Chromosoma (2004) 112: 308–315
DOI 10.1007/s00412-004-0275-7

RESEARCH ARTICLE

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Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in *Arabidopsis thaliana*

Received: 2 January 2004 / Accepted: 17 February 2004 / Published online: 10 March 2004
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Abstract The Arabidopsis *KRYPTONITE* gene encodes a member of the Su(var)3-9 family of histone methyltransferases. Mutations of *kryptonite* cause a reduction of methylated histone H3 lysine 9, a loss of DNA methylation, and reduced gene silencing. Lysine residues of histones can be either monomethylated, dimethylated or trimethylated and recent evidence suggests that different methylation states are found in different chromatin domains. Here we show that bulk Arabidopsis histones contain high levels of monomethylated and dimethylated, but not trimethylated histone H3 lysine 9. Using both immunostaining of nuclei and chromatin immunoprecipitation assays, we show that monomethyl and dimethyl histone H3 lysine 9 are concentrated in heterochromatin. In *kryptonite* mutants, dimethyl histone H3 lysine 9 is nearly completely lost, but monomethyl histone H3 lysine 9 levels are only slightly reduced. Recombinant KRYPTONITE can add one or two, but not three, methyl groups to the lysine 9 position of histone H3. Further, we identify a KRYPTONITE-related protein, SUVH6, which displays histone H3 lysine 9 methylation activity with a spectrum similar to that of KRYPTONITE. Our results suggest that multiple Su(var)3-9 family members are active in Arabidopsis and that dimethylation of histone H3 lysine 9 is the critical mark for gene silencing and DNA methylation.

Communicated by P. Shaw

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Introduction

Epigenetic gene silencing in eukaryotic organisms is generally associated with the formation of heterochromatin, a complex of histone and non-histone proteins that combine to package the DNA tightly. The histones found in heterochromatin are characterized by specific post-translational modifications (Strahl and Allis 2000; Turner 2000). One of the best characterized modifications is methylation of lysine 9 of histone H3 (H3K9) (reviewed in Jenuwein and Allis 2001; Richards and Elgin 2002; Turner 2002). Additionally, in many eukaryotic organisms, including plants and mammals, cytosine DNA methylation is a necessary component of epigenetic gene silencing (reviewed in Martienssen and Colot 2001).

Genetic screens in *Arabidopsis thaliana* have identified a number of components that are required for initiating and maintaining these epigenetic marks. The *clark kent* alleles of the *SUPERMAN* gene are silenced by DNA methylation, resulting in a *superman* like mutant phenotype—flowers develop additional stamens and unfused carpels. KRYPTONITE (KYP), a histone methyltransferase specific for H3K9, was identified in a screen for second site suppressors of the *clark kent-stable* allele (Jackson et al. 2002). *KYP* mutants were also uncovered independently in a screen for second site suppressors of

gene silencing of the Arabidopsis *PAI* loci (Malagnac et al. 2002).

KYP is a member of the Su(var)3-9 family of histone methyltransferases. This group of proteins is characterized by the presence of an approximately 130 amino acid SET domain which was originally identified in, and named after, three Drosophila proteins known to be involved in epigenetic processes, Su(var)3-9, Enhancer of zeste, and Trithorax (Tschiersch et al. 1994). A mammalian homolog of Su(var)3-9 was the first to be identified as a histone methyltransferase specific for H3K9 (Rea et al. 2000). Subsequently, other SET domain containing proteins have been shown to methylate K4, K9, K27, or K36 of H3 and K20 of H4. At least 29 SET domain proteins have been identified in Arabidopsis. Based on sequence identity and overall domain architecture, nine of these genes were grouped into the Su(var)3-9 subfamily, identified as *SUVH1* through *SUVH9* with *KYP* listed as *SUVH4* (Baumbusch et al. 2001). The function of the other eight *KYP* related genes is unknown, but since they are expressed, it is likely that some of them are producing active gene products (Baumbusch et al. 2001).

Mutations in *KYP* result in the suppression of *SUPERMAN* gene silencing as well as reactivation of the expression of several silent retrotransposons (Jackson et al. 2002). The *kyp* mutations reduce H3K9 methylation at affected loci in vivo (Johnson et al. 2002) and cause a decrease in DNA methylation at CpNpG sites, suggesting that H3K9 methylation controls CpNpG DNA methylation. Studies in *Neurospora crassa* showed a similar link between DNA and histone methylation. Mutating the Neurospora H3K9 specific methyltransferase DIM5, or mutating lysine 9 of H3 to arginine, resulted in a complete loss of DNA methylation (Tamaru and Selker 2001). Thus a relationship between histone methylation and DNA methylation is likely to be conserved.

Lysines can accept three methyl groups, and can therefore be monomethylated, dimethylated or trimethylated (hereafter denoted as m, m^2, and m^3), and recent evidence suggests that there may be functional differences between these methylation states (Dutnall 2003). For instance, H3K4m^2 correlates with inactive regions of euchromatin in yeast, while H3K4m^3 correlates with actively transcribed chromatin (Krogan et al. 2003; Ng et al. 2003; Santos-Rosa et al. 2002). Furthermore, evidence from immunostaining of chromosomes suggests that H3K9m^3 is localized to heterochromatic regions in animals (Cowell et al. 2002). More recently, in mammalian cells it was shown that H3K9m^3 is preferentially localized to pericentromeric heterochromatin, while H3K9m and H3K9m^2 are localized to euchromatin (Peters et al. 2003; Rice et al. 2003). In Neurospora, silent and DNA methylated loci that have recently undergone repeat-induced point mutation are specifically associated with H3K9m^3 (Tamaru et al. 2003).

In this report, we investigate the association of H3K9m, H3K9m^2, and H3K9m^3 with gene silencing in Arabidopsis. We find abundant H3K9m and H3K9m^2 but little if any H3K9m^3 in bulk histones and at silent loci. The *kyp* mutants show a large reduction of H3K9m^2 and more minor effects on H3K9m. This correlates well with in vitro data showing that both KYP, and the related Arabidopsis gene product SUVH6, can only add two methyl groups to H3K9 peptides. These data suggest that H3K9m^2 is likely the predominant mark for gene silencing in Arabidopsis.

Materials and methods

Histone preparations

Histones were isolated from wild-type and *kyp-2* plants using sulfuric acid extraction of nuclei followed by acetone precipitation. Three grams of tissue was ground with a mortar and pestle then resuspended in 10 ml of NIB buffer [15 mM PIPES pH 6.8, 5 mM $MgCl_2$, 60 mM KCl, 0.25 M sucrose, 15 mM NaCl, 1 mM $CaCl_2$, 0.8% Triton X100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.7 μg/ml pepstatin, and complete mini-Tab protease inhibitors (Roche)]. The slurry was filtered through mira cloth and the filtrate was centrifuged at 10,000 *g* for 20 min. The nuclei were then extracted twice with 0.4 M H_2SO_4 and precipitated with 12 volumes of acetone. The precipitate was collected by centrifugation and the pellet resuspended in 200 μl of 4 M urea.

Protein blot analysis

Proteins were separated by electrophoresis in 15% SDS-polyacrylamide gels and then transferred to PVDF membrane in CAPS/methanol buffer. Primary antibodies specific for H3K9m (1:1000; α-H3K9m-TJ), H3K9m^2 (1:1000; α-H3K9m^2-DA) and H3K9m^3 (α-H3K9m^3-PS) were used to probe the blot (see Table 1 for description of antibodies).

Immunostaining

Nuclei from young rosette leaves of *A. thaliana* Landsberg *erecta* (Ler), and the *kyp* mutant (*kyp-2* in *clark kent-3gl1-1* background) in Ler were isolated as described (Jasencakova et al. 2003). Nuclear suspensions were stained with 4′,6-diamidino-2-phenylindole

Table 1 Rabbit polyclonal antibodies against methylated lysine 9 of histone H3 (H3K9m) used in this study

Name	Reported specificity	Source	Reference
α-H3K9m-TJ	H3K9m	T. Jenuwein	Peters et al. (2003)
α-H3K9m^2-TJ	H3K9m^2	T. Jenuwein	Peters et al. (2003)
α-H3K9m^2-UBI	H3K9m^2	Upstate Biotechnology	Catalog no. 07-212
α-H3K9m^2-DA	H3K9m^2	David Allis	Nakayama et al. (2001)
α-H3K9m^3-TJ	H3K9m^3	T. Jenuwein	Peters et al. (2003)
α-H3K9m^3-PS	H3K9m^3	P. Singh	Cowell et al. (2002)
α-H3K9m^3-AB	H3K9m^3 and H3K27m^3	Abcam	Catalog no. ab8898

(DAPI, 1 μg/ml) and processed for flow-sorting according to Jasencakova et al. (2003). Nuclei of 4C ploidy level representing the major fraction of leaf nuclei were used in most experiments. Rabbit polyclonal antisera against H3K9m, H3K9m^2, and H3K9m^3 were used (see Table 1 for description of antibodies). The immunolabeling procedure was as described (Jasencakova et al. 2003). After post-fixation in 4% paraformaldehyde/PBS, subsequent washes in PBS, and blocking at 37°C, slides were exposed to primary antisera (1:300–1:600) overnight at 4°C. After washes in PBS (at room temperature), the incubation with secondary antibody, goat anti-rabbit conjugated with rhodamine (1:200, Jackson Immuno Research Laboratories), was done at 37°C. Nuclei were counter-stained with DAPI (1 g/ml in Vectashield mounting medium, Vector Laboratories). The slides were inspected using a Zeiss Axiophot 2 epifluorescence microscope equipped with cooled CCD camera (Photometrics). Images were captured using IPLab Spectrum software under identical exposure conditions for Ler and *kyp* for each respective antibody.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed exactly as described previously (Johnson et al. 2002). The polymerase chain reaction was used to determine the amounts of genomic DNA immunoprecipitated in the ChIP experiments. The amount of immunoprecipitate used in each assay was determined empirically such that an equal amount of *ACTIN* gene was amplified.

In vitro methyltransferase assay

The glutathione *S*-transferase (GST) fusion constructs GST:SUVH1, GST:SUVH5, and GST:SUVH6 were made by cloning SUVH1 (amino acids 336–790) into pGEX4T and full-length SUVH5 and SUVH6 into pGEX2TK. GST-DIM5 was a gift of E. Selker. Recombinant proteins were expressed and purified using a modified RIPA buffer [20 mM TRIS pH 7.7, 150 mM NaCl, 1% NP-40, Protease Cocktail-EDTA (Pierce), 0.25 mg/ml lysozyme] then washed and resuspended in superdex 75 buffer [20 mM glycine pH 9.8, 150 mM NaCl, 1 mM dithiothreitol (DTT), 5% glycerol]. Methylase assays on calf thymus histones (Roche), GST-H3 fusion proteins, and peptides (Upstate) were performed as described (Jackson et al. 2002). GST-H3 (mouse) fusion proteins were a gift of Y. Shinkai (Tachibana et al. 2001).

Mass spectrometry of in vitro methylation products

The GST-KYP and GST-SUVH6 fusion proteins were eluted from glutathione beads by 20 mM reduced glutathione in 100 mM TRIS pH 8.5. Methylation reactions were initiated by adding 10 μM unmodified H3K9 peptide substrate (residues 1–15, ARTKQ-TARKSTGGKA) to a 50 μl mixture of 50 mM glycine pH 9.8, 10 mM DTT, 1 mM AdoMet and ~10 μg of GST-KYP or ~0.5 μg GST-SUVH6 recombinant protein. After incubation at room temperature for the indicated times, the reaction was stopped by adding trifluoroacetic acid to 0.5%. Peptide masses were measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry on an Applied Biosystems Voyager System 4258 using α-cyano-4-hydroxycinnamic acid as the matrix.

Results

H3K9m and H3K9m^2 but not H3K9m^3 are found in vivo

We tested for the presence of monomethylation, dimethylation, and trimethylation at H3K9 in vivo, using immunoblot analysis of histones extracted from whole plants (Fig. 1), and a series of antibodies directed against different forms of methylated H3K9 (antibodies are described in Table 1). We found abundant H3K9m (using the α-H3K9m-TJ antibody) and H3K9m^2 (using the α-H3K9m^2-DA antibody), but did not detect H3K9m^3 (using the α-H3K9m^3-PS antibody). However, we did detect abundant H3K9m^3 in calf thymus histone control samples (Fig. 1). We repeated the immunoblotting using two additional H3K9m^2 antibodies (α-H3K9m^2-TJ, and H3K9m^2-UBI) and two additional H3K9m^3 antibodies (α-H3K9m^3-TJ and α-H3K9m^3-AB), and obtained similar results. These data suggest that bulk histones in Arabidopsis show a significant amount of monomethylation and dimethylation, but trimethylation of H3K9 is at very low levels, if present at all.

To assay the localization of H3K9m, H3K9m^2, and H3K9m^3 in vivo, we performed immunostaining of nuclei using several antibodies. Using the α-H3K9m-TJ and α-H3K9m^2-TJ antibodies we found that both H3K9m and H3K9m^2 staining are predominantly localized to heavily DAPI staining chromocenters (Fig. 2a,b). The pattern of H3K9m^2 localization is similar to that previously reported using α-H3K9m^2-UBI antibody (Jasencakova et al. 2003; Soppe et al. 2002). Using two different H3K9m^3 antibodies, H3K9m^3-TJ and H3K9m^3-PS, we found no enrichment of signal in chromocenters, and instead observed speckles evenly distributed throughout the nuclei (Fig. 2c,d). Since these antibodies did not detect H3K9m^3 signal on immunoblots of total isolated histones, we hypothesize that the immunostaining observed with these antibodies is due to the cross-reactivity of these antibodies to other non-histone proteins. Indeed, using immunoblot analysis of crude total protein preparations, we found that

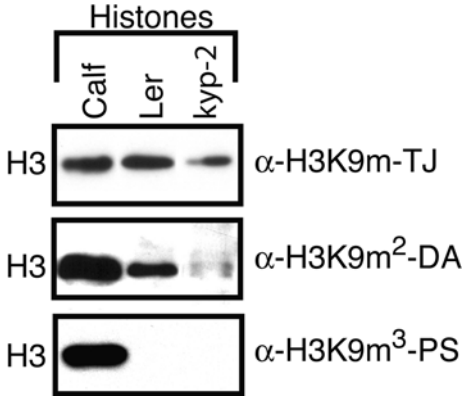

Fig. 1 Analysis of total H3K9 methylation. Immunoblot of histones isolated from wild-type *Arabidopsis thaliana* Landsberg *erecta* (*Ler*) and *kyp-2* plants, and from calf thymus histones (*Calf*) were probed with the indicated antibodies

both the α-H3K9m^3-PS antibody (Fig. 2e) and the α-H3K9m^3-TJ antibody (data not shown) cross-react with several other proteins, including the protein RuBisCO, which has been shown to be methylated at lysine 14 (Ying et al. 1999).

KYP controls the levels of H3K9m^2 and to a lesser extent H3K9m

To determine the role of *KYP* in maintaining H3K9m and H3K9m^2 we compared the levels in histones isolated from either wild-type plants or *kyp-2* mutant plants (Fig. 1). We found that *kyp-2* caused a large reduction in the amount of H3K9m^2 and a small but reproducible reduction in the levels of H3K9m. Thus, *KYP* appears to encode the major enzyme controlling H3K9m^2 in Arabidopsis.

We next tested the effect of *KYP* on the distribution of H3K9 methylation by antibody staining of nuclei isolated from either the wild type or *kyp-2* mutants. Using both the α-H3K9m^2-TJ antibody (Fig. 2b) and the α-H3K9m^2-UBI antibody (Jasencakova et al. 2003) we found that the concentrated signals of H3K9m^2 in chromocenters were abolished in the *kyp* mutants. What remained were small speckles of signal distributed evenly throughout the nucleus. Thus, KYP is a major enzyme controlling H3K9m^2 in chromocenters. In contrast, using the α-H3K9m-TJ antibody, we did not observe a difference between the staining patterns in wild-type and *kyp-2* nuclei (Fig. 2a). This suggests that KYP mainly controls H3K9m^2, and that another enzyme is responsible for H3K9m in chromocenters. The fact that we observed a small decrease in H3K9m in isolated histones, but did not see a difference using immunofluorescence, may suggest that the H3K9m lost in the *kyp* mutant is from outside the chromocenters. Alternatively, our immunofluorescence technique may not be able to detect subtle quantitative differences in signal strength. The *kyp-2* mutation did not affect the speckled pattern of staining observed with the α-H3K9m^3 antibodies (Fig. 2c,d).

Finally, we tested the effect of the *kyp-2* mutation on H3K9 and H3K9m^2 at specific loci using ChIP assays. Using primers specific to the silent *Ta3* retrotransposon, the methylated and silenced *FWA* gene, and the silent hypermethylated *SUPERMAN* gene, we found strong enrichment of both H3K9m and H3K9m^2 relative to the euchromatic gene *ACTIN* (Fig. 3). Using the α-H3K9m^2-TJ antibody, we found that levels of H3K9m^2 were strongly reduced at *Ta3*, *FWA*, and *SUPERMAN* in the *kyp-2* mutant. This is consistent with previously published results using the α-H3K9m^2-UBI antibody (Johnson et al. 2002). In contrast, using the α-H3K9m-TJ antibody, we observed only a slight reduction of H3K9m at *Ta3* and *FWA*, and a moderate reduction of H3K9m at *SUPERMAN*

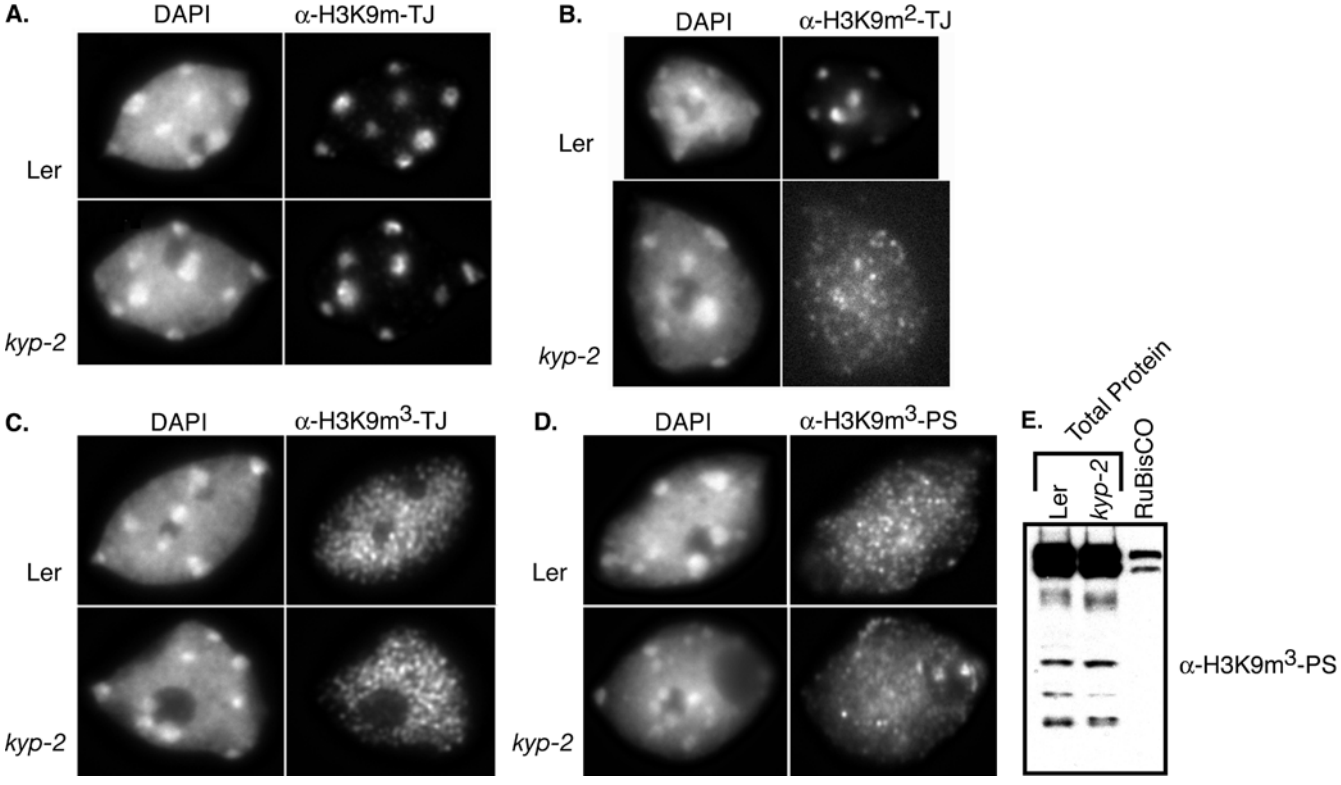

Fig. 2 Immunofluorescence staining for monomethyl, dimethyl, and trimethyl H3K9. **a**–**d** *Left panels* show 4′,6-diamidino-2-phenylindole (DAPI)-stained interphase nuclei isolated from wild-type (*top*) or *kyp-2* plants (*bottom*). *Right panels* show immunofluorescence staining with the indicated antibody. Note that the speckles of signals within euchromatin appear brighter when strong signals at chromocenters are lacking. On longer exposures, these euchromatic speckles can also be seen in samples that have brightly labeled chromocenters. **e** Immunoblot of total proteins isolated from wild-type (*left*) and *kyp-2* plants (*right*) and purified RuBisCO, indicating cross-reactivity of the α-H3K9m^3-PS antibody with non-histone proteins

in the *kyp-2* mutant (Fig. 3). These results show that at silenced loci KYP plays a greater role in maintaining levels of H3K9m^2 than in maintaining H3K9m.

Recombinant KYP can catalyze H3K9m and H3K9m^2

To characterize the enzymatic activity of KYP with regard to the number of methyl groups transferred, we used mass spectrometry to measure the results of in vitro methyltransferase assays on a histone H3 peptide substrate (residues 1–15) (Fig. 4). We found that KYP initially adds a single methyl group to lysine 9 then slowly proceeds to add a second group (Fig. 4a, b). Within 1 h, KYP had converted the majority of the unmethylated peptide to the monomethylated form. However, when the reactions were allowed to proceed for longer periods of time, a significant amount of dimethylated product was formed. The kinetics of these in vitro methyltransferase reactions suggests that KYP is a very efficient monomethylase and a moderately efficient dimethylase. However, KYP did not catalyze a detectable level of trimethylation. To confirm the lack of trimethylation activity using a different method, we tested the activity of recombinant KYP on either unmodified or H3K9 dimethylated histone H3 peptides (amino acids 1–17). *S*-adenosyl-[methyl-^{14}C]-L-methionine was included in the reactions so that enzymatically labeled peptides could be detected by fluorography. We found that KYP methylated an unmodified H3 peptide (Fig. 4c). However, the methyltransferase activity was blocked by the K9 dimethylated peptide. As a control we tested the DIM5 enzyme, which preferentially trimethylates H3K9 (Tamaru et al. 2003). DIM5 efficiently methylated both the unmodified and the dimethylated peptide (Fig. 4c). Thus our in vitro methylation data suggest that KYP can catalyze H3K9m and H3K9m^2 but not H3K9m^3.

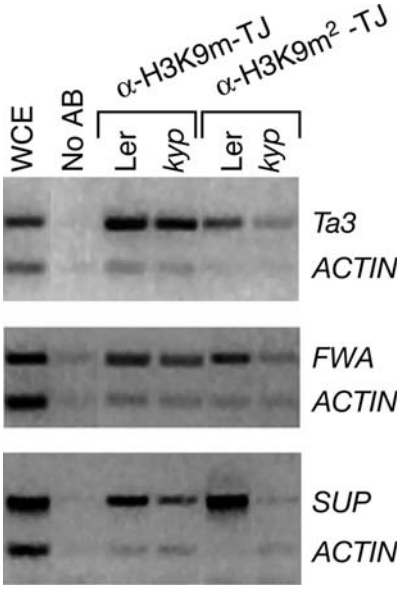

Fig. 3 Results of chromatin immunoprecipitation assays using the indicated H3K9m or H3K9m^2 antibodies. Primers specific for *ACTIN* (*lower band*) and either the *Ta3* retrotransposon (*top panel*), *FWA* gene (*middle*), or *SUPERMAN* gene (*bottom*) were used. Whole cell extract (*WCE*) with no immunoprecipitation, and no antibody (*no AB*) controls are shown

SUVH6 is an active H3K9 methyltransferase

The residual H3K9m and H3K9m^2 present in the *kyp-2* mutant [a strong loss-of-function allele (Jackson et al. 2002)] suggests that there are other active H3K9 methyltransferases in Arabidopsis. The Arabidopsis genome encodes eight proteins with a high level of sequence identity and the same basic domain architecture as KYP. These were named SUVH proteins, since they are most closely related to the Su(var)3-9 family of proteins (Baumbusch et al. 2001). We performed a limited survey of the activity of these KYP-related proteins by cloning and expressing *SUVH1*, *SUVH5*, and *SUVH6*. Each was cloned as a GST fusion protein and expressed in bacteria. The fusion proteins were purified on glutathione-Sepharose matrices and the recombinant enzymes were tested on calf thymus histones. Using an in vitro methyltransferase assay described previously (Rea et al. 2000), we found that, like KYP, SUVH6 methylates histone H3 (Fig. 5a). However, SUVH1 and SUVH5 were unable to methylate any of the five histones tested (Fig. 5a). Next we tested the specificity of SUVH6 using GST:H3 tail fusion proteins as substrates (Tachibana et al. 2001). We found that, like KYP (Jackson et al. 2002), the methyltransferase activity of SUVH6 is blocked by a mutation of residue 9 from lysine to arginine (Fig. 5b). Therefore, SUVH6 appears to be a second H3K9 methyltransferase. We tested the specificity of H3K9 methylation by using mass spectrometry to analyze the products of SUVH6 in vitro reactions (Fig. 4). We found that, like KYP, SUVH6 was a very efficient monomethylase and a moderately efficient dimethylase, but did not catalyze trimethylation. Together, these results suggest that SUVH6 and possibly other members of the Arabidopsis Su(var)3-9-related protein family are good candidates for enzymes controlling the residual H3K9 methylation observed in the *kyp-2* mutant.

Discussion

We found that the majority of methylation at H3K9 in Arabidopsis is either monomethylation or dimethylation. Using three different H3K9m^3 antibodies, we did not detect H3K9m^3 on immunoblots of total histones. Using immunostaining of nuclei, we found that H3K9m and H3K9m^2 but not H3K9m^3 were localized to chromocenters. Further, using ChIP assays, we found that H3K9m and H3K9m^2 were enriched at silent loci. Finally, we found that two Arabidopsis H3K9 methyltransferases, KYP and SUVH6, caused monomethylation and dimethylation of histone H3 peptides, but no detectable trimethylation. Thus H3K9m^3 seems unlikely to play a significant role in the maintenance of heterochromatin in Arabidopsis. Our results are consistent with an earlier study of the

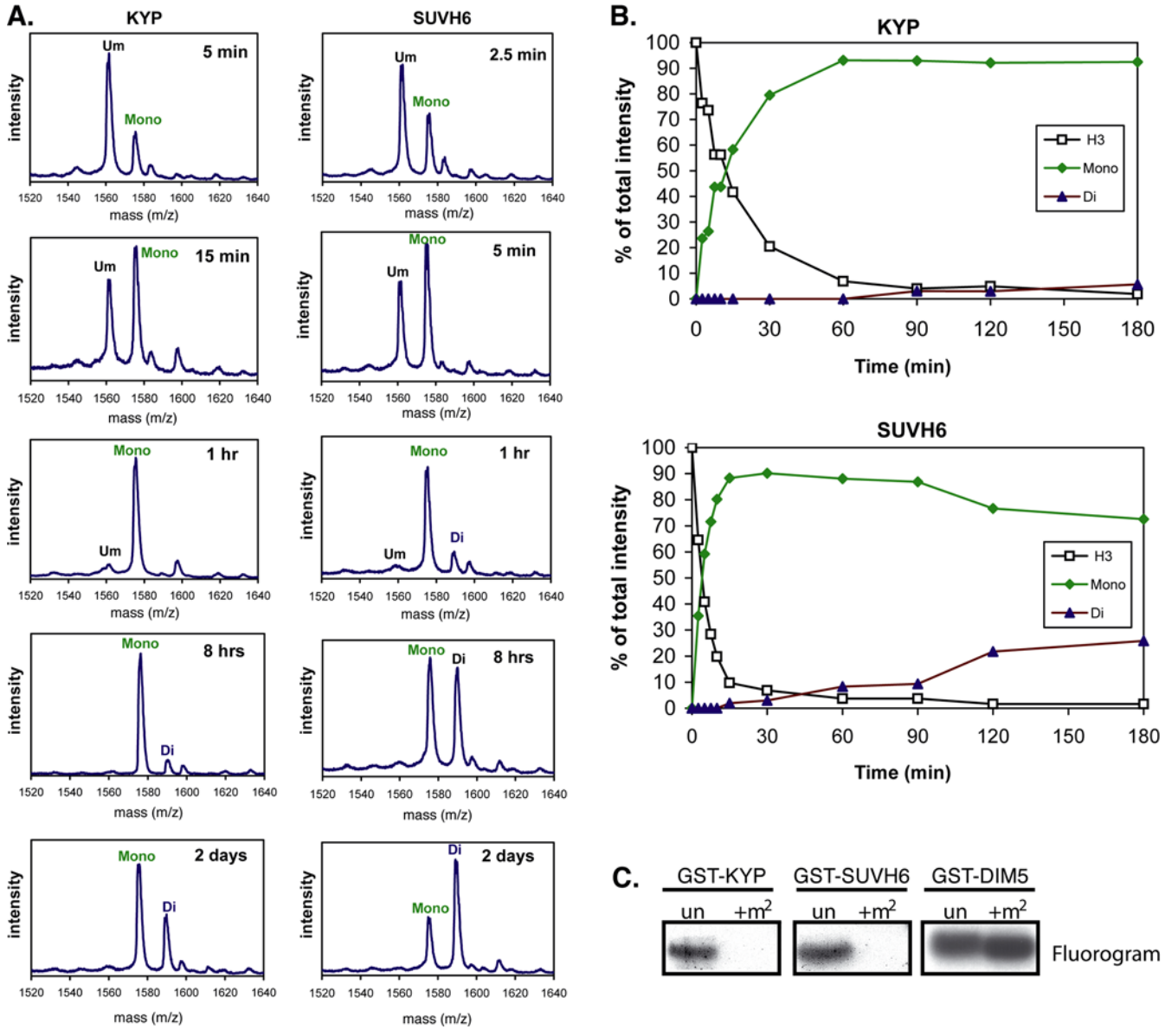

Fig. 4 Mass spectrometry analysis of methylation activity of glutathione *S*-transferase (GST) fusion proteins GST-KYP and GST-SUVH6 on unmodified H3 peptide substrate (residues 1–15). **a** Time course of methylase activity. Profiles show arbitrary intensity (intensity) vs mass. Time of measurement is shown in *upper right*. **b** Relative intensity of each mass was plotted vs time. Mass identities of the H3 peptide with different K9 methylation status are indicated. Reactions were stopped after incubation for the times shown. **c** Fluorogram of methylase assays using unmethylated (*un*) H3 peptide (1–17) or dimethylated ($+m^2$) peptide as substrate

steady state levels of H3K9 methylation in alfalfa histones. In particular using automated protein sequencing, it was found that the majority of H3K9 methylation found in alfalfa is H3K9m or H3K9m^2 (Waterborg 1990). H3K9m^3 was not found in the major histone H3 variant in alfalfa, H3.1, and was found in only a very small percentage of the minor variant, H3.2. In fact, the author of this study points out that data for H3K9m^3 were difficult to obtain and accurately quantitate (Waterborg 1990). Therefore, it is possible that there is an insignificant level of H3K9m^3 methylation in plants.

H3K9m^2 is the critical mark for gene silencing

Immunostaining of nuclei showed that both H3K9m and H3K9m^2 are strongly enriched at DAPI-staining chromocenters, and ChIP assays show that both H3K9m and H3K9m^2 are preferentially localized to the silent retrotransposon *Ta3* and the silent *FWA* and *SUPERMAN* genes. However, a comparison of wild-type and *kyp* mutant plants showed that only H3K9m^2 is drastically reduced by *kyp* mutations. H3K9m levels were decreased only to a small extent as measured by immunoblot or ChIP assays, and not at all as measured by immunofluorescence of chromocenters. Since the majority of H3K9m remains in the *kyp* mutant, which shows derepression of normally

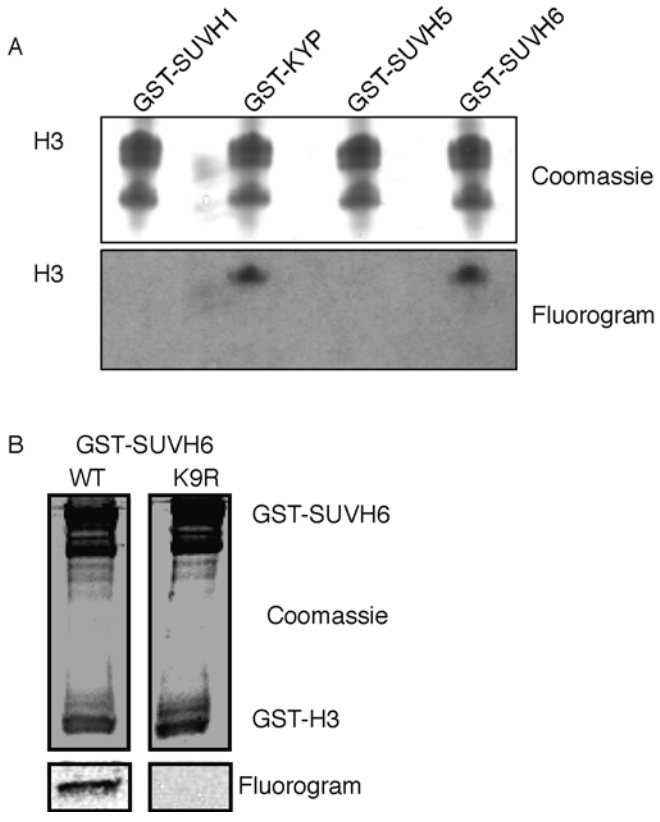

Fig. 5 Analysis of four Su(var)3-9 family members in *Arabidopsis thaliana*. **a** Methyltransferase activity of SUVH1, KYP (SUVH4), SUVH5 and SUVH6 on calf thymus histones. Coomassie Blue staining of histones and recombinant proteins (*top*) and fluorogram of ^{14}C-labeled methyl groups transferred to histone H3 (*bottom*). Position of histone H3 is marked. **b** SUVH6-GST (glutathione-S-transferase) fusion protein activity on recombinant GST:histone H3 tails (1–57) that were either unmodified (*WT*) or had a substitution of arginine for lysine at position 9 (*K9R*). *Top panel* shows Coomassie Blue staining of the GST-SUVH6 and GST-H3 fusion proteins in the reaction. *Bottom panel* shows fluorogram of ^{14}C-labeled methyl group transferred to wild-type GST-H3 fusion protein, but not the mutant fusion protein

silent genes, these data suggest that H3K9m alone is not sufficient for gene silencing and DNA methylation. Rather, these data suggest that H3K9m^2 is the necessary mark for gene silencing in *Arabidopsis*.

Our results are in stark contrast to findings in animal and fungal systems. For instance immunolocalization studies in mammals and *Drosophila* show that H3K9m^3 marks heterochromatin and to a large extent co-localizes with the binding of HETEROCHROMATIN PROTEIN1 (Cowell et al. 2002; Peters et al. 2003; Rice et al. 2003). Additionally, in Neurospora, it is H3K9m^3, not H3K9m^2 that is present at high levels at silent loci, and that seems to be required for the maintenance of DNA methylation (Tamaru et al. 2003). In contrast to the plant enzymes KYP and SUVH6, which do not show trimethylation activity, the DIM5 enzyme of Neurospora is a very efficient H3K9 trimethylase (Zhang et al. 2003). Thus, while the general phenomenon of H3K9 methylation controlling DNA methylation is found in both Arabidopsis and Neurospora, there are differences that define an interesting divergence between species. The first is that while H3K9 methylation is required for all DNA methylation in Neurospora, it is only required for the non-CpG methylation in Arabidopsis, especially CpNpG methylation. The second is that while H3K9m^3 is required for DNA methylation in Neurospora, H3K9m^2 is required in Arabidopsis.

The finding that both H3K9m and H3K9m^2 are present at silent loci implies that a combination of marks may be necessary for proper heterochromatinization. In support of this idea, *kyp* mutants cause a release of epigenetic gene silencing (Jackson et al. 2002), but DAPI-staining chromocenters remain intact (Jasencakova et al. 2003). Thus the overall compaction of chromatin into chromocenters can still occur despite the loss of H3K9m^2. One speculation therefore is that, while H3K9m^2 is critical for the maintenance of silencing, H3K9m may be more important for the compaction of constitutive heterochromatin.

The finding that the *kyp-2* mutation results in greater reduction of H3K9m^2 in vivo than of H3K9m is somewhat surprising given the in vitro activity of the gene product, since KYP was much more efficient at monomethylation than dimethylation. One possible explanation for this is that KYP could be targeted to and persistently localized to regions of heterochromatin. This stable localization could allow for increased local concentrations of KYP and therefore higher concentrations of H3K9m^2. A second possibility is that a cofactor changes the enzyme dynamics of KYP in vivo, thus allowing it rapidly to add a second methyl group to histones found in heterochromatin. An example of this phenomenon is the mAM protein, which is required for the conversion of H3K9m^2 to H3K9m^3 by the SET domain protein ESET/SETDB1 (Wang et al. 2003). Finally, it is possible that the in vitro conditions used for these reactions are suboptimal, and do not accurately mimic the in vivo capacity of KYP for dimethylation.

Multiple methylases control H3K9 methylation in Arabidopsis

Despite the existence of eight genes in the Arabidopsis genome that are similar to *KYP* in sequence identity and domain architecture, the *kyp* mutant eliminates the majority of H3K9m^2, showing that KYP is the predominant H3K9m^2 methylase. It is interesting to note that *KYP* is the only one of the nine genes in this family that contains introns within its coding region. This suggests that KYP was the ancestral member of the gene family, and that other paralogous genes may have evolved by gene duplication, and taken on more specialized roles.

However, examination of the levels of methylation in isolated histones by immunoblot analysis showed that the *kyp-2* mutation did not completely eliminate H3K9m^2. This suggests that at least one additional H3K9m^2 methyltransferase is active in Arabidopsis. This remaining H3K9m^2 could be present at silent loci, but be undetectable by the immunofluorescence and ChIP methods used in this study. Alternatively, the remaining

H3K9m^2 could be present at genes or intergenic regions in euchromatin. This later situation would be analogous to that found in mammals where one type of methylase (Suv39H1 and Suv39H2) controls H3K9 methylation at pericentromeric heterochromatin, while a second SET domain protein, G9a, controls H3K9 methylation in euchromatin (Peters et al. 2003; Rice et al. 2003; Tachibana et al. 2002). Further, the finding that the majority of H3K9m is retained in a *kyp* mutant background suggests that at least one additional H3K9 monomethylase is encoded in the Arabidopsis genome. Our limited survey of the activity of KYP-related proteins identified SUVH6 as an enzyme with a catalytic profile similar to KYP. SUVH6 specifically methylated H3K9, and caused monomethylation and dimethylation but not trimethylation. Therefore, future genetic analysis of SUVH6 and other Su(var)3-9 family members may provide further insight into the functions of H3K9m and H3K9m^2 in Arabidopsis.

Acknowledgments We thank L. Cahoon, E. Huang, J. Bruder, and C. Hyun for technical assistance, A. Meister for flow-sorting of nuclei, E. Selker and H. Tamaru for providing the GST-DIM5 fusion construct, and members of the Jacobsen laboratory for discussions and critical review of the manuscript. This work was supported by NIH grant GM60398 (S.E.J.), NIH training grant GM07104 (J.P.J.), grants from the Land Sachsen-Anhalt (3233A/0020L) and DFG (Schu 951/8-2) (Z.J. and I.S.), and NIH grants GM49245 and GM61355 (X.Z. and X.C.).

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