry **39,** 1683-1692.

Lee, H.-S., Wang, J., Tian, L., Black, M., Jiang, H., Madlung, A., Watson, B., Lukens, L., Pires, J.C., Wang, J.J., Comai, T., Osborn, R., Doerge, R.W., and Chen, Z.J. (2004). Sensitivity of 70-mer oligonucleotides and cDNAs for microarray analysis of gene expression in A*rabidopsis* and its related species. Plant Biotechnology J. **2**, 45-57.

Lemercier, C., Verdel, A., Galloo, B., Curtet, S., Brocard, M.P., and Khochbin, S. (2000). mHDA1/HDAC5 histone deacetylase interacts with and represses MEF2A transcriptional activity. J Biol Chem **275,** 15594-15599.

Li, G., Hall, T.C., and Holmes-Davis, R. (2002). Plant chromatin: development and gene control. Bioessays **24,** 234-243.

Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S., and Jacobsen, S.E. (2001). Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. Science **292,** 2077-2080.

Lippman, Z., May, B., Yordan C., Singer, T., and Martienssen R. (2003). Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification. PLoS Biol **1**, 420-428.

Litt, M.D., Simpson, M., Gaszner, M., Allis, C.D., and Felsenfeld, G. (2001). Correlation between histone lysine methylation and developmental changes at the chicken beta-globin locus. Science **293,** 2453-2455.

Lo, W.S., Duggan, L., Tolga, N.C., Emre, Belotserkovskya, R., Lane, W.S., Shiekhattar, R., and Berger, S.L. (2001). Snf1--a histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. Science **293,** 1142-1146.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature **389,** 251-260.

Lundblad, J.R., Kwok, R.P., Laurance, M.E., Harter, M.L., and Goodman, R.H. (1995). Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. Nature **374,** 85-88.

Luo, J., Su, F., Chen, D., Shiloh, A., and Gu, W. (2000a). Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature **408,** 377-381.

Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell **107,** 137-148.

Luo, M., Bilodeau, P., Dennis, E.S., Peacock, W.J., and Chaudhury, A. (2000b). Expression and parent-of-origin effects for FIS2, MEA, and FIE in the endosperm and embryo of developing *Arabidopsis* seeds. Proc Natl Acad Sci U S A **97,** 10637-10642.

Luo, R.X., Postigo, A.A., and Dean, D.C. (1998). Rb interacts with histone deacetylase to repress transcription. Cell **92,** 463-473.

Lusser, A., Brosch, G., Loidl, A., Haas, H., and Loidl, P. (1997). Identification of maize histone deacetylase HD2 as an acidic nucleolar phosphoprotein. Science **277,** 88-91.

Lusser, A., Kolle, D., and Loidl, P. (2001). Histone acetylation: lessons from the plant kingdom. Trends Plant Sci **6,** 59-65.

Lusser, A. (2002). Acetylated, methylated, remodeled: chromatin states for gene regulation. Curr Opin Plant Biol **5,** 437-443.

Lynn, K., Fernandez, A., Aida, M., Sedbrook, J., Tasaka, M., Masson, P., and Barton, M.K. (1999). The PINHEAD/ZWILLE gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the ARGONAUTE1 gene. Development **126,** 469-481.

Lyon, M.F. (1993). Epigenetic inheritance in mammals. Trends Genet **9**, 123-8.

Madisen, L., Krumm, A., Hebbes, T.R., and Groudine, M. (1998). The immunoglobulin heavy chain locus control region increases histone acetylation along linked c-myc genes. Mol Cell Biol **18,** 6281-6292.

Marmorstein, R. (2001). Structure and function of histone acetyltransferases. Cell Mol Life Sci **58,** 693-703.

Martens, J.H., Verlaan, M., Kalkhoven, E., and Zantema, A. (2003). Cascade of distinct histone modifications during collagenase gene activation. Mol Cell Biol **23,** 1808-1816.

Martienssen, R., and Henikoff, S. (1999). The House & Garden guide to chromatin remodelling. Nat Genet **22,** 6-7.

Martienssen, R.A., and Colot, V. (2001). DNA methylation and epigenetic inheritance in plants and filamentous fungi. Science **293,** 1070-1074.

Mayer, K., Schuller, C., Wambutt, R., Murphy, G., Volckaert, G., Pohl, T., Dusterhoft, A., Stiekema, W., Entian, K.D., Terryn, N., Harris, B., Ansorge, W., Brandt, P., Grivell, L., Rieger, M., Weichselgartner, M., de Simone, V., Obermaier, B., Mache, R., Muller, M., Kreis, M., Delseny, M., Puigdomenech, P., Watson, M., McCombie, W.R., and et al. (1999). Sequence and analysis of chromosome 4 of the plant *Arabidopsis thaliana*. Nature **402,** 769-777.

Mewes, H.W., Albermann, K., Bahr, M., Frishman, D., Gleissner, A., Hani, J., Heumann, K., Kleine, K., Maierl, A., Oliver, S.G., Pfeiffer, F., and Zollner, A. (1997). Overview of the yeast genome. Nature **387,** 7-65.

Meyerowitz, E.M. (1997). Genetic control of cell division patterns in developing plants. Cell **88,** 299-308.

Miska, E.A., Karlsson, C., Langley, E., Nielsen, S.J., Pines, J., and Kouzarides, T. (1999). HDAC4 deacetylase associates with and represses the MEF2 transcription factor. EMBO J **18,** 5099-5107.

Moazed, D. (2001). Common themes in mechanisms of gene silencing. Mol Cell **8,** 489-498.

Morata, G., and Kerridge, S. (1981). Sequential functions of the bithorax complex of *Drosophila*. Nature **290,** 778-781.

Morel, J.B., Godon, C., Mourrain, P., Beclin, C., Boutet, S., Feuerbach, F., Proux, F., and Vaucheret, H. (2002). Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell **14,** 629-639.

Moussian, B., Schoof, H., Haecker, A., Jurgens, G., and Laux, T. (1998). Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during *Arabidopsis* embryogenesis. EMBO J **17,** 1799-1809.

Muchardt, C., and Yaniv, M. (1999). ATP-dependent chromatin remodelling: SWI/SNF and Co. are on the job. J Mol Biol **293,** 187-198.

Murai, K., Takumi, S., Koga, H., and Ogihara, Y. (2002). Pistillody, homeotic transformation of stamens into pistil-like structures, caused by nuclear-cytoplasm interaction in wheat. Plant J **29,** 169-181.

Murfett, J., Wang, X.J., Hagen, G., and Guilfoyle, T.J. (2001). Identification of *Arabidopsis* histone deacetylase HDA6 mutants that affect transgene expression. Plant Cell **13,** 1047-1061.

Muth, V., Nadaud, S., Grummt, I., and Voit, R. (2001). Acetylation of TAF(I)68, a subunit of TIF-IB/SL1, activates RNA polymerase I transcription. EMBO J **20,** 1353-1362.

Nagy, P.L., Griesenbeck, J., Kornberg, R.D., and Cleary, M.L. (2002). A trithorax-group complex purified from *Saccharomyces cerevisiae* is required for methylation of histone H3. Proc Natl Acad Sci U S A **99,** 90-94.

Nakao, M. (2001). Epigenetics: interaction of DNA methylation and chromatin. Gene **278,** 25-31.

Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N., and Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature **393,** 386-389.

Narlikar, G.J., Fan, H.Y., and Kingston, R.E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell **108,** 475-487.

Newell-Price, J., Clark, A.J., and King, P. (2000). DNA methylation and silencing of gene expression. Trends Endocrinol Metab **11,** 142-148.

Ng, H.H., Zhang, Y., Hendrich, B., Johnson, C.A., Turner, B.M., Erdjument-Bromage, H., Tempst, P., Reinberg, D., and Bird, A. (1999). MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. Nat Genet **23,** 58-61.

Ng, H.H., and Bird, A. (2000). Histone deacetylases: silencers for hire. Trends Biochem Sci **25,** 121-126.

Nicolas, E., Morales, V., Magnaghi-Jaulin, L., Harel-Bellan, A., Richard-Foy, H., and Trouche, D. (2000). RbAp48 belongs to the histone deacetylase complex that associates with the retinoblastoma protein. J Biol Chem **275,** 9797-9804.

Nishioka, K., Chuikov, S., Sarma, K., Erdjument-Bromage, H., Allis, C.D., Tempst, P., and Reinberg, D. (2002a). Set9, a novel histone H3 methyltransferase that facilitates transcription by precluding histone tail modifications required for heterochromatin formation. Genes Dev **16,** 479-489.

Nishioka, K., Rice, J.C., Sarma, K., Erdjument-Bromage, H., Werner, J., Wang, Y., Chuikov, S., Valenzuela, P., Tempst, P., Steward, R., Lis, J.T., Allis, C.D., and Reinberg, D. (2002b). PR-Set7 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and is associated with silent chromatin. Mol Cell **9,** 1201-1213.

Noma, K., Allis, C.D., and Grewal, S.I. (2001). Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science **293,** 1150-1155.

Nomura, T., Khan, M.M., Kaul, S.C., Dong, H.D., Wadhwa, R., Colmenares, C., Kohno, I., and Ishii, S. (1999). Ski is a component of the histone deacetylase complex required for transcriptional repression by Mad and thyroid hormone receptor. Genes Dev **13,** 412-423.

North, B.J., Marshall, B.L., Borra, M.T., Denu, J.M., and Verdin, E. (2003). The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase. Mol Cell **11,** 437-444.

Nowak, S.J., and Corces, V.G. (2000). Phosphorylation of histone H3 correlates with transcriptionally active loci. Genes Dev **14,** 3003-3013.

Ogas, J., Kaufmann, S., Henderson, J., and Somerville, C. (1999). PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in *Arabidopsis*. Proc Natl Acad Sci U S A **96,** 13839-13844.

Ohlsson, R., Renkawitz, R., and Lobanenkov, V. (2001). CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends Genet **17,** 520-527.

Onate, S.A., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science **270,** 1354-1357.

Palladino, F., Laroche, T., Gilson, E., Axelrod, A., Pillus, L., and Gasser, S.M. (1993). SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. Cell **75,** 543-555.

Pandey, R., Muller, A., Napoli, C.A., Selinger, D.A., Pikaard, C.S., Richards, E.J., Bender, J., Mount, D.W., and Jorgensen, R.A. (2002). Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis thaliana* suggests functional diversification of chromatin modification among multicellular eukaryotes. Nucleic Acids Res **30,** 5036-5055.

Papa, C.M., Springer, N.M., Muszynski, M.G., Meeley, R., and Kaeppler, S.M. (2001). Maize chromomethylase Zea methyltransferase2 is required for CpNpG methylation. Plant Cell **13,** 1919-1928.

Pazin, M.J., and Kadonaga, J.T. (1997). What's up and down with histone deacetylation and transcription? Cell **89,** 325-328.

Perrod, S., Cockell, M.M., Laroche, T., Renauld, H., Ducrest, A.L., Bonnard, C., and Gasser, S.M. (2001). A cytosolic NAD-dependent deacetylase, Hst2p, can modulate nucleolar and telomeric silencing in yeast. EMBO J **20,** 197-209.

Peters, A.H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schofer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., Opravil, S., Doyle, M., Sibilia, M., and Jenuwein, T. (2001). Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. Cell **107,** 323-337.

Peterson, C.L. (2002). HDAC's at work: everyone doing their part. Mol Cell **9,** 921-922.

Phelan, M.L., Schnitzler, G.R., and Kingston, R.E. (2000). Octamer transfer and creation of stably remodeled nucleosomes by human SWI-SNF and its isolated ATPases. Mol Cell Biol **20,** 6380-6389.

Pirrotta, V. (1998). Polycombing the genome: PcG, trxG, and chromatin silencing. Cell **93,** 333-336.

Pollard, K.J., and Peterson, C.L. (1997). Role for ADA/GCN5 products in antagonizing chromatin-mediated transcriptional repression. Mol Cell Biol **17,** 6212-6222.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature **406,** 593-599.

Reik, W., and Murrell, A. (2000). Genomic imprinting. Silence across the border. Nature **405,** 408-409.

Reik, W., and Dean, W. (2001). DNA methylation and mammalian epigenetics. Electrophoresis **22,** 2838-2843.

Reyes, J.C., Hennig, L., and Gruissem, W. (2002). Chromatin-remodeling and memory factors. New regulators of plant development. Plant Physiol **130,** 1090-1101.

Richards, E.J., and Elgin, S.C. (2002). Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. Cell **108,** 489-500.

Robyr, D., Suka, Y., Xenarios, I., Kurdistani, S.K., Wang, A., Suka, N., and Grunstein, M. (2002). Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. Cell **109,** 437-446.

Ronchi, A., Petroni, K., and Tonelli, C. (1995). The reduced expression of endogenous duplications (REED) in the maize R gene family is mediated by DNA methylation. EMBO J **14,** 5318-5328.

Ronemus, M.J., Galbiati, M., Ticknor, C., Chen, J., and Dellaporta, S.L. (1996). Demethylation-induced developmental pleiotropy in *Arabidopsis*. Science **273,** 654-657.

Rossi, V., Hartings, H., and Motto, M. (1998). Identification and characterisation of an RPD3 homologue from maize (*Zea mays* L.) that is able to complement an rpd3 null mutant of *Saccharomyces cerevisiae*. Mol Gen Genet **258,** 288-296.

Rossi, V., and Varotto, S. (2002). Insights into the G1/S transition in plants. Planta **215,** 345-356.

Rossi, V., Locatelli, S., Lanzanova, C., Boniotti, M.B., Varotto, S., Pipal, A., Goralik-Schramel, M., Lusser, A., Gatz, C., Gutierrez, C., and Motto, M. (2003). A maize histone deacetylase and retinoblastoma-related protein physically interact and cooperate in repressing gene transcription. Plant Mol Biol **51,** 401-413.

Rundlett, S.E., Carmen, A.A., Kobayashi, R., Bavykin, S., Turner, B.M., and Grunstein, M. (1996). HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc Natl Acad Sci U S A **93,** 14503-14508.

Rundlett, S.E., Carmen, A.A., Suka, N., Turner, B.M., and Grunstein, M. (1998). Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. Nature **392,** 831-835.

Sakai, H., Medrano, L.J., and Meyerowitz, E.M. (1995). Role of SUPERMAN in maintaining *Arabidopsis* floral whorl boundaries. Nature **378,** 199-203.

Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J., and Kouzarides, T. (2002). Active genes are tri-methylated at K4 of histone H3. Nature **419,** 407-411.

Scherl, A., Coute, Y., Deon, C., Calle, A., Kindbeiter, K., Sanchez, J.C., Greco, A., Hochstrasser, D., Diaz, J.J. (2002). Functional proteomic analysis of human nucleolus. Mol Biol Cell **13,** 4100-9.

Selker, E.U. (1998). Trichostatin A causes selective loss of DNA methylation in *Neurospora*. Proc Natl Acad Sci U S A **95,** 9430-9435.

Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Charbonneau, H., and Deshaies, R.J. (1999). Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell **97,** 233-244.

Simpson, G.G., and Dean, C. (2002). *Arabidopsis*, the rosetta stone of flowering time?. Science **296,** 285-289.

Singer, T., Yordan, C., and Martienssen, R.A. (2001). Robertson's mutator transposons in *A. thaliana* are regulated by the chromatin-remodeling gene decrease in DNA methylation (DDM1). Genes Dev **15,** 591-602.

Smith, J.S., Brachmann, C.B., Celic, I., Kenna, M.A., Muhammad, S., Starai, V.J., Avalos, J.L., Escalante-Semerena, J.C., Grubmeyer, C., Wolberger, C., and Boeke, J.D. (2000). A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc Natl Acad Sci U S A **97,** 6658-6663.

Soppe, W.J., Jacobsen, S.E., Alonso-Blanco, C., Jackson, J.P., Kakutani, T., Koornneef, M., and Peeters, A.J. (2000). The late flowering phenotype of fwa mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. Mol Cell **6,** 791-802.

Soppe, W.J., Jasencakova, Z., Houben, A., Kakutani, T., Meister, A., Huang, M.S., Jacobsen, S.E., Schubert, I., and Fransz, P.F. (2002). DNA methylation

controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*. EMBO J **21,** 6549-6559.

Souer, E., van Houwelingen, A., Kloos, D., Mol, J., and Koes, R. (1996). The *No Apical Meristem* gene of petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell **85**, 1127-1135.

Spotswood, H.T., and Turner, B.M. (2002). An increasingly complex code. J Clin Invest **110,** 577-582.

Srinivas, S. (2000). Development: nature and nurture. J Cell Sci **113 (Pt 20),** 3549-3550.

Stam, M., Belele, C., Dorweiler, J.E., and Chandler, V.L. (2002). Differential chromatin structure within a tandem array 100 kb upstream of the maize b1 locus is associated with paramutation. Genes Dev **16,** 1906-1918.

Sterner, D.E., and Berger, S.L. (2000). Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev **64,** 435-459.

Stockinger, E.J., Mao, Y., Regier, M.K., Triezenberg, S.J., and Thomashow, M.F. (2001). Transcriptional adaptor and histone acetyltransferase proteins in *Arabidopsis* and their interactions with CBF1, a transcriptional activator involved in cold-regulated gene expression. Nucleic Acids Res **29,** 1524-1533.

Stokes, T.L., and Richards, E.J. (2002). Induced instability of two *Arabidopsis* constitutive pathogen-response alleles. Proc Natl Acad Sci U S A **99,** 7792-7796.

Stokes, T.L., Kunkel, B.N., and Richards, E.J. (2002). Epigenetic variation in *Arabidopsis* disease resistance. Genes Dev **16,** 171-182.

Strahl, B.D., Ohba, R., Cook, R.G., and Allis, C.D. (1999). Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in *Tetrahymena*. Proc Natl Acad Sci U S A **96,** 14967-14972.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature **403,** 41-45.

Straight, A.F., Shou, W., Dowd, G.J., Turck, C.W., Deshaies, R.J., Johnson, A.D., and Moazed, D. (1999). Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. Cell **97,** 245-256.

Struhl, K. (1999). Fundamentally different logic of gene regulation in eukaryotes and prokaryotes. Cell **98,** 1-4.

Suka, N., Carmen, A.A., Rundlett, S.E., and Grunstein, M. (1998). The regulation of gene activity by histones and the histone deacetylase RPD3. Cold Spring Harb Symp Quant Biol **63,** 391-399.

Suka, N., Suka, Y., Carmen, A.A., Wu, J., and Grunstein, M. (2001). Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin. Mol Cell **8,** 473-479.

Suka, N., Luo, K., and Grunstein, M. (2002). Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. Nat Genet **32,** 378-383.

Sung, S., and Amasino, R.M. (2004). Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature **427,** 159-64.

Takami, Y., and Nakayama, T. (2000). N-terminal region, C-terminal region, nuclear export signal, and deacetylation activity of histone deacetylase-3 are essential for the viability of the DT40 chicken B cell line. J Biol Chem **275,** 16191-16201.

Tanner, K.G., Landry, J., Sternglanz, R., and Denu, J.M. (2000). Silent information regulator 2 family of NAD- dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. Proc Natl Acad Sci U S A **97,** 14178-14182.

Tate, P., Skarnes, W., and Bird, A. (1996). The methyl-CpG binding protein MeCP2 is essential for embryonic development in the mouse. Nat Genet **12,** 205-208.

Taunton, J., Hassig, C.A., and Schreiber, S.L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science **272,** 408-411.

ten Lohuis, M., Galliano, H., Heidmann, I., and Meyer, P. (1995). Treatment with propionic and butyric acid enhances expression variegation and promoter methylation in plant transgenes. Biol Chem Hoppe Seyler **376,** 311-320.

Thiagalingam, S., Cheng, K.H., Lee, H.J., Mineva, N., Thiagalingam, A., and Ponte, J.F. (2003). Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci **983,** 84-100.

Thitamadee, S., Tuchihara, K., and Hashimoto, T. (2002). Microtubule basis for left-handed helical growth in *Arabidopsis*. Nature **417,** 193-196.

Thomas, J.O. (1984). The higher order structure of chromatin and histone H1. J Cell Sci Suppl **1,** 1-20.

Thorne, A.W., Sautiere, P., Briand, G., and Crane-Robinson, C. (1987). The structure of ubiquitinated histone H2B. EMBO J **6,** 1005-1010.

Tian, L., and Chen, Z.J. (2001). Blocking histone deacetylation in *Arabidopsis* induces pleiotropic effects on plant gene regulation and development. Proc Natl Acad Sci U S A **98,** 200-205.

Tian, L., Wang, J., Fong, M.P., Chen, M., Cao, H., Gelvin, S.B., and Chen, Z.J. (2003). Genetic control of developmental changes induced by disruption of *Arabidopsis* histone deacetylase 1 (AtHD1) expression. Genetics **165,** 399-409.

Tong, J.J., Liu, J., Bertos, N.R., and Yang, X.J. (2002). Identification of HDAC10, a novel class II human histone deacetylase containing a leucine-rich domain. Nucleic Acids Res **30,** 1114-1123.

Tong, J.K., Hassig, C.A., Schnitzler, G.R., Kingston, R.E., and Schreiber, S.L. (1998). Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. Nature **395,** 917-921.

Tse, C., Sera, T., Wolffe, A.P., and Hansen, J.C. (1998). Disruption of higher-order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase III. Mol Cell Biol **18,** 4629-4638.

Turner, B.M. (1991). Histone acetylation and control of gene expression. J Cell Sci **99 (Pt 1),** 13-20.

van der Vlag, J., and Otte, A.P. (1999). Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat Genet **23,** 474-478.

Varga-Weisz, P. (2001). ATP-dependent chromatin remodeling factors: nucleosome shufflers with many missions. Oncogene **20,** 3076-3085.

Varotto, S., Locatelli, S., Canova, S., Pipal, A., Motto, M., and Rossi, V. (2003). Expression profile and cellular localization of maize Rpd3-type histone deacetylases during plant development. Plant Physiol **133,** 606-617.

Varshavsky, A., Levinger, L., Sundin, O., Barsoum, J., Ozkaynak, E., Serdlow, P., and Finley, D. (1982). Cellular and SV40 chromatin: replication, segregation, ubiquitination, nuclease hypersensitive sites, HMG-containing nucleosomes and

heterochromatin-specific protein. Cold Spring Harb Symp Quant Biol **47,** 511-528.

Verbsky, M.L., and Richards, E.J. (2001). Chromatin remodeling in plants. Curr Opin Plant Biol **4,** 494-500.

Verdin, E., Dequiedt, F., and Kasler, H.G. (2003). Class II histone deacetylases: versatile regulators. Trends Genet **19,** 286-293.

Vermaak, D., Ahmad, K., and Henikoff, S. (2003). Maintenance of chromatin states: an open-and-shut case. Curr Opin Cell Biol **15,** 266-274.

Verreault, A., Kaufman, P.D., Kobayashi, R., and Stillman, B. (1998). Nucleosomal DNA regulates the core-histone-binding subunit of the human Hat1 acetyltransferase. Curr Biol **8,** 96-108.

Vidal, M., and Gaber, R.F. (1991). RPD3 encodes a second factor required to achieve maximum positive and negative transcriptional states in *Saccharomyces cerevisiae*. Mol Cell Biol **11,** 6317-6327.

Vlachonasios, K.E., Thomashow, M.F., and Triezenberg, S.J. (2003). Disruption mutations of ADA2b and GCN5 transcriptional adaptor genes dramatically affect *Arabidopsis* growth, development, and gene expression. Plant Cell **15,** 626-38.

Vogelauer, M., Wu, J., Suka, N., and Grunstein, M. (2000). Global histone acetylation and deacetylation in yeast. Nature **408,** 495 - 498.

Vongs, A., Kakutani, T., Martienssen, R.A., and Richards, E.J. (1993). *Arabidopsis thaliana* DNA methylation mutants. Science **260,** 1926-1928.

Wade, P.A., Pruss, D., and Wolffe, A.P. (1997). Histone acetylation: chromatin in action. Trends Biochem Sci **22,** 128-132.

Wade, P.A., Jones, P.L., Vermaak, D., and Wolffe, A.P. (1998). A multiple subunit Mi-2 histone deacetylase from *Xenopus laevis* cofractionates with an associated Snf2 superfamily ATPase. Curr Biol **8,** 843-846.

Wade, P.A. (2001). Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin. Hum Mol Genet **10,** 693-698.

Walbot, V. (1996). Sources and consequences of phenotypic and genotypic plasticity in flowering plants. Trends Plant Sci **1,** 27-32.

Wang, A., Kurdistani, S.K., and Grunstein, M. (2002). Requirement of Hos2 histone deacetylase for gene activity in yeast. Science **298,** 1412-1414.

Wang, A.H., Kruhlak, M.J., Wu, J., Bertos, N.R., Vezmar, M., Posner, B.I., Bazett-Jones, D.P., and Yang, X.J. (2000). Regulation of histone deacetylase 4 by binding of 14-3-3 proteins. Mol Cell Biol **20,** 6904-6912.

Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Borchers, C., Tempst, P., and Zhang, Y. (2001a). Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase. Mol Cell **8,** 1207-1217.

Wang, H., Huang, Z.Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Strahl, B.D., Briggs, S.D., Allis, C.D., Wong, J., Tempst, P., and Zhang, Y. (2001b). Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. Science **293,** 853-857.

Waterborg, J.H., Harrington, R.E., and Winicov, I. (1990). Dynamic histone acetylation in alfalfa cells. Butyrate interference with acetate labeling. Biochim Biophys Acta **1049,** 324-330.

Waterborg, J.H. (1992). Identification of five sites of acetylation in alfalfa histone H4. Biochemistry **31,** 6211-6219.

Weidle, U.H., and Grossmann, A. (2000). Inhibition of histone deacetylases: a new strategy to target epigenetic modifications for anticancer treatment. Anticancer Res **20,** 1471-1485.

Wolffe, A.P., and Matzke, M.A. (1999). Epigenetics: regulation through repression. Science **286,** 481-486.

Wolffe, A.P., Jones, P.L., and Wade, P.A. (1999). DNA demethylation. Proc Natl Acad Sci U S A **96,** 5894-5896.

Wu, J., Suka, N., Carlson, M., and Grunstein, M. (2001). TUP1 utilizes histone H3/H2B-specific HDA1 deacetylase to repress gene activity in yeast. Mol Cell **7,** 117-126.

Wu, K., Malik, K., Tian, L., Brown, D., and Miki, B. (2000a). Functional analysis of a RPD3 histone deacetylase homologue in *Arabidopsis thaliana*. Plant Mol Biol **44,** 167-176.

Wu, K., Tian, L., Malik, K., Brown, D., and Miki, B. (2000b). Functional analysis of HD2 histone deacetylase homologues in *Arabidopsis thaliana*. Plant J **22,** 19-27.

Wu, K., Tian, L., Zhou, C., Brown, D., and Miki, B. (2003). Repression of gene expression by *Arabidopsis* HD2 histone deacetylases. Plant J **34,** 241-247.

Wutz, A. (2003). RNAs templating chromatin structure for dosage compensation in animals. Bioessays **25**, 434-42.

Xue, Y., Wong, J., Moreno, G.T., Young, M.K., Cote, J., and Wang, W. (1998). NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Mol Cell **2,** 851-861.

Yang, W.M., Inouye, C., Zeng, Y., Bearss, D., and Seto, E. (1996). Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. Proc Natl Acad Sci U S A **93,** 12845-12850.

Yoshida, M., Horinouchi, S., and Beppu, T. (1995). Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. Bioessays **17,** 423-430.

Zaret, K., and Wolffe, A. (2001). Chromosomes and expression mechanisms. The post-genomic era of gene control. Curr Opin Genet Dev **11,** 121-123.

Zegerman, P., Canas, B., Pappin, D., and Kouzarides, T. (2002). Histone H3 lysine 4 methylation disrupts binding of nucleosome remodeling and deacetylase (NuRD) repressor complex. J Biol Chem **277,** 11621-11624.

Zemach, A., and Grafi, G. (2003). Characterization of *Arabidopsis thaliana* methyl-CpG-binding domain (MBD) proteins. Plant J **34,** 565-572.

Zhang, J., Kalkum, M., Chait, B.T., and Roeder, R.G. (2002). The N-CoR-HDAC3 nuclear receptor corepressor complex inhibits the JNK pathway through the integral subunit GPS2. Mol Cell **9,** 611-623.

Zhang, Y., LeRoy, G., Seelig, H.P., Lane, W.S., and Reinberg, D. (1998a). The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell **95,** 279-289.

Zhang, Y., Ng, H.H., Erdjument-Bromage, H., Tempst, P., Bird, A., and Reinberg, D. (1999). Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev **13,** 1924-1935.

Zhang, Y., Sun, Z.W., Iratni, R., Erdjument-Bromage, H., Tempst, P., Hampsey, M., and Reinberg, D. (1998b). SAP30, a novel protein conserved between

human and yeast, is a component of a histone deacetylase complex. Mol Cell **1,** 1021-1031.

Zhang, Y., and Reinberg, D. (2001). Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev **15,** 2343-2360.

Zhou, X., Marks, P.A., Rifkind, R.A., and Richon, V.M. (2001). Cloning and characterization of a histone deacetylase, HDAC9. Proc Natl Acad Sci U S A **98,** 10572-10577.

Zhou, Y.X., Newton, R.J., and Gould, J.H. (1997). A simple method for identifying plant/T-DNA junction sequence resulting from *Agrobacterium*-mediated DNA transformation. Plant Mol.Biol.Rep. **15**.

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PUBLICATIONS

1. **Tian, L., and Chen, Z. J.** (2001). Blocking histone deacetylation in *Arabidopsis* induces pleiotropic effects on plant gene regulation and development. Proc. Natl. Acad. Sci. USA **98**, 200-205.
2. **Tian, L., Wang, J., Fong, M., K., Chen, M., Cao, H., Gelvin, S., B., and Chen, Z. J.** (2003) Genetic control of developmental changes induced by disruption of *Arabidopsis* histone deacetylase 1 (*AtHD1*) expression. Genetics **165**, 399-409.
3. **Lee, H.-S., Wang, J., Tian, L., Black, M., Jiang, H., Madlung, A., Watson, B., Lukens, L., Pires, J. C., Wang, J., J., Comai, L., Osborn, T., Doerge, R. W., and Chen, Z. J.** (2004). Sensitivity of 70-mer oligonucleotides and cDNAs for microarray analysis of gene expression in *Arabidopsis* and its related species. Plant Biotechnology J **2**: 45-57.
4. **Chen, Z. J., Wang, J., Tian, L., Lee, H.-S., Wang, J., J., Chen, M., Lee, J., J., Josefsson, C., Madlung, A., Watson, B., Pires, J., C., Lippman, Z., Vaughn, M., Colot, V., Doerge, R. W., Martienssen, R. A., Comai, L., and Osborn, T., C.** (2004). The development of an *Arabidopsis* model system for genome-wide analysis of polyploidy effects. Biol. J. Linn. Soc. (in press).
5. **Wang, J., Lee, J., J., Lee, H.-S., Tian, L., Chen, M., Rao, S., Wei, E., N., Comai, L., and Chen, Z., J.** (2004). Methods for genome-wide analysis of gene expression in polyploids. Submitted.