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# Fertilization induces strong accumulation of a histone deacetylase (HD2) and of other chromatin-remodeling proteins in restricted areas of the ovules

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**Abstract**

Fertilization triggers a unique and complex developmental program leading to embryogenesis and seed set. Recently, mutations affecting chromatin-remodeling enzymes in plants have shown their key roles in development as demonstrated before in animal cells. Using a negative selection screen to isolate genes expressed in ovary tissues upon fertilization, we have identified a histone deacetylase gene (named *ScHD2a*) of the plant-specific HD2 family, which is predominantly expressed in ovaries of the self-incompatible species *Solanum chacoense.* The *ScHD2a* is the probable orthologue of the *Arabidopsis thaliana AtHD2a* gene, which upon antisense suppression leads to aborted seeds formation. Transcription of the *ScHD2a* gene is strongly triggered by fertilization and transcripts accumulate predominantly in the micropylar region of the ovule's integument. Interestingly, this fertilization-induced accumulation pattern was also observed for other genes involved in transcriptional repression but not for a MYST-family histone acetyltransferase. The strong increase in *ScHD2a* mRNA levels in ovules after fertilization suggests an important and localized role for transcriptional repression in seed development, and indicates why silencing of the *AtHD2a* gene leads to aborted seed formation.

*Abbreviations:* HAT, Histone AcetylTransferase; HDAC, Histone Deacetylase.

## Introduction

Chromatin remodeling by acetylation and deacetylation plays an important role in gene regulation through the post-translational modification of histones, either transiently in promoter-proximal nucleosomes, or for longer-term modulation over large chromatin domains (Turner, 2000). The overall pattern of histone modifications is proposed to constitute a code, the histone code, that defines the nature of the surrounding chromatin in either a repressive or an active state (Strahl and Allis, 2000). The basic unit of chromatin, the nucleosome, is composed of a histone octamer core that contains a total of about 26 highly conserved potentially acetylated lysine residues. Crystallographic analysis has revealed that the N-terminal tail of histones protrude from the octamer (Luger *et al.*, 1997). Histone acetyltransferases (HATs) transfer the acetyl moiety of acetyl-CoA to the $\varepsilon$-amino group within this N-terminal extension; this reaction can be reversed by the action of histone deacetylases (HDACs). It has been demonstrated that HDACs can interact with specific DNA-binding activator or repressor proteins, thereby modulating transcriptional activity of specific promoters by locally changing chromatin structure (Heinzel *et al.*, 1997; Pazin and Kadonaga, 1997).

The nucleotide sequence data reported will appear in the GenBank, EMBL and DDBJ Nucleotide Sequence Databases under the accession numbers AY346455 (ScHD2a), AY346456 (ScP18), AY346457 (ScSWIb), AY346458 (ScHAT) and AY346459 (ScH4).

The transcriptional repression of specific genes via targeted deacetylation of histones appears to be equally important for cell proliferation and development as the acetylation itself. HDACs have been grouped into four classes: the RPD3-like and HDA1-like superfamily, the SIR2-like family, and the plant-specific HD2-like family (Lusser *et al.*, 2001). This latter class forms multigene families of highly similar members within the plant kingdom, but no closely related proteins have been identified so far in animals or fungi (Dangl *et al.*, 2001). The first cloned plant HDAC was isolated as an acidic nucleolar phosphoprotein from maize embryo, and belonged to the plant-specific HD2 class (Lusser *et al.*, 1997). In *Arabidopsis thaliana*, three RPD3-related HDAC have been shown to be involved in various developmental processes. Antisense *AtRPD3A* transgenic *Arabidopsis* plants show delayed flowering, suggesting that histone deacetylation plays a role in vegetative to reproductive phase transition (Wu *et al.*, 2000a). Furthermore, when targeted to a normally active promoter, the AtRPD3 histone deacetylase protein repressed gene expression from a reporter gene construct. The *Arabidopsis HD6* histone deacetylase was isolated in a mutant screen and was shown to affect transgene silencing (Murfett *et al.*, 2001). Reduction of the *AtHD1* histone deacetylase levels by an antisense approach induced pleiotropic effects and resulted in plants with various phenotypes including early senescence, ectopic expression of silenced genes, homeotic conversions, and floral defects including male and female sterility (Tian and Chen, 2001). The antisense *AtHD1* plants also accumulated hyperacetylated histones, demonstrating the direct role of plant HDACs on histone acetylation levels (Tian and Chen, 2001). Apart from the maize nucleolar *HD2* gene, another HD2-related histone deacetylase has been shown to be involved in reproductive development. Silencing of the *AtHD2A* gene results in aborted seeds in transgenic *Arabidopsis* plants (Wu *et al.*, 2000b). In a Gal4-AtHD2A tethering system, it was also shown that this histone deacetylase could repress transcription when targeted to an active promoter.

In order to characterize early responses upon pollination and fertilization, we have used a modified negative selection screen that targets weakly expressed genes. In this study, we report the isolation and characterization of a fertilization-induced histone deacetylase of the HD2 family as well as of other chromatin-remodeling protein expressed in restricted areas of the ovules after fertilization in the self-incompatible wild potato *Solanum chacoense.*

## Materials and methods

### *Plant material*

The diploid (2n=2x=24) *Solanum chacoense* Bitt. self-incompatible genotypes used include line PI 458314 ($S_{11}$ and $S_{12}$ self-incompatibility alleles) and line PI 230582 ($S_{13}$ and $S_{14}$ self-incompatibility alleles), and were originally obtained from the Potato Introduction Station (Sturgeon Bay, WI). Plants were grown in greenhouses with an average of 14 h of light per day.

### *Library construction and virtual subtraction*

The cDNA library used was as described in Lantin *et al.* (1999). The cDNA phage library was converted to plasmid cDNA library by mass excision. Plasmid cDNA library colonies were transferred onto positively charged nylon membranes and denatured in 0.5 M NaOH and 1.5 M NaCl. The resulting membranes were hybridized with a probe derived from $\alpha$-[$^{32}$P]-dATP random-labeled cDNA leftovers (library target tissues) obtained during the library construction (the complete procedure will be described elsewhere). The membranes were exposed at $-85$ °C with one or two intensifying screens on Kodak Biomax MR film (Interscience, Markham, Ontario).

### *Isolation and gel blot analysis of RNA and DNA*

Total RNA was isolated as described previously (Jones *et al.*, 1985). RNA concentration was determined by measuring its absorbance at 260 nm and verified by agarose gel electrophoresis and ethidium bromide staining. Equal loading of total RNA on RNA gel blots was verified with a *S. chacoense* 18S RNA probe. Genomic DNA isolation was performed via a modified CTAB extraction method (Reiter *et al.*, 1992). DNA gel blot analysis, including restriction, electrophoresis, and capillary transfer onto a positively charged nylon membrane (Hybond $N^+$, Amersham Pharmacia Biotech, Baie D'Urfé, Quebec) were performed as described. (Sambrook *et al.*, 1989). Hybridization of the membranes was performed at 42 °C for 16–24 h in 50 % deionized formamide, 5× Denhardt's solution, 0.5% SDS, 200 $\mu$g/ml denatured salmon sperm DNA, and 6× SSC. After hybridization, the membranes were washed twice for 30 min in a 2× SSC/0.1% SDS solution at 35 °C, followed by washes in 1× SSC/0.1% SDS at 45 °C and 55 °C for 30 min, and finally once in 0.1× SSC/0.1% SDS at 55 °C for 10 min (1× SSC is

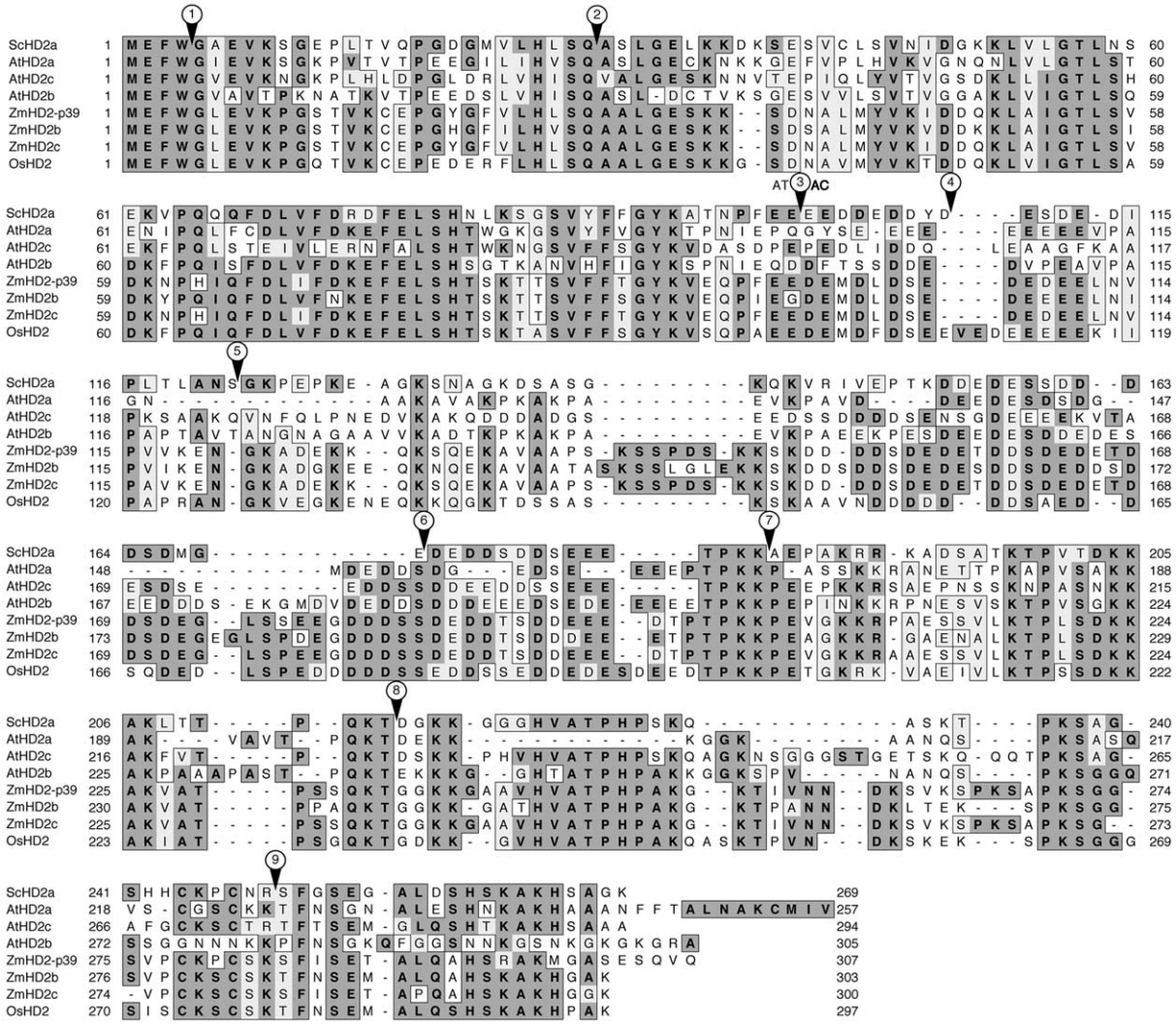


*Figure 1.* Alignment of the ScHD2a deduced protein sequence with closely related histone deacetylases. The deduced amino acid sequence of ScHD2a was aligned (with MacVector 7.2) with those of the following HD2-type histone deacetylases: AtHD2a (GenBank accession number AAB70032), AtHD2b (T52287), AtHD2c (AAF70197), ZmHD2-p39 (T04141), ZmHD2b (AAF68624), ZmHD2c (AAF68625) and OsHD2 (AAF70196). The arrows and circled numbers represent the intron position of the *ScHD2a* gene as determined by PCR amplification and sequencing of genomic DNA and comparison with cDNA sequence data.

0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). RNA gel blot analyses were performed as described by Sambrook *et al.* (1989), following the formaldehyde denaturing protocol. RNAs were capillary-transferred and cross-linked (120 mJ/cm$^2$) to Hybond N+ nylon membranes. Hybridization of the membranes was performed under high-stringency conditions at 45 °C in the same solution as for DNA gel blots. The membranes were washed as mentioned for the DNA gel blots. Probes for both DNA and RNA gel blot analyses were synthesized from random-labeled isolated DNA inserts (Roche Diagnostic, Laval, Quebec) or from the StripEX DNA kit (Ambion, Austin, TX) with $\alpha$-[$^{32}$P]-dATP (ICN Biochemicals, Irvine, CA). The membranes were exposed at −85 °C with one intensifying screen on Kodak Biomax MR film (Interscience, Markham, Ontario) or exposed to a Europium phosphor screen and detected with a Typhoon 9200 phosphorimager.

### In situ *hybridization*

Ovaries (cut in halves) and shoot apex were fixed in FAA (60% ethanol, 5% acetic acid, 5% formalin) at

4 °C overnight. After dehydration with *tert*-butanol and embedment in paraffin, samples were cut into 10 $\mu$m sections and mounted on slides coated with AES (3-aminopropyltriethoxy-silane; Sigma, Oakville, Ontario). Tissue sections were treated and hybridized as described (Lantin *et al.*, 1999).

## Results

### *Isolation of* ScHD2a, ScSAP18, ScSWIb, ScHAT, *and* ScH4 *from Solanum chacoense ovaries*

Key regulatory genes involved in development are generally weakly and transiently expressed as well as being highly tissue-specific. We initiated a negative selection screen based on the virtual subtraction procedure of Li and Thomas (1998), targeting only weakly expressed genes, with the aim of identifying genes involved in fertilization and in seed initiation in *Solanum chacoense*, a wild potato species close to potato and tomato. *S. chacoense* is a self-incompatible species and therefore pollination time can be manually controlled. After selection, colonies representing weakly expressed genes were targeted for 5′-end sequencing and RNA expression analyses were conducted on those genes identified as potentially involved in chromatin remodeling. The *S. chacoense* HD2a histone deacetylase, the SAP18 homologue (part of the histone deacetylase SIN3 repression complex), a SWIb domain containing protein (part of the SWI/SNF complex), a histone H4, and a MYST family acetyltransferase were isolated during that screen. In this paper we focus on the characterization of the *ScHD2a* histone deacetylase gene and on the expression domains of some of these genes involved in chromatin remodeling during early fertilization events.

### *Sequence analysis and phylogeny*

The ScHD2a clone contains a cDNA of 1091 bp (excluding the poly(A) tail) with a short 5′-untranslated leader of 50 nucleotides (nt) and a 3′-UTR of 231 nt (accession number AY346455). The size of the ScHD2a cDNA corresponds to the size of the mRNA, as determined by RNA gel blot analysis (1.1 kb, Figure 3), suggesting that the ScHD2a cDNA is full-length or near full-length. An in-frame stop codon is found in the 5′-UTR suggesting that the first ATG encountered is the translation start site. The ScHD2a cDNA is predicted to encode a 269 amino acid polypeptide of 29.1 kDa with an acidic pI of 4.72. Identity searches in DNA and protein databases revealed strong similarities with the plant-specific histone deacetylase HD2 family (Dangl *et al.*, 2001). A protein alignment with the histone deacetylases closest to the ScHD2a deduced protein is shown in Figure 1. The ScHD2a deduced protein has all the structural features of the plant HD2 protein family including a highly conserved $NH_2$-terminal region (always starting with the pentapeptide MEFWG), a centrally located extended acidic region (the overall content of ScHD2a in aspartic acid and glutamic acid residues is 21.2%), a basic region immediately downstream of the acidic domain, and a single COOH-terminal $C_2H_2$ zinc finger (Dangl *et al.*, 2001). Another striking feature is the high percentage of serine and threonine residues (11.2% and 5.2% respectively). Table 1 shows the amino acid identity and similarity between ScHD2a and the most closely related histone deacetylases. Surprisingly, the histone deacetylases from monocot species were more similar to ScHD2a than the *A. thaliana* HD2s, with a mean identity score of around 50% for the former, and of 40% for the latter. To determine the most probable orthologue, a ClustalX alignment followed by phylogenic analysis was performed (Figure 2). This analysis showed that, although sequence identity was significantly higher with the four monocot sequences from maize and rice, the ScHD2a HDAC is clearly related to the *A. thaliana* HD2 family, and more specifically to AtHD2a (or AtHDT1 from the Plant Chromatin Database).

An interesting feature of the plant HD2 family (except for *AtHD2a*) is the presence of an unusual U12-type intron located precisely at the third intron position (the overall intron position is well conserved in the plant HD2 family (Dangl *et al.*, 2001). These rare introns are characterized by 5′-AT and 3′-AC boundaries instead of the canonical 5′-GT and 3′-AG boundaries found in U2-type introns, and must be spliced through a U12-type spliceosome (Burge *et al.*, 1998). In order to determine if the ScHD2a also possess a U12-type intron, we determined the position of the ScHD2a introns, and fully or partially sequenced the introns from the ScHD2a corresponding genomic DNA through PCR amplification with primers derived from the cDNA sequence. Genomic organization of maize and *Arabidopsis* HD2 homologues had revealed the presence of seven or eight introns (Dangl *et al.*, 2001). Nine introns were found in the *ScHD2a* gene (arrowheads in Figure 1). All except intron 3 followed the canonical GT-AG U2-type rule. The small *ScHD2a* intron 3 (110 bp, data not shown) had the 5′-AT and 3′-AC boundaries of

*Table 1.* Histone deacetylase (HD2) sequence identities and similarities (in parenthesis).

| | ScHD2 | ZmHD2b | OsHD2 | ZmHD2-p39 | ZmHD2c | AtHD2a | AtHD2c | AtHD2b |
|---|---|---|---|---|---|---|---|---|
| ScHD2 | 100 | 50 (65) | 49 (64) | 49 (63) | 49 (62) | 39 (55) | 40 (53) | 36 (50) |
| ZmHD2b | | 100 | 67 (76) | 77 (84) | 78 (85) | 41 (52) | 42 (59) | 36 (49) |
| OsHD2 | | | 100 | 63 (71) | 64 (73) | 40 (52) | 42 (59) | 40 (53) |
| ZmHD2-p39 | | | | 100 | 95 (95) | 38 (51) | 39 (57) | 34 (48) |
| ZmHD2c | | | | | 100 | 39 (52) | 40 (58) | 35 (49) |
| AtHD2a | | | | | | 100 | 35 (47) | 54 (63) |
| AtHD2c | | | | | | | 100 | 32 (46) |
| AtHD2b | | | | | | | | 100 |

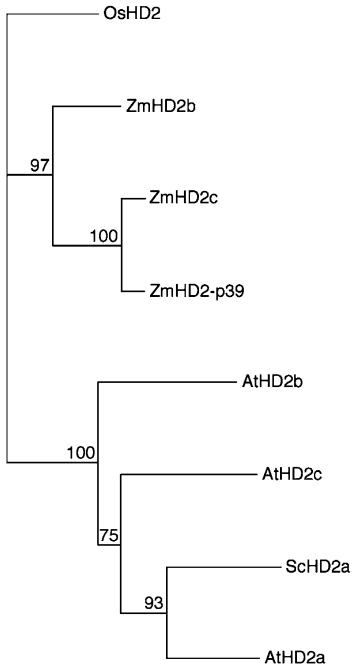


*Figure 2.* Phylogenic analysis of the HD2 histone deacetylase deduced protein sequences aligned in Figure 1. A ClustalX alignment was used to produce a jacknife analysis with Paup 4.08b (Swofford, 1998). The bootstrap support values (1000 replicates) are indicated on the left.

U12-type introns and, furthermore, showed a perfect 5′ border consensus sequence (ATATCCTT, consensus ATATCCTY), and branch point consensus (TCCTTGAC, consensus TCCTTRAY) typical of U12-type introns (Burge *et al.*, 1998).

To determine the *ScHD2a* gene copy number, a DNA gel blot of *S. chacoense* genomic DNA was probed with the ScHD2a cDNA insert (data not shown). The *Bam*HI and *Kpn*I sites are absent from the cDNA sequence, while the *Pvu*II restriction enzyme cut only once. A *Kpn*I site was also found in intron 6 (data not shown). Since one (*Bam*HI) or two hybridizing fragments (*Kpn*I and *Pvu*II) were seen on the DNA gel blot, this suggests that *ScHD2a* is a single-copy gene in *S. chacoense.*

### ScHD2a *mRNA expression in mature tissues*

Tissue-specific expression of *ScHD2a* was determined with total RNA extracted from different tissues of *S. chacoense.* The RNA gel blot analysis in Figure 3A indicates that *ScHD2a* is only weakly expressed in mature roots, stems, leaves and petals. No signal could be detected in mature anthers and in *in vitro* grown pollen tubes. Strongest expression levels were detected in pistils and in ovaries. A 5–6-fold increase in *ScHD2a* mRNA levels was detected 48 h after pollination in pistils, consistent with fertilization-induced expression. This transient increase was followed by a gradual decrease in *ScHD2a* mRNA levels. Densitometric scans indicated that *ScHD2a* mRNA levels were about 12-fold less abundant in roots, stems and leaves, and 21-fold less in petals as compared to pollinated pistils 48 h after pollination.

We have previously shown that some genes are activated at a distance in the ovary upon pollination and that this could be mediated by wounding (through pollen tube growth in the style) or wound hormones like jasmonic acid or methyl jasmonate (Lantin *et al.*, 1999). In order to determine if pollination *per se* triggered the accumulation of *ScHD2a* mRNAs, or if fertilization was necessary, an incompatible pollination was performed. *S. chacoense* is a gametophytic self-incompatible species that expresses stylar ribonucleases (S-RNases) in the transmitting tissue of the style (Matton *et al.*, 1997). Figure 3B shows that the strong *ScHD2a* mRNA increase observed in ovaries

was indeed the result of fertilization since no such increase in *ScHD2a* mRNA levels could be detected after self-incompatible pollination. Furthermore, mechanical wounding of the style, as well as incomplete pollen tube growth from an incompatible pollination, could not induce such an elevated expression in the ovary. The RNA gel blot in Figure 3B also shows that *ScHD2a* expression in pistil was restricted to the ovary since no signal could be detected in mature styles. *ScHD2a* mRNA levels were also monitored in ovules from the day of pollination until 16 days later, corresponding to ovules bearing embryos at the late torpedo stage (Figure 3C). As for earlier time points after fertilization, after an initial increase due to fertilization, *ScHD2a* mRNA levels in ovules decreased rapidly from six days after pollination. To determine more precisely in the ovary where the *ScHD2a* mRNA increase could be detected, ovules and placenta were separated from the surrounding pericarp tissue. Figure 3D shows that the strong accumulation of *ScHD2a* mRNA after compatible pollination and effective fertilization could be equally detected in both the isolated ovules and in the surrounding pericarp tissue.

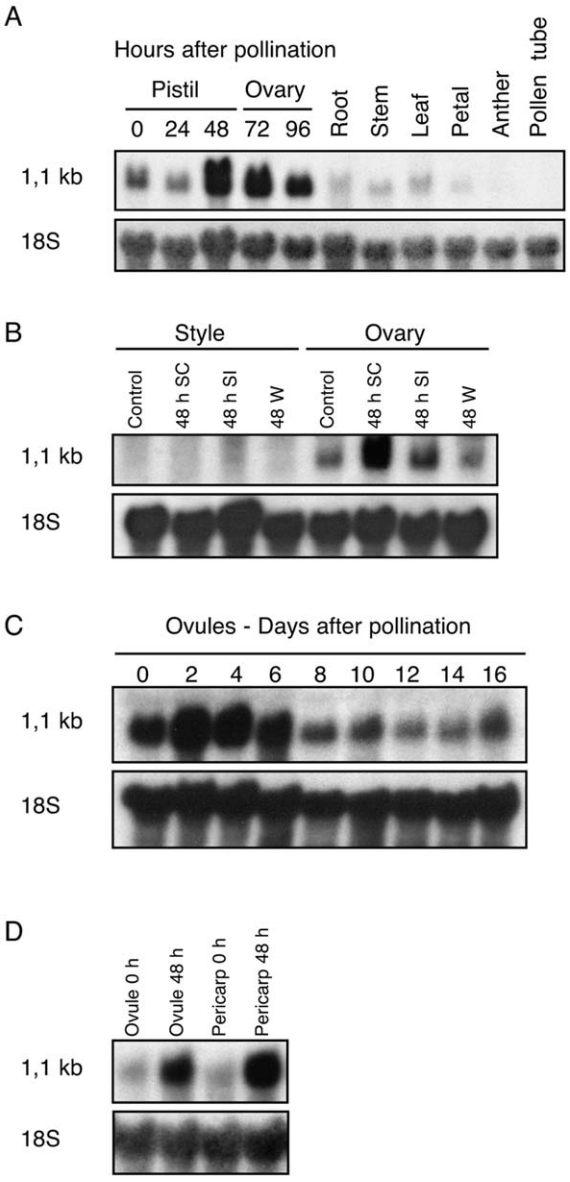


*Figure 3.* RNA expression analysis of *ScHD2a* transcript levels. A. Tissue-specific expression pattern of *ScHD2a* mRNA levels in vegetative and reproductive tissues. *ScHD2a* transcript levels were determined by RNA gel blot analysis of mature vegetative tissues and in pollinated pistils and ovaries from the time of pollination (0) to four days (96) after pollination. Total RNA (10 $\mu$g) from each tissue was probed with the ScHD2a cDNA insert, stripped and re-probed with the 18S rRNA gene as a control. B. Fertilization-induced expression of *ScHD2a* in pistil tissues. *ScHD2a* transcript levels were determined by RNA gel blot analysis in non-pollinated styles and ovaries at anthesis day (0), in styles and ovaries collected 48 h after a fully compatible ($S_{11}S_{12}$x $S_{13}S_{14}$) pollination (48 h SC), in styles and ovaries collected 48 h after a fully incompatible ($S_{13}S_{14}$ x $S_{13}S_{14}$) pollination (48 h SI), and in styles and ovaries collected 48 h after wounding of the upper style (48 W). Conditions were the same as in A. C. RNA expression analysis of *ScHD2a* transcript levels in isolated ovules. ScHD2a transcript levels were determined by RNA gel blot analysis in isolated ovules from anthesis day (0) until 16 days after pollination. Conditions were the same as in A. D. RNA expression analysis of *ScHD2a* transcript levels in ovary tissues. ScHD2a transcript levels were determined by RNA gel blot analysis in isolated ovules or from the surrounding pericarp tissue isolated on anthesis day (0) or collected after a fully compatible pollination (48 h). Conditions were the same as in A.

*Chromatin-remodeling enzyme and related proteins mRNA expression profile in mature tissues*

Since we observed a strong increase in *ScHD2a* mRNA levels in ovaries after fertilization, we decided to test whether other chromatin-remodeling enzymes and related proteins would behave similarly upon fertilization. A homologue of the SIN3-associated protein p18 (SAP18) involved in histone deacetylase complex in animals was isolated and completely sequenced. Comparison of the full-length cDNA clone with homologues from various species showed that the *S. chacoense* p18 deduced protein shared 70% sequence identity (78% similarity) with the *A. thaliana* homologue and 45% sequence identity (56% similarity) with chicken, mouse and human p18 homo-

logues. A BAF60b domain-containing protein of the SWI/SNF complex was also identified in our screen (partial cDNA). Both the *S. chacoense* SAP18 homologue and the SWIb domain protein displayed an mRNA expression pattern identical to the *ScHD2a* gene (compare Figures 4A and 3A). Messenger RNA levels were low in vegetative tissues and barely detectable in pollen tube, slightly higher in unfertilized ovules and strongly increased following fertilization. To test whether such a strong increase in mRNA levels after fertilization was also typical of genes involved in transcriptional activation such as histone acetyltransferase (HAT), we also probed the same tissues with a partial cDNA highly similar (overall sequence similarity 90%) to the *Arabidopsis HAG4* and *HAG5* genes (also known as *HAC6* and *HAC11* respectively), and to the maize *HAG104* gene (also known as *HAC108*). All four above HATs are part of the MYST superfamily of HATs that also possess an N-terminal chromodomain (Utley and Côte, 2003). In strong contrast with the *ScHD2a*, *ScP18* and *ScSWIb* cDNAs, the *S. chacoense HAT* was only slightly up-regulated in ovary tissues after fertilization. One target of both HDAC and HAT activity is the histone H4. Histone H4 is also often used as a marker of cell division (Brandstadter *et al.*, 1994). A histone H4 cDNA was also isolated and tested for its expression profile in various tissues as well as after fertilization. With the exception of a detectable signal in *in vitro* grown pollen tube, histone H4 expression pattern was identical to the profile observed for the *ScHD2a*, the *ScP18* and the *ScSWIb* genes.

### ScHD2a, ScP18, ScSWIb, *and* ScH4 *expression profiles in rapidly dividing tissues*

Since other genes involved in chromatin remodeling have also been shown to be actively transcribed in rapidly dividing tissues (Wagner and Meyerowitz, 2002), we also compared the expression profiles of the *ScHD2a*, *ScP18*, *ScSWIb*, and *ScH4* genes in shoot apex and callus tissues. Expression in young callus derived from cut leaf disks was not significantly different from expression in mature leaves (Figure 4B). In shoot apex containing actively dividing cells, expression of all the genes tested was only slightly increased. Densitometric analysis calibrated against the 18S rRNA control hybridization revealed that the steady-state mRNA level of *ScHD2a*, *ScSWIb*, *ScP18*, and *ScH4* in shoot apex tissues was increased by a factor of 2.3, 1.6, 1.2, and 1.2 respectively (Figure 4B).

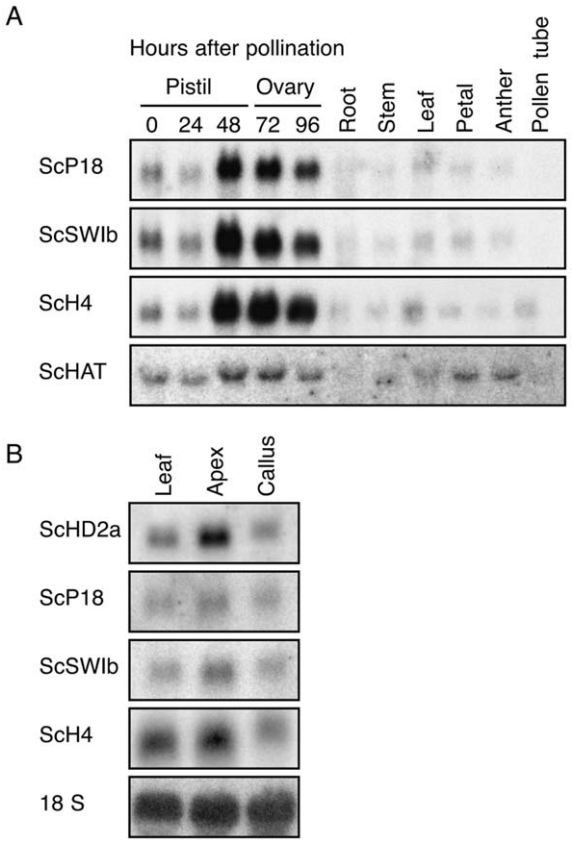


*Figure 4.* RNA expression analysis of other proteins involved in chromatin-remodeling. A. Tissue-specific expression pattern of *ScP18*, *ScSWIb*, *ScH4* and *ScHAT* mRNA levels in vegetative and reproductive tissues. Transcript levels were determined by RNA gel blot analysis of mature vegetative tissues and in pollinated pistils and ovaries from the time of pollination (0) to four days (96) after pollination. Total RNA (10 $\mu$g) from each tissue was probed with the ScHD2a cDNA insert. The control was the same as in Figure 3A. B. Expression analysis of *ScHD2a*, *ScP18*, *ScSWIb*, and *ScH4* in rapidly dividing tissues. Conditions were the same as in Figure 3A.

The expression profile of the *ScHD2a* gene was also examined by *in situ* hybridization in the shoot apex. Figure 5 shows that *ScHD2a* is strongly expressed in both shoot apical meristem and floral meristem, as well as in the procambium, which contain actively dividing cells.

### *Micropylar accumulation of* ScHD2a, ScP18 *and* ScSWI *mRNA in the ovule*

In order to characterize the cellular expression pattern of *ScHD2a*, *ScP18* and *ScSWIb* in ovary tissues, *in situ* hybridizations were performed on ovary sections 48 h after pollination. In a larger view of the whole ovary (Figure 6B, C), mRNA expression of the

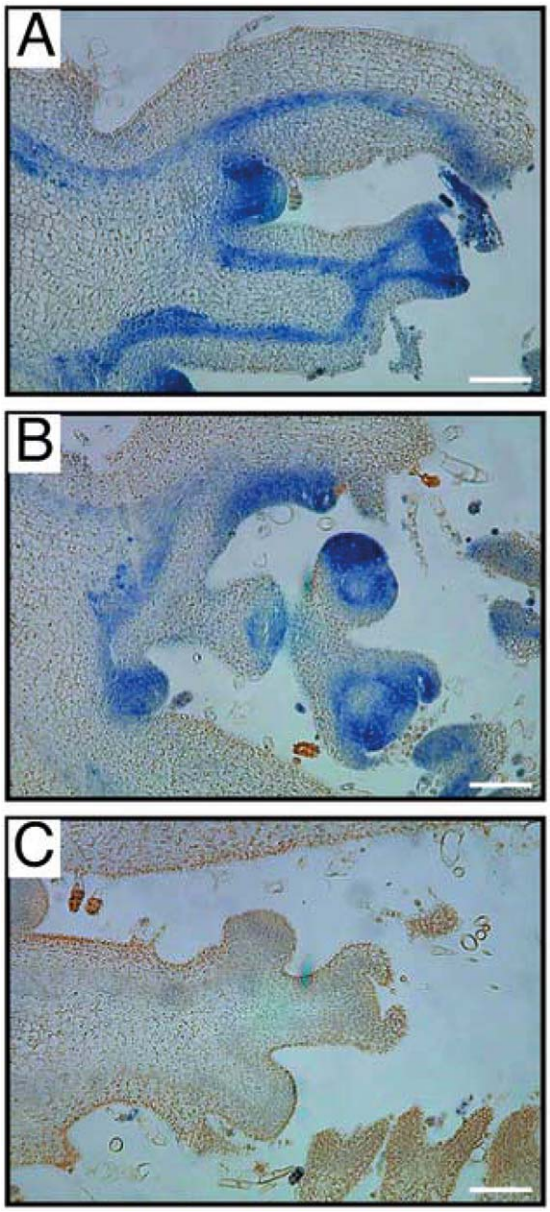


*Figure 5. In situ* localization of *ScHD2a* in shoot apex tissues. A and B. Sections of *S. chacoense* shoot apex. Anti-sense ScHD2a probe. C. Section of *S. chacoense* shoot apex. Sense (control) probe. White bar represents 100 $\mu$m.

*ScHD2a* mRNA can be detected mostly in the ovules, in the pericarp, and in the placenta. Hybridization with reduced concentration of the ScHD2a probe or decreased enzymatic reaction time after antibody detection of the probe revealed that the area of strongest expression was localized in the integument around the micropylar region of the ovule (Figure 6C). A magnified view of the ovules confirmed that *ScHD2a* is expressed more strongly in the micropylar region of the integument, where the pollen tube enters the ovule (Figures 6D and 6E). In these magnified views, mRNA expression of the *ScHD2a* gene can also be detected, albeit only weakly, in the region immediately adjacent to the ovules in the placenta, as well as more diffusely in the pericarp. *In situ* analyses were also conducted for the *ScP18* (Figure 6F and G) and *ScSWIb* genes (Figure 6H and I). Both showed a strong preferential accumulation also in the ovule's integument around the micropyle. The cell layer of the placenta tissue closest to the attached ovules also showed a significant expression of both these genes. Control sense-labeled sections for *ScHD2a*, *ScP18* and *ScSWIb* gave no hybridization signal (Figure 6A, F, I, L).

## Discussion

In recent years, numerous plant chromatin-remodeling factors have been identified in mutational studies and through sequence similarity with their animal counterparts. The role of chromatin remodeling in plant development has recently been reviewed in detail (Goodrich and Tweedie, 2002; Reyes *et al.*, 2002; Wagner, 2003). Contrary to animals, where such mutations are lethal, in plants most of these mutants are viable but the mutations affect various aspects of plant growth and development. Many chromatin-remodeling enzymes have been shown to specifically affect seed development. The three polycomb-group proteins FIS2, MEA and FIE have been isolated in genetic screens for plants showing seed development without pollination. They have been shown to be involved in various aspects of seed development, and mutations in these genes all lead to developmental arrest of the seeds (recently reviewed in Chaudhury and Berger, 2001; Lohe and Chaudhury, 2002). Mutation in the *SPLAYED* gene, a member of the SWI/SNF chromatin-remodeling ATPases (SNF2/Brm), also affects seed formation due to ovule growth arrest at megagametogenesis (Wagner and Meyerowitz, 2002). The *SPLAYED* gene is also strongly expressed in both vegetative and inflorescence meristems, as showed here for the *ScHD2a* gene (Figure 5). The PICKLE chromodomain protein, also similar to another member of the SWI/SNF chromatin-remodeling ATPases complex, has been shown to be necessary for the repression of *LEC1*, a gene implicated as a critical activator of embryo development (Ogas *et al.*, 1999). As mentioned in the Introduction, histone deacetylase genes have also been found to have a strong effect on plant growth and

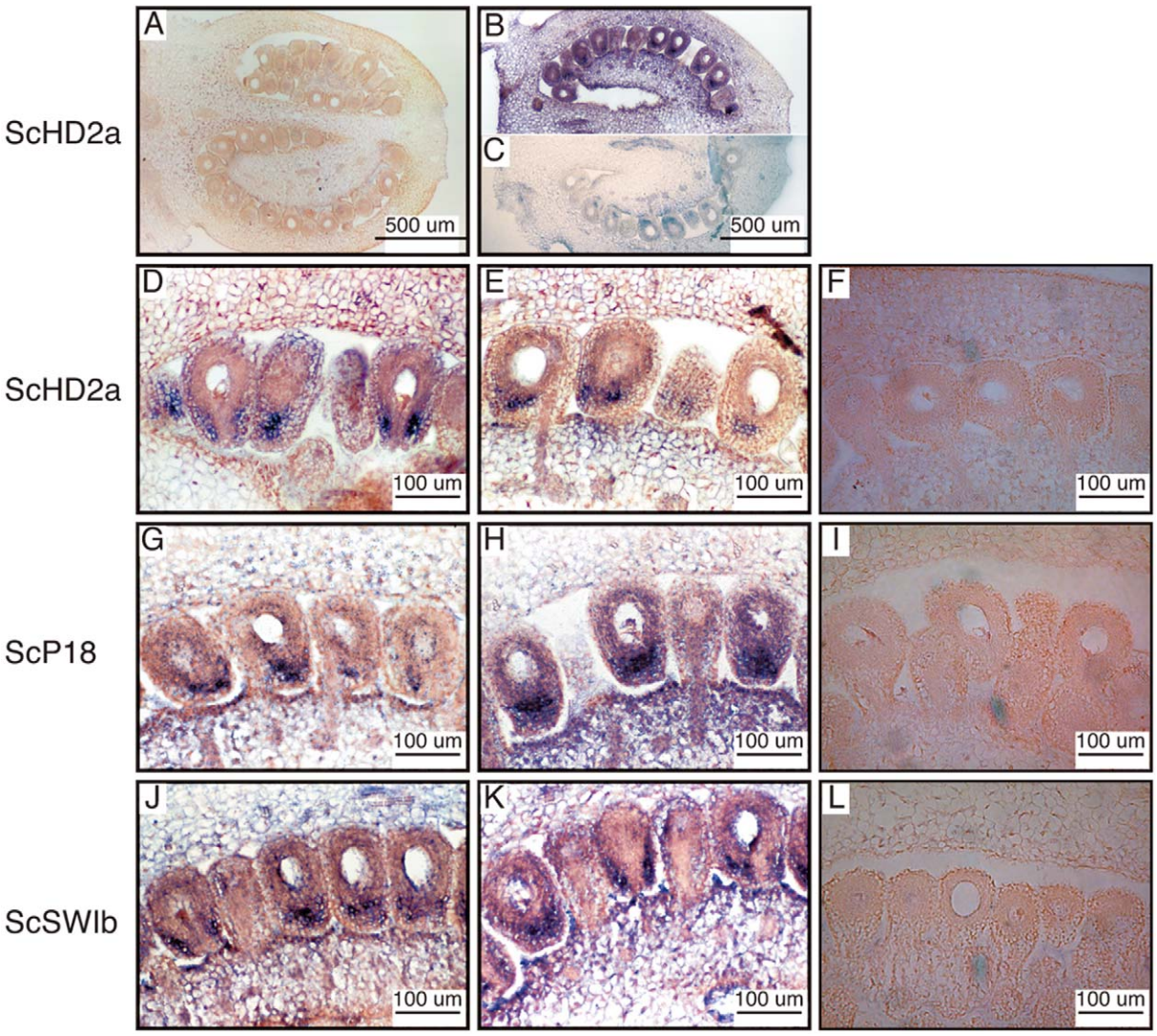


*Figure 6. In situ* localization of *ScHD2a*, *ScP18* and *ScSWIb* transcripts in fertilized ovaries 48 h after pollination. A. Longitudinal section of complete ovary 48 h after pollination. Sense (control) probe. B. Longitudinal section of complete ovary 48 h post-pollination. Antisense ScHD2a probe. Longer staining time. C. Longitudinal section of complete ovary 48 h after pollination. Antisense ScHD2a probe. Shorter staining time showing areas of strongest expression. D and E. Magnification of the ovules in ovary 48 h after pollination showing *ScHD2a* expression in the micropylar region of the ovule's integument. Antisense ScHD2a probe. F. Magnification of the ovules in ovary 48 h after pollination. Sense ScHD2a probe. G and H. Magnification of the ovules in ovary 48 h after pollination showing *ScP18* expression in the micropylar region of the ovule's integument. Antisense ScP18 probe. I. Magnification of the ovules in ovary 48 h after pollination. Sense ScP18 probe. J and K. Magnification of the ovules in ovary 48 h after pollination showing *ScSWIb* expression in the micropylar region of the ovule's integument. Antisense ScSWIb probe. L. Magnification of the ovules in ovary 48 h after pollination. Sense ScSWIb probe. Digoxigenin labeling is visible as blue staining. All hybridizations used 10 $\mu$m thick sections and an equal amount of either sense or antisense probe was used.

development. Antisense *AtHD2a* plants show a seedless silique phenotype but no other strong phenotypes could be observed in these plants, suggesting a highly specific role for this HDAC (Wu *et al.*, 2000b). In this paper we have focused on the *ScHD2a* histone deacetylase, a gene strongly expressed in specific regions of the ovule after fertilization. The phylogenic analysis showed that *ScHD2a* is most closely related to the *AtHD2a* gene (Figure 2), although all characterized HD2 genes, except the *Arabidopsis AtHD2a*, but including *ScHD2a*, possess an unusual third intron with AT-AC borders (Dangl *et al.*, 2001). It is most probable that the *AtHD2a* gene has suffered a conversion from a U12-type intron to a more common

U2-type intron, as shown in many species where U12-type introns have switched, possibly through a simple sequential modification of the donor and acceptor sites (AT-AC → AT-AG → GT-AG) (Burge *et al.*, 1998). Furthermore, the expression pattern observed for the *ScHD2a* gene in the ovule's integument combined with the seedless silique phenotype observed in antisense *AtHD2a* plants suggest that they are probably orthologous (Wu *et al.*, 2000b).

*ScHD2a* expression analyses showed that this gene was only very weakly expressed in vegetative tissues, but was strongly induced in ovaries upon fertilization. Peak accumulation was observed in the integument of the ovules, mainly in the micropylar region where the pollen tube enters to deliver its two sperm nuclei. Contrary to the result obtained from RNA gel blot analysis (Figure 4D), where an equal hybridization signal could be detected both in the ovules and the pericarp, *in situ* analysis clearly showed that expression is localized much more in the ovules than in the pericarp. This could explain why the more global albeit weaker expression in the pericarp, as detected by *in situ* hybridization, can be as strong as the one observed from the ovules, determined from the RNA gel blot analysis, where the signal is stronger but more localized to a particular area of the ovule. The normally stronger expression of *ScHD2a* gene after fertilization in the ovule's integument and around the mycropylar end suggests that the seedless phenotype of the antisense *AtHD2a* plants is probably related to the absence of this specific surge of expression in restricted areas of the ovules (Figures 4 and 6). Closer anatomical analyses of the *AtHD2a* mutant seeds would be needed to reveal the developmental defect produced by the suppression of this HDAC in the ovule's integument. To determine if this strongly fertilized-induced expression pattern could also be true for other chromatin-remodeling proteins, we also tested if homologues of proteins shown to be associated with repression complex in animals were also regulated by fertilization. A homologue of the SIN3-associated protein p18 (SAP18) showed an identical pattern of expression as for the *ScHD2a* gene (Figures 5 and 6). The SAP18 protein is part of the SIN3 repressor complex in animal and yeast cells, which also includes HDACs and associated proteins (Ahringer, 2000). Many subunits of the SIN3 complex and of the NuRD repression complex have now been found in plants, and it is reasonable to assume that they possess a similar gene repression function. SAP18 has been found to interact with Polycomb group proteins (Wang *et al.*, 2002) and homeotic genes in animal cells (Zhu *et al.*, 2001). The same results were also obtained with a BAF60b domain-containing protein, similar to proteins of the SWI/SNF complex (Figures 5 and 6). Although such complex was originally identified as being involved in transcriptional activation (Sudarsanam and Winston, 2000), whole-genome expression analysis in yeast has revealed that it was also involved in repression of transcription, as almost half of the genes affected in *swi/snf* mutants have increased mRNA levels (Holstege *et al.*, 1998; Sudarsanam *et al.*, 2000). Conversely, no such strong increase was observed for a MYST-family histone acetyltransferase, which also expressed preferentially in female reproductive tissues (Figure 4). All these results point to a specific role in transcriptional repression in restricted areas of the ovule, immediately after fertilization has occurred. The *ScHD2a* gene expression in maternal tissues also suggests an important role for maternal tissue to embryo communication, as fertilization occurs in the embryo sac around 36 h after pollination. These events could also be related to an increase in transcription rate in the ovule's integument after fertilization. Induction of the histone *H*4 gene expression at G1/S-phase transition has been previously shown to be the consequence of such an increase in transcription rate at the initiation step of DNA replication (Reichheld *et al.*, 1998). In fact, histone induction preceded the burst of DNA synthesis in synchronized tobacco cells. Similarly, we also observed a strong increase in histone *H*4 mRNA levels immediately following fertilization, although we did not monitor the replication state of the integument's cells. Furthermore, during replication, histone H4 was observed as being highly acetylated (Jasencakova *et al.*, 2000). This and the fact that we observed a strong increase in mRNA levels for *ScHD2a*, *ScP18* and *ScSWIb* and not for the MYST-HAT, suggest that this increase in chromatin-remodeling protein involved in transcriptional repression is probably targeting specific genes and is not related to replication events. This specific rather than general or global role has also been shown for the SIN3 and NuRD complex in animal systems (Ahringer, 2000), and illustrate the complex but specific nature of the activity of histone deacetylases and chromatin-remodeling proteins in plants.

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