Mass spectrometry analysis of *Arabidopsis* histone H3 reveals distinct combinations of post-translational modifications

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ABSTRACT

Chromatin is regulated at many different levels, from higher-order packing to individual nucleosome placement. Recent studies have shown that individual histone modifications, and combinations thereof, play a key role in modulating chromatin structure and gene activity. Reported here is an analysis of Arabidopsis histone H3 modifications by nanoflow-HPLC coupled to electrospray ionization on a hybrid linear ion trap-Fourier transform mass spectrometer (LTQ/FTMS). We find that the sites of acetylation and methylation, in general, correlate well with other plants and animals. Two well-studied modifications, dimethylation of Lys-9 (correlated with silencing) and acetylation of Lys-14 (correlated with active chromatin) while abundant by themselves were rarely found on the same histone H3 tail. In contrast, dimethylation at Lys-27 and monomethylation at Lys-36 were commonly found together. Interestingly, acetylation at Lys-9 was found only in a low percentage of histones while acetylation of Lys-14 was very abundant. The two histone H3 variants, H3.1 and H3.2, also differ in the abundance of silencing and activating marks confirming other studies showing that the replication-independent histone H3 is enriched in active chromatin.

INTRODUCTION

Gene expression is intricately linked to chromatin structure, whose foundation in eukaryotic cells is the nucleosome. The nucleosome consists of 146 bp of DNA wrapped around a histone octamer (two each of histones H2A, H2B, H3 and H4) (1). Recent evidence suggests that this octamer is assembled first as two dimers of H3/H4 on the DNA, followed by addition of two dimers of H2A/H2B (2). This assembly happens both during replication (3) and outside of replication primarily during active transcription (4,5). Variation in the composition of the nucleosome octamer by incorporation of histone variants and in post-translational modifications of the unstructured N-terminal tails of the individual histones allows fine-tuning of the chromatin structure at several different levels. First, higher-order packing or condensation of the chromatin can be modulated both at specific times in the cell cycle (mitotic condensation) and continuously through the cell cycle (heterochromatic regions) (6). Second, the strength of interaction between the DNA and the histone octamer can be altered, influencing the sliding or movement of nucleosomes and thereby affecting gene regulation (7–10). Finally, specific modifications have been shown to serve as binding sites for proteins involved in silencing or activation while others have been shown to inhibit binding of certain proteins (11–14).

Over the past several years, many studies examining histone modifications and their role in gene expression have been reported [reviewed in (15)]. Modifications on the histone H3 N-terminus that have been associated with transcriptional activation are methylation of Lys-4 (K4), acetylation of Lys-9 (K9), acetylation of Lys-14 (K14), methylation of Arg-17 (R17), acetylation of Lys-18 (K18) and acetylation of Lys-23 (K23). On the other hand, methylation of K9 and methylation of K27 have been correlated with gene silencing or heterochromatin. Phosphorylation of Ser-10 (S10) plays a role in chromatin condensation during mitosis as well as an involvement in transcriptional activation (12,16). The role of methylation of K36 remains somewhat elusive; it has been reported as both an active mark and a repressive mark with respect to transcription (17,18).

Recent results have indicated that the combination of modifications on a single nucleosome may be the key in determining chromatin structure and function. For example,

in Drosophila methylation at K9 of H3 is not a mark for silencing when found on the same nucleosome as methylation at K4 of H3 and K20 of H4 (19). In Arabidopsis, methylation at both K9 and K27 is required for binding of the DNA methyltransferase CMT3 (20). In addition to the specific sites of modification, methylation of lysines can exist as monomethylation, dimethylation or trimethylation (21). Therefore, not only are the combinations of these modifications on a single nucleosome important for regulating chromatin structure and function, but also the number of methyl groups added at a particular site (11,12,14,22).

Arabidopsis thaliana is an excellent system to analyze histone variants and their modifications given its facile forward and reverse genetics along with its small and completely sequenced genome. There are two predominant histone H3 variants in the genome in addition to the centromeric histone CenH3: H3.1 (five copies) and H3.2 (three copies). H3.1 is similar to the S phase-dependent variant found in animals and H3.2 is similar to the replacement histone H3.3 expressed throughout the cell cycle (23). These two proteins differ in only a few amino acids (24), however in HeLa cells H3.1 is found exclusively associated with the CAF-1 complex involved in nucleosome assembly during DNA replication while H3.3 is found exclusively associated with the HIRA complex involved in assembly outside of S phase (2). This is consistent with the finding that the H3.3 replacement histone is found primarily in actively transcribed regions, whereas the H3.1 may be found in all regions but at higher concentration in heterochromatic loci (5,25).

The Arabidopsis genome encodes homologs of most major classes of histone methyltransferases and acetyltransferases found in animals (http://www.chromdb.org). From a screen for mutants that cause derepression of a silent gene called *SUPERMAN*, mutant alleles were isolated in the gene *KRYPTONITE* (*KYP*), one of the histone methyltransferases homologous to Su(var)3-9 (a histone H3 K9 specific methyltransferase) (26). In a similar independent screen, additional alleles of this gene were also identified (27). Using antibodies, we showed that *kyp* mutants reduce dimethylation (but not monomethylation) of H3 K9 at heterochromatic loci (28,29). However, it is not clear whether there is residual methylation at K9 which could be controlled by one of the other Su(var)3-9 homologs in Arabidopsis or what effect this change might have on neighboring modifications.

In this report, we describe the first characterization of histone H3 N-terminal modifications in Arabidopsis using mass spectrometry, eliminating problems inherent in using antibodies [differences in strength, specificity, interference by neighboring modifications and cross-reaction with other proteins (12,29)]. In addition, mass spectrometry allows us to determine multiple modifications on a given fragment. We find certain modifications are essentially mutually exclusive on the same N-terminus, whereas others can be found together. In addition, we find that the replication-dependent histone H3 (H3.1) is enriched in modifications associated with silencing while the replication-independent histone H3 (H3.2) has a lower abundance of the silencing modifications and higher abundance of methylation at K36. Finally, we compare histones isolated from plants containing the mutant *kyp* to wild-type plants and observe

a 3- to 4-fold decrease in dimethylation and trimethylation at H3 K9.

MATERIALS AND METHODS

Histone isolation for western blots and mass spectrometric analysis

Crude histone preparations were made from 1 g of inflorescences from *Arabidopsis thaliana* Landsberg *erecta* (L*er*) containing the *SUPERMAN* inverted repeat (*clk-st*) (30) and *kyp* plants (26) as described previously (31). In addition to the guanidine hydrochloride used in the lysis buffer to inhibit proteases, deacetylases, phosphatases, etc., the following inhibitors were added: PMSF, pepstatin, complete miniTab (Roche), NaOrthovanadate, hydrogen peroxide, calyculin and sodium butyrate. The histone-enriched extract was then used directly for dot blot or western blot analysis. Primary antibodies specific for AcK9 were obtained from Upstate Biotechnology (Catalog #06-942, Lot #21897). Dot blots containing three levels of AcK9 peptide, AcK14 peptide (both at 50, 10 and 2 pmol) and L*er* nuclear extract (5, 2.5 and 1.2 μg) were probed with either α-AcK9 antibodies preincubated with either a 10× molar excess of AcK14 peptide or AcK9 peptide. For the western blot, proteins were separated by electrophoresis on 15% SDS–PAGE and then transferred to PVDF membrane in CAPS/methanol buffer. Two identical blots were prepared and probed with either α-AcK9 antibodies or α-AcK9 antibodies preincubated with a 10× molar excess of AcK9 peptide.

Purification of histone H3 from the crude histone sample was accomplished by HPLC with a C8 column (250 mm × 4.6 mm i.d., Keystone Scientific, Inc.) on an Agilent 1100 series instrument (Palo Alto, CA). A gradient of 0–100% B in 100 min (solvent A = 5% MeCN in 0.1% TFA, solvent B = 90% acetonitrile in 0.085% TFA, flow rate = 0.8 ml/min) was employed for the separation. Fractions were collected every minute and then analyzed by gel electrophoresis to locate those that contained histone H3. Gel electrophoresis experiments were performed for 1 h at 150 V using 18% Tris–HCl gels (Bio-Rad, Hercules, CA). Protein bands were visualized by staining with Coomassie blue for 25 min. Histone H3 proteins from pooled HPLC fractions were digested with Glu-C protease and fragments containing residues 1–50 were purified by HPLC, derivatized by propionylation, and then digested with trypsin (enzyme:protein = 1/15) in 100 mM NH_4HCO_3 (pH = 8) for 8 h at 37°C, as described previously (32). Newly generated N-termini were propionylated and the reaction mixture was then lyophilized. Derivatized peptides from the L*er* sample were converted to d_0–ethyl esters with 100 mM solution of HCl in d_0-ethanol (32). Peptides from the *kyp* sample were converted to d_5-ethyl esters with 100 mM HCl in d_6-ethyl alcohol. Both reactions were allowed to proceed at room temperature for 1 h. Solvents were then removed by lyophilization. Esterification procedures were repeated a second time with fresh reagents and lyophilized peptide ethyl esters were reconstituted in 50 μl of 0.1% acetic acid. Aliquots (10 μl) of each solution were mixed to prepare samples for comparative analysis by mass spectrometry (Figure 5).

Mass spectrometry

Mixtures of derivatized tryptic peptides from histone H3 proteins were analyzed by nanoflow HPLC/micro-ESI (micro electrospray ionization) interfaced to a Finnigan LTQ-FT mass spectrometer (Thermo Electron Corp.) (32). Spectra were acquired with the instrument operating in the data dependent mode of operation. Every 3 s, the LTQ-FT acquired a full mass spectrum with the FTMS as the analyzer, a full mass spectrum with the linear ion trap as the analyzer, and MS/MS spectra on the ten most abundant peptide ions in the full MS scan. The linear ion trap was used as the analyzer for this latter experiment. All MS/MS spectra were interpreted manually. Mass measurements made with the FTMS in the full mass spectrum were all within 2–3 p.p.m. (parts per million) of the theoretical masses. Since the fragment containing residues 41–50 of H3 is not known to contain post-translational modifications, ion abundances observed for this fragment were used to normalize all other fragment ion abundances observed in the L*er* and *kyp* samples.

As phosphorylation was not initially observed, we enriched our sample for phosphopeptides by using IMAC (immobilized metal affinity chromatography). Then we looked for the parent ion with an addition of 80 Da and for abundant fragment masses in the MS/MS spectra resulting from a neutral loss of phosphoric acid (MH^+ −98 or MH^{+2} −49) from the parent mass.

RESULTS

Analysis of N-terminal modifications on Histone H3

It has been noted previously that Arabidopsis histone variants H3.1 and H3.2, unlike Alfalfa variants, are not separable by HPLC due to a sequence difference at position 90 (23). Hence, we proceeded to purify and analyze a mixture of the two variants. To isolate specific peptides from the N-terminus for analysis by mass spectrometry, histone H3 proteins were digested with the protease Glu-C and the N-terminal peptides containing residues 1–50 were then separated and derivatized by propionylation as described in Materials and Methods. Propionylation of the peptide N-terminus and the epsilon amino groups on lysine residues blocks trypsin from cleaving residues 1–50 on the C-terminal side of lysine. As a result, proteolysis with trypsin occurs only C-terminal to arginine (Figure 1), and generates fragments containing the following residues: 3–8, 9–17, 18–26, 27–40 and 41–50. Peptides containing residues 3–8 elute early in the HPLC gradient and are often detected with low efficiency.

Methylation of K9 and acetylation of K14 are essentially mutually exclusive

Three well-characterized modifications are found on the 9–17 fragment: methylation at K9 (normally associated with silent chromatin) and acetylation at K9 and K14 (normally associated with active chromatin). We found that methylation of H3 K9 was predominantly monomethyl K9 (20% 1MK9) or dimethyl K9 (10% 2MK9; Figure 2A). Trimethylation at K9 (3MK9) was 100-fold lower than 1MK9. This is consistent with analysis of heterochromatic chromocenters using antibodies to 1MK9, 2MK9 and 3MK9 (29) and with the
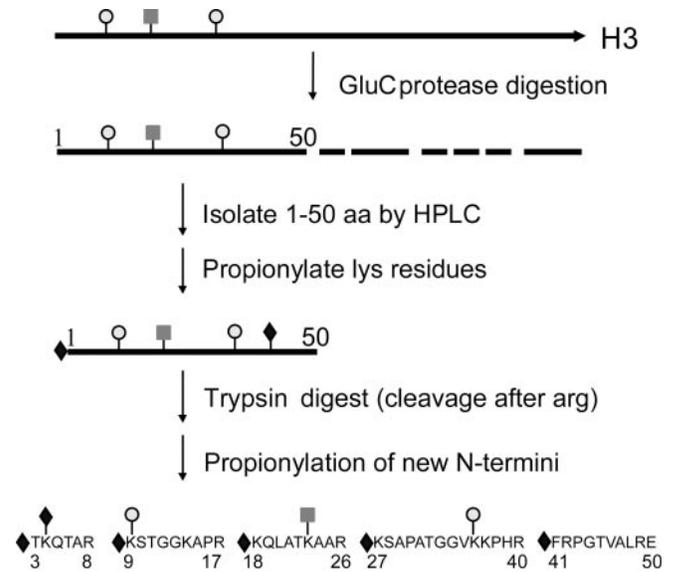

Figure 1. Cleavage of histone H3 for analysis by mass spectrometry. Schematic representation of the protocol used to generate tryptic peptides from residues 1–50 of histone H3. The resulting fragments contained residues 3–8, 9–17, 18–26, 27–40 and 41–50. Circles and squares above the line represent hypothetical sites of methylation and acetylation, respectively. Diamonds indicate that peptide N-termini and lysine epsilon amino groups have been derivatized with a C_2H_5CO-moiety (propionylation). Monomethyl lysines are also propionylated, but dimethyl-, trimethyl- and acetylated lysines are not.

estimates for the amount of heterochromatin found in Arabidopsis (33).

Interestingly, acetylation appears to occur primarily at K14 (~30% of the 9–17 peptide isoforms). Fragments containing residues 9–17 that are mono-acetylated at either K14 or K9 co-elute into the mass spectrometer. However, MS/MS spectra were used to estimate the abundances for each species and we conclude that ratio of the AcK14/AcK9 peptides in the chromatic peak is 9/1 (data not shown). The abundance of diacetylated species (AcK14 & AcK9) was ~30-fold lower than that of the singly acetylated species. We also observed very low levels of peptides modified by methylation at K9 along with acetylation at K14 (Figure 2A). We did not observe peptides that contained S10 phosphorylation. These may have been destroyed by phosphatases during histone purification or may have been below our level of detection because they are only produced transiently during the cell cycle (21,23).

Although the very low levels of acetylation at K9 that we observed are consistent with what was found in Alfalfa (34), this site is of particular interest since acetylation at K9 could compete with methylation preventing gene silencing (35). In addition, several studies have detected AcK9 in Arabidopsis using commercially available antibodies (36,37). Hence, we tested the presence of AcK9 in our preparations using antibodies. We first determined the specificity of the antibodies by dot-blots using an excess of AcK9 or AcK14 peptide as competitor. We found that α-AcK9 antibodies indeed recognized peptides acetylated at K9 and not K14, and interaction with the peptide was competed by preincubation with AcK9 peptide but not AcK14 peptide. However, we found that they also cross-react with other nuclear proteins in Arabidopsis that
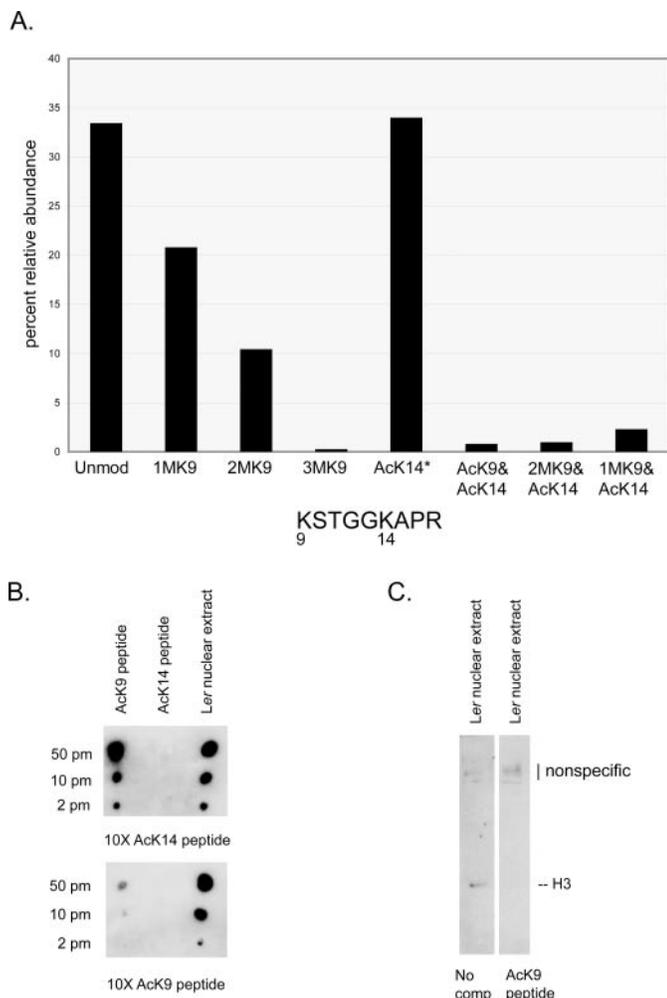
A.

KSTGGKAPR
9   14

B.

10X AcK14 peptide

10X AcK9 peptide

C.

Figure 2. Analysis of peptide isoforms containing residues 9–17 of histone H3. (**A**) Percent relative abundances observed for eight different isoforms of the tryptic peptide containing residues 9–17 of histone H3. The amino acid sequence for residues 9–17 is shown below the graph with K9 and K14 labeled. The eight isoforms include the unmodified sequence plus peptides containing mono-, di- and tri-methylated K9, acetyl-K14, acetyl-K9 plus acetyl-K14, dimethyl-K9 plus acetyl-K14 and monomethyl-K9 plus acetyl-K14. Percent relative abundance data was generated by mass spectrometry and is calculated by dividing the ion current (peak area) observed for each peptide by the total ion current (peak areas) for all fragments containing residues 9–17. *Peptide isoforms containing a single site of acetylation at K14 or K9 could not be separated by HPLC and co-eluted into the mass spectrometer. Analysis of the MS/MS spectra recorded on the mixture indicated that the two peptides were present in the following ratio; AcK14/AcK9 = 9/1. (**B**) Dot blots determining specificity of α-AcK9 antibodies using AcK9 peptide (1–12), AcK14 peptide (8–19), and L*er* nuclear extract using either AcK9 or AcK14 peptide as a competitor. (**C**) Western blot of L*er* nuclear extract probed with either α-AcK9 antibodies or α-AcK9 antibodies preincubated with AcK9 peptide competitor.

are not competed out by excess peptide (Figure 2B). Western blots of Arabidopsis nuclear proteins show clear recognition of histone H3 as well as higher molecular weight proteins (Figure 2C). Only the H3 band is competed out by prior incubation of the antibody with a 10× molar excess of AcK9 peptide indicating that there are detectable levels of AcK9 in Arabidopsis. It is not possible by western blotting to calculate a percentage of histones modified by acetylation at K9, but
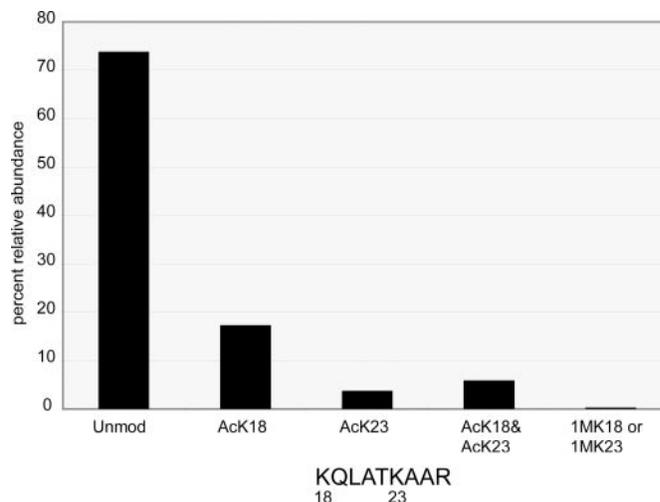

KQLATKAAR
18   23

Figure 3. Analysis of peptide isoforms containing residues 18–26 of histone H3. Percent relative ion abundances observed for five different isoforms of the tryptic peptide containing residues 18–26, KQLATKAAR. Low ion abundance for the monomethylated peptide isoform made it impossible to assign the site of modification as either 1MK18 or 1MK23.

antibodies are more sensitive than mass spectrometry, so even if the level is only a few percent of the total histone H3, it is still easily detectable. Nonetheless, AcK14 seems to be the predominant mark on histone H3, not AcK9.

Acetylation is observed at both K18 and K23

For the H3 fragment containing residues 18–26, we observed the unmodified peptide plus isoforms acetylated at K18 (AcK18), K23 (AcK23) and both K18 and K23 (AcK18 & AcK23) (Figure 3). Monomethylated fragments containing residues 18–26 were also detected but at levels <0.5% of that for the acetylated. Assignment of the methylation site to K18, K23 or a mixture of both was not possible in this analysis.

Histone H3 variants differ in the extent of methylation at K27 and K36

Histone H3.1 and H3.2 differ by four amino acids located at amino acids 31 (A versus T), 41 (F versus Y), 87 (S versus H) and 90 (A versus L). Although the two variants cannot be separated by HPLC, the sequence differences in fragments 27–40 and 41–50 allowed us to distinguish between the variants by mass spectrometry. The only modifications observed on the 27–40 fragment of these two variants involved methylation at K27 and K36 (MK27, MK36 and MK27 & MK36). Acetylation was not detected at either of these sites. As shown in Figure 4, the percent relative abundance of methylated isoforms derived from the two variants differ significantly. H3.1 has a mixture of peptide isoforms dominated by those methylated at K27 (60% 1MK27, 16% 2MK27 and 5% 3MK27), no detectable 1MK36 or 2MK36 and 3% 3MK36. Fragments methylated at both K36 and K27 were also observed (~3% 1MK36 & 2MK27 and 3% 2MK36 & 1MK27, see Figure 4A). In contrast, the peptide isoforms observed for H3.2 show lower levels of methylation at K27 (36% 1MK27 and 6% 2MK27 with no detectable 3MK27) and higher levels of methylation at
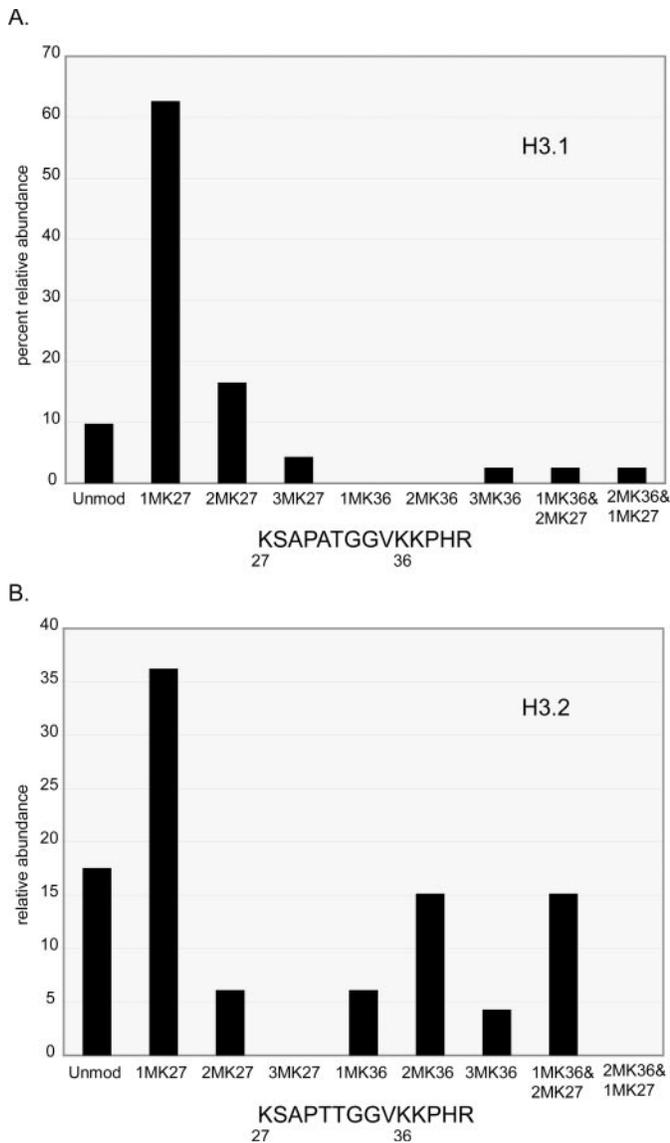
Figure 4. Analysis of peptide isoforms containing residues 27–40 of histone H3.1 and H3.2. Percent relative ion abundances observed for nine peptide isoforms of the tryptic peptide containing residues 27–40, (**A**) KSAPATGGVKKPHR from histone H3.1 and (**B**) KSAPTTGGVKKPHR from histone H3.2.

K36 (6% 1MK36, 15% 2MK36, 3% 3MK36). In addition, 15% of the peptide isoforms had two modifications, 2MK27 & 1MK36 (see Figure 4B).

Di- and tri-methylation of K9 is reduced in the *kyp* mutant

K9 methylation has been shown to be catalyzed by the KYP histone methyltransferase *in vitro* and mutations in KYP result in a decrease 2MK9 *in vivo* (26,29). However, quantitation of the changes observed *in vivo* was not possible in these studies. To compare post-translational modifications on histone H3 proteins isolated from wild-type and *kyp* mutant plants, we digested propionylated fragments containing residues 1–50 and then tagged the resulting peptides from each source with
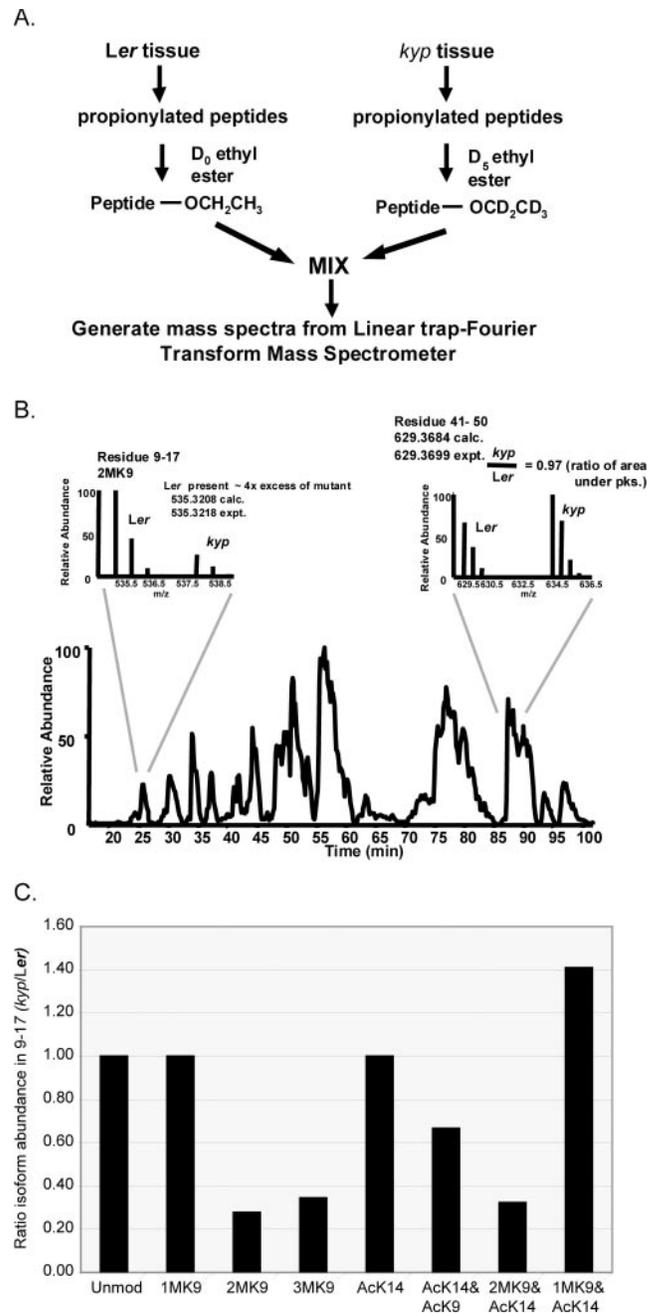

Figure 5. Comparative analysis H3 peptide isoforms (residues 9–17) derived from wild-type (L*er*) and mutant (*kyp*) Arabidopsis tissues. (**A**) Diagram of the method employed to derivatize and isotopically label peptides isolated from the above two sources. H3 peptides from the *kyp* mutant were converted to d5-ethyl esters and those from L*er* (wild-type) were converted to d0-ethyl esters. A mixture containing equal amounts of these two samples was analyzed by mass spectrometry. (**B**) Total ion chromatogram and individual mass spectra that highlight the ion abundances observed for the 2MK9 and 41–50 peptides isolated from the two plant tissues. Theoretical and experimentally measured masses for the doubly charged 2MK9 and 41–50 peptides obtained from the L*er* sample are also shown. (**C**) Plot of ion abundance ratios (*kyp*/L*er*) observed for peptide isoforms containing residues 9–17.

different isotopic labels (Figure 5A). H3 peptides from the *kyp* mutant were converted to d5-ethyl esters and those from L*er* (wild-type) were converted to d0-ethyl esters. A mixture containing equal quantities of the above two samples was analyzed

nano-flow HPLC interfaced to a linear-trap-Fourier transform mass spectrometer. By converting H3 fragments derived from Ler and *kyp* to d_0- and d_5-ethyl esters, respectively, signals for peptides containing identical post-translational modifications appear as doublets in the resulting mass spectra (Figure 5B). Since we have yet to detect a post-translational modification on the peptide containing residues 41–50, the ratio of ion abundances observed for d_0- and d_5-ethyl esters of this fragment (*kyp*/Ler = 0.97) was used to normalize the ratio of ion abundances for all other fragments.

Plotted in Figure 5C is the ratio of ion abundances (*kyp*/Ler) observed for peptide isoforms containing residues 9–17 of histone H3. Ratios of unmodified and 1MK9 peptide isoforms were the same in the two samples. Levels for the 2MK9, 3MK9 and 2MK9 & AcK14 peptide isoforms were found to be reduced in the *kyp* sample by factors of 4, 3 and 3, respectively. Note, that the percent relative abundance of the 3MK9 isoform is considerably lower than the other methylated 9–17 peptides (Figure 2A). These results confirm our analysis of *kyp* H3 isoforms using antibodies (28). Evidence for the presence of peptides containing additional nearby modifications (phosphorylation of S10) that would block antibody access to the K9 methylated peptides was not observed.

DISCUSSION

The combination of different post-translational modifications on histones plays an important role in regulating chromatin activity and providing epigenetic marks that are carried through cellular generations (13,22). Many of these modifications have been analyzed using highly specific antibodies in chromatin immunoprecipitations, immunofluorescence or western blot analyses. Although the sensitivity of these techniques is high, it can be difficult to interpret the data without prior knowledge of the overall levels and presence of specific modifications. In addition, certain combinations of modifications may interfere with antibody detection, and cross-reactivity with non-histone proteins can complicate the results. Mass spectrometry is being used increasingly to determine the exact sites of modifications as well as the abundance of different species (38). We provide here the first description of modifications found on the Arabidopsis histone H3 N-terminus as determined by mass spectrometry. This analysis allows the determination of sites and types of modifications, their relative abundance, and specific combinations of modifications found on individual fragments.

Overall, we found that the major sites of methylation were K4, K9, K27 and K36, with minor levels detected at K18 and K23. Major sites of acetylation were K14 and K18, with minor sites detected at K23 and K9. No acetylation was observed at K27 or K36, and no methylation was detected at K14. No phosphorylation was detected at S10 or S28, though it is possible that phosphorylated serine residues suffered hydrolysis during the isolation protocol despite the use of inhibitors or were present only transiently and in amounts too low to detect by mass spectrometry. Arginine methylation was not analyzed in the present experiments.

The only other plant species that has been analyzed using a method that does not rely on antibodies is Alfalfa (*Medicago sativa*), where the two histone variants were separated by RP-HPLC and analyzed by Edman degradation (34). Consistent with our observations, only low levels of acetylation at K9 were detected whereas acetylation at K14 was abundant. In addition, a clear increase in the levels of silencing modifications (MK9 and MK27) and a decrease in the levels of active modifications (MK4, AcK14, AcK18 and AcK23) were observed on H3.1 compared to H3.2. This difference in the levels of specific modifications between histone variants has also been observed recently in Drosophila (39) and lends support to the model that the replication-independent variant H3 is enriched in actively transcribed regions of the genome (5,34,40).

Interestingly, we found that on a single histone H3 N-terminus some modifications were rarely found together and others were commonly found together. Of particular significance was the observation that amino acids 9–17 could be modified by either methylation at K9 or acetylation at K14, but only in 3% of the H3 proteins did we find both methylation at K9 and acetylation at K14. Several models come to mind to explain these results. One simple model is that the histones are found in different compartments, euchromatic versus heterochromatic, and that differential targeting of modifying enzymes can account for this seeming exclusivity. Alternatively, preexisting modifications may affect the ability of a modifying enzyme to bind to the histone tail. *In vitro* studies of SUV39H1 showed little difference between K9 methylation of an unmodified peptide versus a peptide acetylated at K14 (41), however mutation of the K14 deacetylase *clr3* in *S.pombe* has been shown to result in a decrease in methylation of K9 as well as a loss of silencing (42). In Arabidopsis, mutations in two different histone deacetylases have recently been identified that implicate deacetylation in gene silencing, however the specificities of these enzymes have not yet been determined (36,43–45).

Alternatively, the K14 acetyltransferase may be affected by prior methylation at K9. GCN5 has been identified as the major acetyltransferase specific for K14 in other systems (46–48). Structure analysis has revealed that contacts between GCN5 and histone H3 occur over a 12 amino acid stretch including K9 (from Arg-8 to Gln-19) (49). Although it has not been determined if methylation of K9 directly interferes with acetylation at K14, there is a substantial amount of evidence supporting a less direct model for how K9 and K14 can influence one another. It has previously been shown that phosphorylation at S10 enhances acetylation of K14 by reducing the K_m of the phosphorylated substrate (50,51). Structural studies indicate an increased interaction between the phosphorylated histone H3 and the GCN5 acetyltransferase (49). In addition, phosphorylation at S10 has also been shown to inhibit methylation at K9 by SUV39H1 *in vitro* (41). This suggests a model whereby phosphorylation of S10 increases acetylation at K14, and inhibits methylation at K9 leading to the opposed marks on H3 N-termini, one primed for silencing and the other for activation (16). Furthermore, Agalioti and colleagues have shown that acetylation of K14 correlates with recruitment of TFIID and that mutation of either K9, S10 or K14 prevented this recruitment (52). In a similar manner, the opposed marks could be set up through methylation at K4. SUV39H1 has been shown *in vitro* to be inhibited by prior methylation at K4 and acetylation at K14 has been shown to be stimulated (53).

Unlike our results, or those obtained in Alfalfa, recent mass spectrometry analysis of H3 in Hela cells indicate that in this

system acetylation at K14 and trimethylation at Lys-9 are not mutually exclusive (54). As trimethylation of Lys-9 is almost undetectable in Arabidopsis, it is clear that variation between plants and animals has evolved with regards to the combinations of histone modifications contributing to gene regulation.

In contrast to this dichotomy observed between K9 and K14, we found that MK27 and MK36 coexist on the same H3 N-terminus (Figure 4). Methylation of K27 has been shown to be associated with gene silencing in many systems (55–57), and we report here that methylation at K27 is approximately twice as abundant in H3.1 than H3.2 (Figure 4). Although methylation at K36 has been reported to cause repression when tethered near a promoter (17), it has recently been found associated with the phosphorylated CTD of Pol II suggesting a role in transcription elongation (18). Our analysis of Arabidopsis histone H3 variants shows clear association between K36 methylation and the replication-independent histone H3.2. No mono- or dimethylation at K36 was detected on H3.1, while H3.2 had 6% 1MK36, 15% 2MK36 and 3% 3MK36. This association with the replacement histone supports a role for methylation at K36 in actively transcribed regions. Surprisingly, 15% of the peptides from H3.2 were modified with both 2MK27 and 1MK36. Although these marks may represent opposite signals when found alone, it is not clear what the combination of marks signify. It is clear however, that these marks do not exclude one another.

We have recently shown that both 1MK27 and 2MK27 are enriched in heterochromatin in Arabidopsis using immunofluorescence and chromatin immunoprecipitations (20), consistent with our findings here that these modifications are more abundant on H3.1 than H3.2. However, the high abundance of 1MK27 suggests that it may also be found in euchromatin, where its role is unknown.

Finally, using differential labeling of L*er* and *kyp* histones, we were able to confirm our earlier analysis of *kyp* mutants and show that the major changes in histone modifications were at K9. Both 2MK9 and 3MK9 decrease, but no change in 1MK9 was observed. The observation that there is 1MK9 and some remaining 2MK9 present even in the absence of KRYPTONITE suggest other Su(var)3-9 homologs must be active in Arabidopsis. We have recently shown that SUVH6 has K9 methyltransferase activity *in vitro* (29). As silencing is lost even when SUVH6 is present in *kyp* strains, the levels and targeting of this enzyme needs to be investigated further. Additionally, the role of monomethylation at K9 is still in question since high levels remain in *kyp* strains.

ACKNOWLEDGEMENTS

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