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Multiplicity of Histone H3 Variants in Wheat, Barley, Rice, and Maize[1]

Jakob H. Waterborg

Division of Cell Biology and Biophysics, School of Basic Life Sciences, University of Missouri-Kansas City, Kansas City, Missouri 64110–2499

ABSTRACT

Histone H3 proteins were purified to near homogeneity from etiolated seedlings of wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rice (*Oryza sativa*), maize (*Zea mays*), and alfalfa (*Medicago sativa*) to determine the number of histone H3 variants. Five distinct histone H3 variants were identified by gradient gel electrophoresis and reversed phase chromatography. These variants occur in various combinations of two to four forms in each plant species. One minor histone variant form (variant III, named H3.2 in alfalfa) appeared present and identical in all mono- and dicotyledonous plant species tested to date. All H3 proteins were acetylated to multiple levels and in every species the variant III form was acetylated most extensively. The level of histone H3 acetylation showed an inverse correlation with plant genome size. These observations support the idea that acetylated histones and especially variant III proteins are an element of transcriptionally active chromatin.

Variants or isoproteins of core histones have been observed in many eukaryotic organisms, but variant-specific functions have been identified in only a few cases (for reviews, see refs. 8, 26). Our knowledge of histone H3 variants in many eukaryotes other than mammals and birds is very fragmentary, but sequence heterogeneities (10, 20, 25) and gel analysis of histone proteins (1, 17) generally indicate the existence of two or three H3 variant forms without providing information on differences in primary sequence, synthesis, or function. In fungi like *Saccharomyces cerevisiae*, histone H3 variants appear not to exist (7, 25).

During a study of histone variants in alfalfa (*Medicago sativa*), two histone H3 variants have been identified (22), purified, and sequenced (21, 24). These two variants exhibited remarkable differences in histone acetylation (21–24). Genes for both histone H3 variants have been cloned (27). The existence of H3 variant genes and proteins has been suggested for other plants from a partial gene sequence in barley (6) and from protein gels of wheat (9), tobacco (11), and pea (11, 16), although protein heterogeneity was not observed in other studies of wheat (18) and *Arabidopsis thaliana* (13). To date, multiple histone H3 clones have been isolated from wheat (19), maize (5), *Arabidopsis* (4), and rice (28), but all code for H3 proteins identical or virtually identical to the major H3.1 variant of alfalfa (27). Only from barley (6) and alfalfa (27) have single, partial cDNA clones coding for minor H3 variant proteins been isolated.

We report here the presence of five distinct histone H3 protein variants that in various combinations occur in wheat, barley, rice, and maize. In all species, one minor histone H3 variant (variant III, identical to alfalfa H3.2) was always present. This variant always was acetylated to the highest extent among the H3 isoproteins. Thus, this most conserved histone H3 variant may play a specific role in the function of transcriptionally active chromatin.

MATERIALS AND METHODS

Wheat (*Triticum aestivum* var GK Zombor), barley (*Hordeum vulgare* var GK Omega), rice (*Oryza sativa* var Ringo [I]), maize (*Zea mays* var Sze DC488) kernels, and seeds of alfalfa (C.S. Brand *Medicago sativa* of mixed California origin, purchased from Planters Seed Co., Kansas City, MO) were germinated into 5 d old etiolated seedlings by the following protocol. Seeds were imbibed overnight in the dark in water at room temperature. Germinating seeds were washed twice a day with ample tepid tap water and stored in the dark at room temperature and near 100% RH. Husks and nongerminated seeds were removed before homogenization of the sprouts in guanidine·HCl buffer at a ratio of 200 mL buffer/100 g sprouts. Total crude histones were prepared from the clarified homogenates as described for alfalfa callus cultures (21) using 5 mL Bio-Rex-70 resin/extract from 100 g sprouts. Histone H3 was partially purified by Bio-Gel P-60 chromatography (22) and fractions, determined to contain histone H3 by SDS and AUT[2] gel electrophoresis, were pooled, dialyzed against 2.5% (v/v) acetic acid with 0.1 mL 2-mercaptoethanol/liter, and lyophilized. Subsequently, histone H3 variant proteins were fractionated and purified by Zorbax Protein Plus high-performance chromatography (21, 24). Total and purified histone preparations were analyzed by acetic acid urea PAGE with a constant concentration of 9 mM or a transverse gradient of 0 to 10 mM Triton X-100, as described previously (21, 23). (BioRex and Bio-Gel are products of Bio-Rad, and Zorbax Protein Plus is from DuPont.)

RESULTS

To evaluate the complexity of histone H3 variant proteins in monocotyledonous plants, the four species of agriculturally

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[2] Abbreviation: AUT, acetic acid urea Triton X-100.

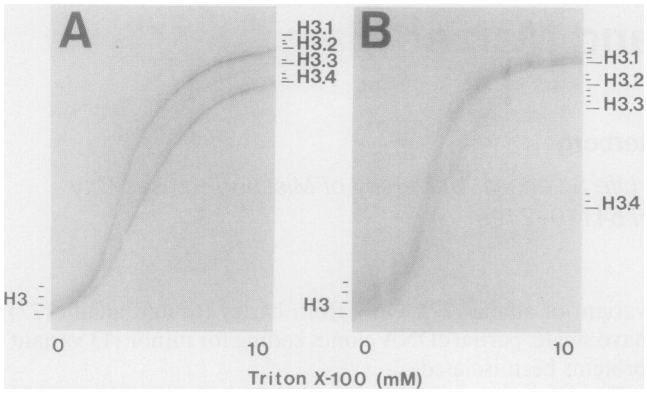

Figure 1. Gradient gel analysis of barley and maize histone H3. Electrophoresis of (A) barley and (B) maize histone H3 purified by Bio-Gel and reversed phase chromatography on an acetic acid urea gel with a transverse gradient of Triton X-100 between 0 and 10 mM. Histone H3 variant forms are indicated with dashes showing acetylated histone bands.

important Gramineae most extensively used in the study of plant histone proteins, histone genes, or chromatin were selected. This study complements a similar analysis of dicotyledonous alfalfa, soybean, *Arabidopsis*, carrot, and tobacco (JH Waterborg, submitted for publication). The existence and relative abundance of histone H3 variants was established by two complementary methods, gel electrophoresis and reversed phase chromatography, applied to histone preparations made by extraction of whole seedlings with guanidine·HCl followed by Bio-Rex-70 chromatography as described elsewhere (JH Waterborg, submitted for publication). Alfalfa histones were prepared from sprouts and analyzed in parallel for comparison.

Electrophoresis of histones in an acid-urea polyacrylamide gel with a transverse gradient of the non-ionic detergent Triton X-100 identifies the four groups of core histones by a characteristic affinity for the detergent (11, 22). Histone H3 proteins bind Triton more weakly than H2A species but more strongly than histones H2B or H4. Multiple H3-like bands in varying proportions with apparent acetylation were observed: two for alfalfa and rice, three for wheat and barley, and three or four for maize (results not shown).

Reversed phase chromatography of histone H3 on Zorbax Protein Plus subsequently yielded purified histone H3, which

Table I. *Multiplicity of Histone H3 Variant Proteins*

Representatives of dicot group I include alfalfa, soybean, and pea (15), and representatives of dicot group II include *Arabidopsis*, carrot, and tobacco.

	Variant Form				
	I	II	III	IV	V
A. Nomenclature of histone H3 variants in plants					
Dicot group I	—[a]	H3.1	H3.2	—	—
Dicot group II	H3.1	—	H3.2	—	—
Wheat	—	H3.1	H3.2	H3.3	H3.4
Barley	H3.1	H3.2	H3.3	H3.4	—
Rice	H3.1	—	H3.2	—	—
Maize	H3.1	H3.2	H3.3	—	H3.4
B. Relative abundance of histone H3 variants[b]					
Wheat (n = 4)[c]	—	85 ± 2%	11 ± 1%	4 ± 1%	0.3%[d]
Barley (n = 5)	0.7 ± 0.4%	51 ± 3%	9 ± 3%	40 ± 4%	—
Rice (n = 3)	75 ± 4%	—	24 ± 6%	—	—
Maize (n = 5)	64 ± 9%	19 ± 6%	11 ± 3%	—	9 ± 4%
C. Acetylated lysine residues per histone H3 variant protein[b]					
Wheat (n = 3)	—	0.13 ± 0.02	0.38 ± 0.03	0.24 ± 0.04	ND
Barley (n = 3)	ND[e]	0.15 ± 0.02	0.35 ± 0.03	0.20 ± 0.01	—
Rice (n = 3)	0.63 ± 0.01	—	0.96 ± 0.07	—	—
Maize (n = 3)	0.18 ± 0.06	0.36 ± 0.20	0.52 ± 0.20	—	0.48 ± 0.26

[a] — = not detectable. [b] The distribution and acetylation of dicotyledonous H3 variants has been reported elsewhere. [c] The number of independent analyses from which the average and standard deviation was calculated is given by n. Relative abundance is the percentage of a variant protein determined by Coomassie brilliant blue staining and densitometry of AUT gels within a nearly linear staining range. The number of analyses with SD are indicated. Analyses were performed on total pooled histone H3 fractions as in Figure 1 and on reversed phase column fractions (Fig. 2), and yielded essentially identical results. Histone acetylation levels are expressed as the average number of acetylated residues per H3 protein molecule, determined by quantitative densitometry of Coomassie stained AUT gels. [d] Wheat variant H3.4 was not always detected. [e] ND = not determined.

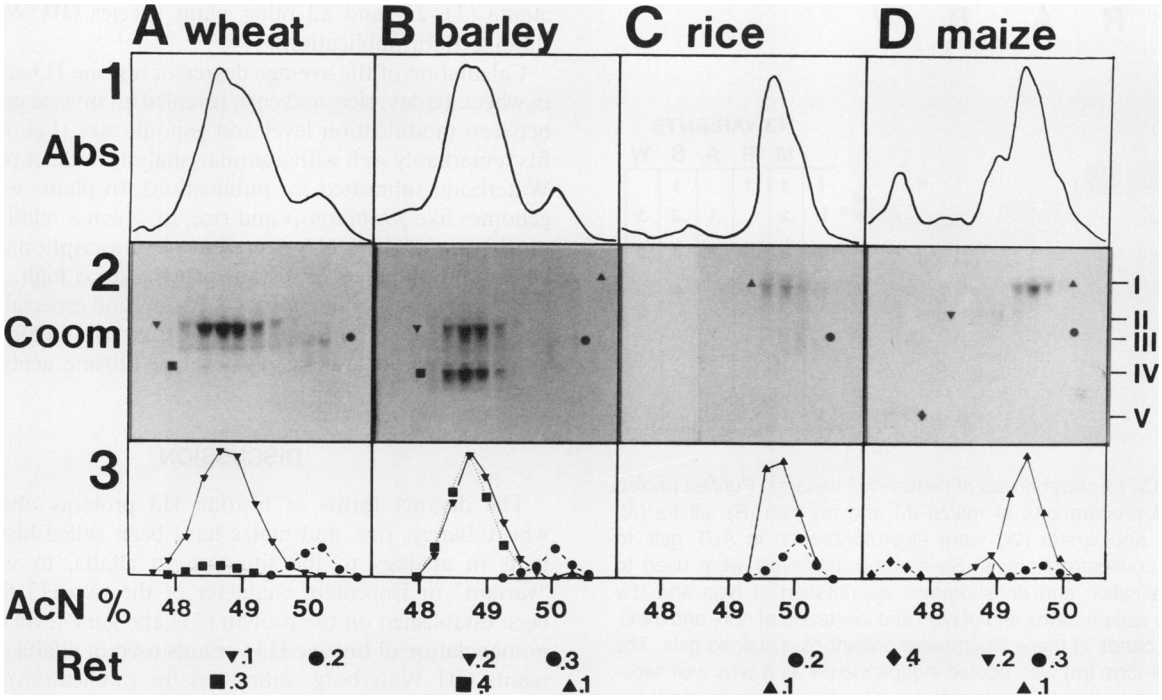

Figure 2. Reversed phase chromatography of histone H3 proteins. Zorbax Protein Plus reversed phase chromatography of Bio-Gel P60 enriched histone H3 preparations of (A) wheat, (B) barley, (C) rice, and (D) maize. Displayed is a 21 min wide window during the chromatography at 1 mL/min and an increase in acetonitrile concentration of 10%/h at 0.1% TFA in water. The concentrations of 48, 49, and 50% acetonitrile (AcN%) in the fractions are indicated. The absorbance pattern (Abs) at 214 nm (row 1), a Coomassie brilliant blue stained AUT gel (Coom) of collected fractions (row 2), and a densitometric analysis of the relative amount of each variant in this gel (row 3) are shown. All plotted data were normalized to facilitate qualitative and relative quantitative comparisons between preparations. The nonacetylated protein forms in row 2 are marked by the same symbols used in row 3: variant I triangle with continuous line, variant II inverted triangle with continuous line, variant III solid circle with dashed line, variant IV solid square with dotted line, and variant V diamond with dashed line. The electrophoretic mobility of each variant form is marked on the right of row 2. A faint shadow of higher mobility below heavy bands can be observed in this gel, which arose during prolonged storage and which may be an oxidation artifact. The reversed phase column retention (Ret) of each form is marked at the bottom by the variant symbol with the name of the variant protein. The retention of histone H3.4 of wheat, undetectable in this experiment, is not given.

again was analyzed by Triton gradient gel analysis. This clearly showed that barley contained, in addition to the three major histone H3 variant species seen in crude preparations, a minor fourth variant (Fig. 1A). All forms had identical mol wt, as demonstrated by co-electrophoresis in the absence of Triton (3). Variants H3.1 through H3.4 were named as before according to their gel mobility, a reflection of hydrophobicity and affinity for Triton X-100 (22) and not of mol wt. Purification of maize H3 confirmed the presence of four variants (Fig. 1B). The H3.1, H3.2, and H3.3 forms showed an affinity typical for histone H3.1 or H3.2 in mammals, birds (29), or dicotyledonous plants (JH Waterborg, submitted for publication) (22). The H3.4 form had a much lower affinity for Triton, although still higher than typically seen for histone H2B species, much like H3.3 in mammals or birds (29). It also had the same apparent mol wt as the other three H3 variants, as determined by gel electrophoresis in SDS (data not shown) and acetic acid urea gels at 0 mM Triton X-100 (Fig. 1B). This makes it likely that maize H3.4 is a real histone H3 variant and did not arise through proteolytic degradation of any of the other forms. Confirmation of this identification awaits purification and sequence analysis. Wheat showed the presence of the same three major H3 variants observed in barley (Table IA), although in different relative proportions (Table IB). Occasionally a trace of a form with low Triton affinity was detected, similar to maize H3.4. As in the case of maize, proteolysis SDS and acid urea gel analysis could exclude the possibility that this form was generated by proteolysis. Purification of rice histone H3 yielded only two protein forms of identical mol wt but with distinctly different affinities for Triton X-100 (Table I).

Reversed phase chromatography of total crude histones was severely limited by the amount of histone H3 that could be purified without loss of column resolution. For preparative analyses, histone H3 was enriched by Biogel P-60 chromatography, which removed some histone H2, all histone H1 and H4, and most nonhistone proteins (22). Several hundred micrograms of histone H3 could then be purified in a single analytical reversed phase column chromatography to near homogeneity (Fig. 2-1). Gel analysis of individual fractions (Fig. 2-2) allowed determination of variant-specific column retention (Fig. 2-3) and quantitation of high and low abundance variant forms (Table IB).

AUT gel electrophoresis of histone H3 preparations from different species allowed identification of apparently identical polypeptide species, based on co-electrophoresis (Fig. 3). Only

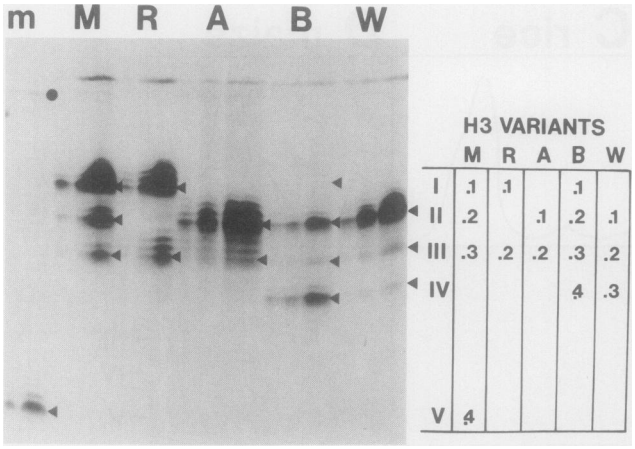

Figure 3. Co-electrophoresis of histone H3 variants. Purified pooled histone H3 preparations of maize (M and m), rice (R), alfalfa (A), barley (B), and wheat (W) were electrophoresed in AUT gels to determine co-electrophoresis. Several loading levels were used to allow visualization and densitometric quantitation of high and low abundance variant forms and of high and low levels of non- and multi-acetylated bands in these Coomassie brilliant blue stained gels. The maize H3.4 form (m) was pooled independently as it was well separated from the other H3 protein forms (Fig. 2D). Triangles mark the nonacetylated species of each histone H3 variant identified. Small amounts of histone H3 dimer are indicated by the solid circles. The faint shadow of higher mobility below heavy bands seen in Figure 2 was also present. The electrophoretic mobilities of variant I through variant V and the names of the histone H3 variants for each species are marked.

the alfalfa H3.2 polypeptide species appeared present in all species. Although named H3.2 in most plants, it is called H3.3 in barley and maize. To facilitate discussion and avoid confusion, a second nomenclature for histone H3 variant proteins is proposed. The five distinct polypeptide species, recognized in this study by their characteristic AUT gel mobility (Fig. 3) and retention during reversed phase chromatography (Fig. 2-3), were named "variant I" through "variant V" (Fig. 3). Only variant III is present in all plants.

Densitometry of Coomassie stained gels allowed quantitation of the relative abundance of each histone H3 variant protein (Table IB) and of the apparent level of steady state acetylation of each species (Table IC). These measurements were necessarily less precise than previously obtained for alfalfa (21, 23) and other dicot plants (JH Waterborg, submitted for publication), because the dynamic range between low and high abundance variants and between multi-acetylated and nonacetylated forms, especially in wheat, barley, and maize, is greater than the linear staining range of Coomassie brilliant blue. In addition, partial overlap between multi-acetylated and nonacetylated bands of closely migrating species has limited quantitation. Despite this degree of inaccuracy, it is clear that the relative abundance of histone H3 variant polypeptides varies widely (Table IB). Also, every H3 form present in significant amounts was acetylated to a significant degree (Table IC) with variant III systematically modified to the highest degree in each species. The same observation has previously been made for variant III (H3.2) in alfalfa (21–23) and all other plant species (JH Waterborg, submitted for publication).

Calculation of the average degree of histone H3 acetylation in wheat, barley, rice, and corn revealed an inverse correlation between modification level and genome size (Fig. 4), which fits remarkably well with a similar analysis in dicot plants (JH Waterborg, submitted for publication). In plants with small genomes like *Arabidopsis* and rice, in which a relatively high proportion of the DNA is present in transcriptionally active chromatin, the level of histone acetylation is high. In plants with large genomes like tobacco, barley, and especially wheat, which have increasing amounts of transcriptionally inactive heterochromatin (14), a low level of histone acetylation is observed.

DISCUSSION

The distinct forms of histone H3 proteins observed in wheat, barley, rice, and maize have been called histone variants in analogy to the situation in alfalfa, in which the "variant" or isoprotein character of the two H3 forms has been established on the protein (21) and gene level (27). The nomenclature of histone H3 variants used in alfalfa and other plants (JH Waterborg, submitted for publication) (24) has also been applied in this study. The variant form with the highest affinity for Triton X-100 in AUT gel electrophoresis is called H3.1, the one with the next highest affinity H3.2, etc. However, this led to different names for apparently identical protein molecules in different species, and an additional numbering system of "variant I" through "variant V" has been introduced (Table I). This system is based on apparent protein identity, as determined by AUT gel co-electrophoresis (Fig. 3) and supported by reversed phase chromatography (Fig. 2).

The distinct forms of histone H3 identified in this study are not products of proteolysis, based on the following observations. All histone H3 preparations purified through HPLC

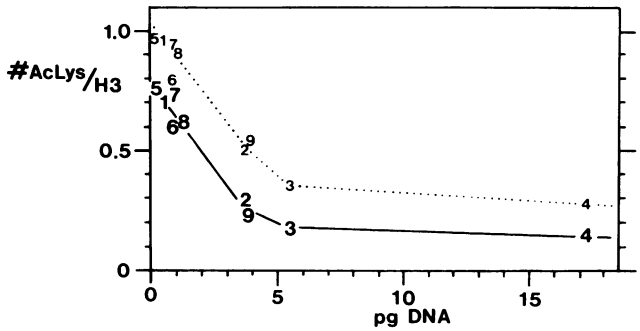

Figure 4. Comparison of histone H3 acetylation between plant species. Histone H3 acetylation, expressed as the average number of acetylated lysine residues per histone H3 protein molecule, combining all variant forms (line with large numeral symbols) and the number of acetylated residues in histone H3 variant III (dotted line with small numeral symbols) was plotted *versus* the size of the plant genome, expressed as the C amount of DNA[3] (2) of: 1, rice; 2, maize; 3, barley; and 4, wheat; and of: 5, *Arabidopsis*; 6, soybean; 7, carrot; 8, alfalfa; and 9, tobacco.

have the same mobility in SDS gels as *bona fide* calf thymus histone H3 with 135 amino acids. Smaller polypeptides, which could have arisen from proteolysis, were not detected in these fractions (results not shown). Similar co-electrophoresis was observed in acetic acid urea gels, a gel system that has been shown to be suited for mol wt determination of basic histones (3) and that readily visualizes proteolysis. As determined for all preparations and shown for barley and maize in Figure 1, A and B, respectively, all histone H3 forms have identical gel mobilities at 0 mM Triton X-100. Increasing amounts of Triton affect the mobility of each variant in a continuous and specific way. This gel system clearly proves that even forms with a low mobility in AUT gels like H3.4 (variant V) of maize (Figs. 2D and 3) are intact histone H3 proteins (Fig. 1B).

The alfalfa histone H3 variant composition obtained from sprouts of mixed genetic origin (this study) was indistinguishable from preparations made from homogeneous callus cultures (21, 23) and from leaves or roots of whole plants. Similarly, maize H3 variant compositions obtained from etiolated seedlings were identical to those obtained from dried leaves of mature maize plants (results not shown). These results suggest that it might be possible to compare the wheat results from this study with previously published data. Spiker (18) has previously detected a single H3 species in wheat by two dimensional gel analysis. Our data suggest that this would be the predominant wheat H3.1 variant (Table IB). More recently, Green and co-workers (9) have detected the existence of the two major H3 variants, H3.1 and H3.2.

Cloning of histone H3 genes from maize (5) and rice (28) has only yielded genes that code for the same protein that was cloned from *Arabidopsis* (4) and that has been identified as *Arabidopsis* histone H3.1 with alanine at position 90 (JH Waterborg, submitted for publication). This protein co-electrophoreses in AUT gels with H3.1 species of barley, rice, maize (results not shown), carrot, and tobacco (JH Waterborg, submitted for publication) and thus must represent variant I (Table II). To date, cloning attempts have failed to isolate representatives of the less abundant variant forms (Table IB). From wheat, the only histone H3 genes isolated (19) have sequences identical to H3.1 (variant II) of alfalfa (21), which differ from variant I only by serine as residue 90 (Table II). Again, only the most abundant H3 variant from wheat has been cloned (Table IB). Only single, partial cDNA clones for the minor H3 variant III form have been isolated from barley (6) and alfalfa (27), in which the protein sequence of the H3.2 form was confirmed (21). The sequences of variants IV and V are unknown. Based on the relative abundance of these proteins (Table IB), cloning of the variant genes from barley and maize, respectively, may be most likely to succeed. Alternatively, purification and sequencing of H3.4 (variant V) protein of maize appears feasible because it is separated from all other histone H3 species during reversed phase chromatography (Fig. 2D).

Histone H3 charge modified forms detected in AUT gels were identified as acetylated by comparison to the exhaustive analysis of alfalfa H3 variant acetylation, which included radioactive acetate labeling *in vivo* (21, 22), sequencing (21), and elimination of phosphorylation as a significant contributor to histone H3 modification (23). In this study, no direct determination of the presence of acetylated lysine residues in histone H3 variant proteins was performed. Also, the absence of significant levels of histone H3 phosphorylation has not been excluded. The low levels of barley H3.1 (variant I) and of the possible variant V form of wheat have precluded determination of steady state acetylation levels. The accuracy of measured acetylation levels in the other variant proteins is less than previously reported for dicot histone H3 variants (21, 23) (JH Waterborg, submitted for publication). This is due to the higher number of histone H3 variants in these species and their partial overlapping patterns of HPLC elution (Fig. 2) and AUT gel electrophoresis (Fig. 3). However, the average levels of histone H3 acetylation relative to genome size of plant species fit remarkably well (Fig. 4) with the more accurate data obtained previously from dicot plants (JH Waterborg, submitted for publication). This extends the correlation between histone acetylation and transcriptional activity of a genome to a wide range of plant species.

Variant III, present in all plants surveyed, has been identified as a replication-independent replacement-type histone in

Table II. *Plant Histone H3 Sequences*

Published sequences of plant histone H3 proteins with numbering of amino acids and capital letter highlights of variable residues. Histone H3 coelectrophoresis (Fig. 3) and cochromatography (Fig. 2) have been used to identify the variant-specific name of all published sequences. 1 = histone H3 variant I: H3.1 of *Arabidopsis*, carrot, tobacco, maize (5) (Fig. 3, M), rice (28) (Fig. 3, R), and barley (Fig. 3, B). 2 = histone H3 variant II: H3.1 of alfalfa (21, 27), soybean, and wheat (Fig. 3, W), H3.2 of barley (Fig. 3, B) and maize (Fig. 3, M), and the major histone H3 of pea (15). 3 = histone H3 variant III: H3.2 of alfalfa (21, 27), *Arabidopsis*, carrot, soybean, tobacco, wheat (Fig. 3, W), and rice (Fig. 3, R), H3.3 of barley (6) (Fig. 3, B) and maize (Fig. 3, M).

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  1                                                                    70
1 artkqtarks tggkaprkql atkaarksap Atggvkkphr Frpgtvalre irkyqkstel lirklpfqrl
2 .......... .......... .......... A......... F......... .......... ..........
3 .......... .......... .......... T......... Y......... .......... ..........

  71                                                                  135
1 vreiaqdfkt dlrfqs SavA alqeaaeayl vglfedtnlc aihakrvtim pkdiqlarri rgera
2 .......... ...... S..S .......... .......... .......... .......... .....
3 .......... ...... H..L .......... .......... .......... .......... .....
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alfalfa (T Kapros, L Bogre, K Nemeth, L Bako, J Gyorgyey, SC Wu, D Dudits, submitted for publication), similar to histone H3.3 in mammals and birds (8). A preferential localization of histone H3.2 (variant III) in alfalfa (22, 23) and other dicot plants (JH Waterborg, submitted for publication) into transcriptionally active chromatin has been deduced from the high level of acetylation. The same is true for wheat, barley, rice, and maize, in which variant III proteins are systematically higher acetylated than average (Fig. 4).

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