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Histone H1 overexpressed to high level in tobacco affects certain developmental programs but has limited effect on basal cellular functions

(chromatin/development)

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ABSTRACT **Histone H1, a major structural component of chromatin fiber, is believed to act as a general repressor of transcription. To investigate *in vivo* the role of this protein in transcription regulation during development of a multicellular organism, we made transgenic tobacco plants that overexpress the gene for *Arabidopsis* histone H1. In all plants that overexpressed H1 the total H1-to-DNA ratio in chromatin increased 2.3–2.8 times compared with the physiological level. This was accompanied by 50–100% decrease of native tobacco H1. The phenotypic changes in H1-overexpressing plants ranged from mild to severe perturbations in morphological appearance and flowering. No correlation was observed between the extent of phenotypic change and the variation in the amount of overexpressed H1 or the presence or absence of the native tobacco H1. However, the severe phenotypic changes were correlated with early occurrence during plant growth of cells with abnormally heterochromatinized nuclei. Such cells occurred considerably later in plants with milder changes. Surprisingly, the ability of cells with highly heterochromatinized nuclei to fulfill basic physiological functions, including differentiation, was not markedly hampered. The results support the suggestion that chromatin structural changes dependent on H1 stoichiometry and on the profile of major H1 variants have limited regulatory effect on the activity of genes that control basal cellular functions. However, the H1-mediated chromatin changes can be of much greater importance for the regulation of genes involved in control of specific developmental programs.**

The transcriptional machinery in eukaryotic cell functions in the chromatin environment with the template DNA packaged by histone proteins into nucleosomes, 30-nm chromatin fibers, and higher-order structures (reviewed in ref. 1).

The linker histone H1 is thought to lie across the dyad axis of the nucleosome, stabilize the nucleosome, and facilitate the folding of the nucleosomal threads into 30-nm fibers (reviewed in refs. 1 and 2). While the results of *in vitro* studies show that both nucleosomal cores and H1 suppress basal transcription, the precise relationship between the effects of cores and H1 is difficult to establish (3). The well-documented case of the H1-mediated suppression of transcription of the oocyte-type 5S RNA genes during development of *Xenopus* (reviewed in ref. 2) involves a small class of genes flanked by specific DNA sequences with high affinity for H1 (4). That there is limited knowledge of the *in vivo* effects of H1 on the transcription of a broader class of genes is partly due to the absence of typical H1 in yeast, a circumstance hampering the possibility of a genetic approach to the problem. The alternative approach has been the manipulation of expression of linker histone genes in living cells. The expression of the transfected histone H5 gene in rat sarcoma cells resulted in the arrest of cell proliferation and changes in genes expression (5). Overexpression of a gene for sea urchin H1 in yeast resulted in severe impediment of cell functions (6, 7). While neither rat sarcoma cells nor yeast provides a native environment for H5 and H1 histone, respectively, the reported results suggest that the change in H1:DNA stoichiometry and/or in the complement of native H1 variants *in vivo* should have serious consequences for the basal cellular functions. This conclusion was strongly contradicted by the results of recent reports on the effects of knock-out of histone H1 genes in *Tetrahymena* and mouse. The elimination of H1-like histones in *Tetrahymena* had no effect on growth and survival of the cells (8). Similarly, $H1^0$, one of the distinct variants of H1, was shown to be dispensable for normal development of mouse (9).

We have succeeded in generation of viable tobacco plants that overexpress a gene for histone H1 from *Arabidopsis thaliana* and have a greatly decreased level of their own H1. The resulting drastic change in H1:DNA stoichiometry and in the complement of H1 variants had a limited effect on the basal cellular functions but affected plant morphology and flowering. These results indicate that, at least in plants, the regulatory function of H1 in respect to transcription may be limited to a class of genes responsible for specific developmental programs.

MATERIALS AND METHODS

Generation of Tobacco Plants That Overexpress a Histone H1 Gene of *Arabidopsis thaliana*. *Arabidopsis* histone H1-2 cDNA (1106 bp) was derived from the pBluescript (Stratagene) plasmid pATH1-2, provided by J. S. Gantt (10). To remove the 222-bp 3′-untranslated region (UTR) of H1-2 cDNA a 16-nucleotide fragment with *Xba*I and *Nsi*I restriction sites was introduced immediately 3′ to the stop codon by site-specific mutagenesis of pATH1-2 according to Kunkel *et al.* (11). The 3′-truncated cDNA of H1-2 was then inserted into the expression cassette derived from plasmid pFF19 containing enhanced cauliflower mosaic virus (CaMV) 35S gene promoter, provided by J. Messing (12). *Agrobacterium tumefaciens* binary plasmid pRok2 (13) was used to prepare the transformation vector. The vector was introduced into leaf discs or into whole 2-week-old seedlings of *Nicotiana tabacum*

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Abbreviations: CaMV, cauliflower mosaic virus; UTR, untranslated region; PCA, perchloric acid; AU, acetic acid/urea.

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cv. SRI plants by using the *Agrobacterium*-mediated procedure (14). Total genomic DNA from leaves of regenerated plants was prepared as described (15) for Southern blot hybridization (16). Total leaf RNA was prepared (17) for Northern blot hybridization (16). Analyses of hybridization with ^{32}P-labeled probes was by autoradiography or with the PhosphorImager (Molecular Dynamics).

Chromatin Analysis with Micrococcal Nuclease. Nuclei isolated from leaves of different plants were digested with micrococcal nuclease for increasing periods of time. Aliquots of DNA from samples taken during the course of digestion were run on 1.5% agarose gels.

Preparation and Analysis of Histones. The isolation of chromatin from tobacco and *Arabidopsis* leaves and the preparation of total tobacco chromatin histones by extraction with 0.2 M H_2SO_4 were as previously described (18). Linker histones were prepared by extraction of leaf tissue with 5% perchloric acid (PCA) according to Mazzolini *et al.* (19). Total *Arabidopsis* histones were isolated by extraction of chromatin with guanidine hydrochloride as previously described for the isolation of *Physarum* histones (20). Extracted proteins were fractionated by SDS/12% PAGE (21) and by acetic acid/urea (AU)/15% PAGE (22) and electroblotted to nitrocellulose membranes. The membranes were incubated with a 1:1000 dilution of a mouse polyclonal anti-wheat H1 antiserum for 4 hr. Goat anti-mouse IgG coupled to alkaline phosphatase (Dako) was used as secondary antibody according to the supplier's recommendations. Washing and detection of bound alkaline phosphatase were as described (23). Stained gels and Western blots were photographed by GDS 5000 (Ultraviolet Products, San Gabriel, CA) video system. Quantitative analyses were done with the IMAGEQUANT (Molecular Dynamics) program.

Microscopic Analyses. 1- to 2-mm^2 squares were cut from leaves (in exactly the same stage) of the analyzed plants and fixed in 2% glutaraldehyde, pH 7.2, in 0.1 M cacodylic acid buffer, followed by 2-hr postfixation in 2% OsO_4. The material was then dehydrated in ethanol and embedded in Epon/Spurr mixture. Semithin (1- to 2-μm) and ultrathin (≈80-nm) sections were cut with an LKB ultramicrotome. Semithin sections were stained in 0.1% toluidine blue for observation in a light microscope (Nikon). Ultrathin sections were contrasted with uranyl acetate and lead citrate as described (24) and observed in a JEOL JEM 1200Ex transmission electron microscope.

RESULTS

Generation of Transgenic Tobacco That Expresses an H1 Gene of *Arabidopsis thaliana*. The genetic construct used for transformation of tobacco contained the cDNA of the *Arabidopsis* histone H1-2 gene (from which the untranslated sequences at the 3′ end carrying potential regulatory signals have been removed) placed under the constitutive viral (CaMV 35S) promoter. The construct in pRok2 vector (Fig. 1A) was stably integrated into the tobacco genome by *Agrobacterium*-mediated transformation of leaf discs or young seedlings. Whole plants were grown from transformed kanamycin-resistant calli by a standard regeneration procedure. DNA isolated from young leaves of 5- to 6-week-old plants was screened for the presence of the H1 transgene by Southern blot analysis. Hybridization with *Arabidopsis* H1-2 cDNA probe (which does not hybridize to native tobacco H1 genes) confirmed the presence of the transgene in genomic DNA of the majority of transformed plants (results not shown). Northern blot analysis with H1-2 cDNA probe was performed on total RNA isolated from young leaves of the transformed plants. In the first experiment, in which we used for transformation the discs cut from developed leaves, the Northern analysis revealed only 1 plant (out of over 20 kanamycin-resistant transformants) in which the transgene was expressed (Fig. 1*B*, blot I).

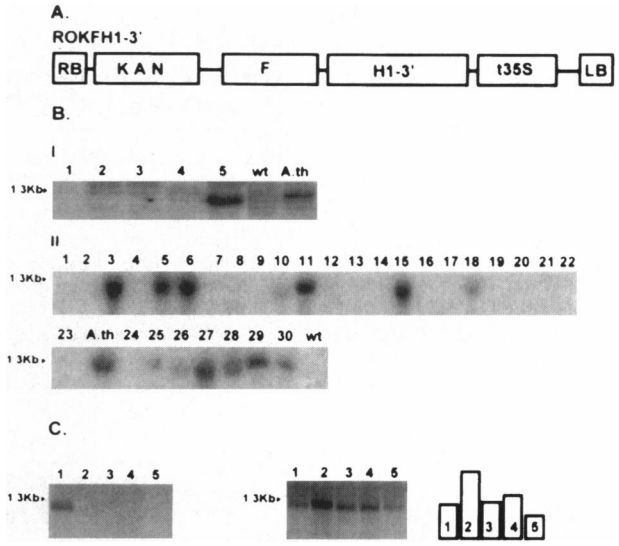

FIG. 1. Construction of transgenic tobacco plants that express the histone H1-2 gene of *Arabidopsis*. (*A*) Construct used for transformation. cDNA without the 3′-UTR of the H1-2 gene (H1-3′) was placed between "enhanced" CaMV 35S promoter (F) and CaMV 35S terminator (t35S). KAN is a kanamycin-resistance cassette. RB and LB are right and left borders, respectively, of the T-DNA in the transformation vector. (*B*) Analysis of transgene expression in transgenic and wild-type plants. Total RNA (10 μg) from leaves of 5- to 8-week-old plants was analyzed by Northern hybridization to H1-2 cDNA probe. Blot I, plants obtained by transformation of leaf discs (only the blot on which the single positive signal was detected is shown); blot II, plants obtained by transformation of young seedlings. In blots I and II each lane represents an individual plant; wt, wild-type tobacco; A.th., wild-type *A. thaliana*. (*C*) Analysis of *Arabidopsis* H1-2 and tobacco H1 mRNA levels in transgene-expressing and -nonexpressing tobacco plants transformed with *Arabidopsis* H1-2 cDNA. Total leaf RNA (10 μg) from plant expressing the transgene (lane 1) and four plants in which expression was not detected (lanes 2–5) was analyzed by Northern hybridization. Equal RNA loads in each lane were checked by quantitative analysis of an ethidium-bromide stained gel. (*Left*) Hybridization using *Arabidopsis* H1-2 cDNA probe. (*Center*) Hybridization of the same blot using tobacco H1 cDNA probe. (*Right*) Densitometric quantification of the bands in *Center*.

In the second experiment we used for transformation the whole young (2-week-old) seedlings. In this experiment, out of 30 regenerated transformed plants, 13 showed expression of the transgene (Fig. 1*B*, blot II). Plants that expressed the transgenic *Arabidopsis* H1 were designated TransH1+. The plants obtained in the same experiments but showing no expression, while containing the transgene cDNA as confirmed by Southern hybridization to genomic DNA, were treated as controls and designated TransH1−.

To examine the effect of the expression of transgenic *Arabidopsis* H1 on the transcription of native tobacco H1 genes we analyzed the total RNA from a TransH1+ plant and from four different TransH1− plants by hybridization to probes specific for *Arabidopsis* and tobacco H1 genes. While no transgene-specific mRNA could be detected in TransH1− plants (Fig. 1*C Left*) the native tobacco mRNA was present in the TransH1+ plant (Fig. 1*C Center*). The amount of native H1 mRNA in the TransH1+ plant was within the range of natural variation detected in four control plants (Fig. 1*C Right*), indicating that the transcription of the transgene is not markedly interfering with the transcription of native tobacco H1 genes.

Linker Histone Proteins in Tobacco Plants That Express Arabidopsis H1 Gene. We compared the proteins extracted with 5% PCA from leaves of the TransH1+ plant obtained in the first transformation experiment (transformation of leaf discs) and a control TransH1− plant. In the TransH1+ plant,

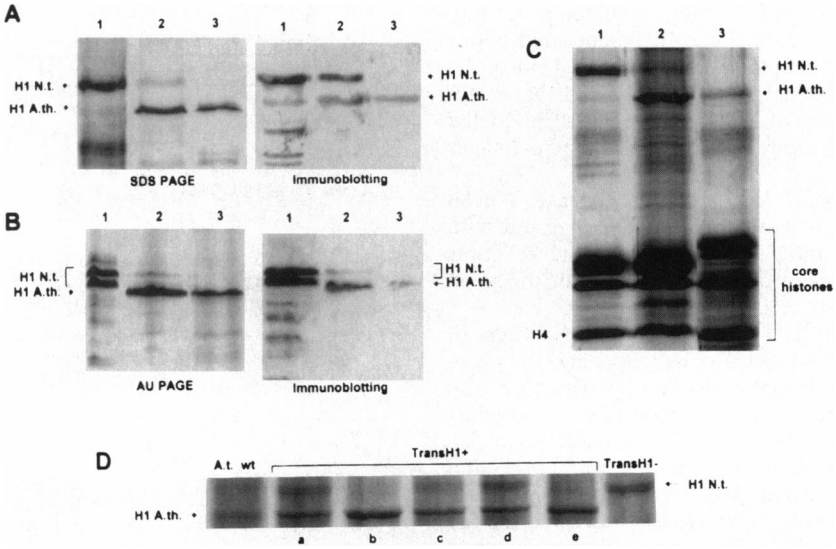

FIG. 2. Electrophoretic and immunoblot analyses of H1 and total chromatin histones in transgene-expressing and -nonexpressing transgenic tobacco plants and in wild-type *Arabidopsis*. N.t., tobacco; A.th., *Arabidopsis*. (*A*) Analysis by SDS/PAGE of proteins extracted from leaves with 5% PCA. Coomassie blue staining (*Left*) and immunoblotting with an anti-wheat H1 polyclonal antiserum (*Right*). Lanes 1, transformed tobacco not expressing the transgene (TransH1− plant); lanes 2, transformed tobacco expressing the transgene (TransH1+ plant); lanes 3, wild-type *Arabidopsis*. (*B*) Analysis by AU/PAGE of proteins extracted from leaves with 5% PCA. Coomassie blue staining (*Left*) and immunoblotting with anti-wheat H1 polyclonal antiserum (*Right*). Lanes as in *A*. (*C*) Analysis by SDS/PAGE of total histones extracted from chromatin isolated from leaves. Coomassie blue-stained gel, lanes as in *A*. The TransH1+ plant analyzed in *A–C* was obtained by transformation of leaf discs. (*D*) Analysis by SDS/PAGE of linker histones in transgene-expressing plants obtained by transformation of young seedlings. Coomassie blue-stained gel. Lanes a–e represent typical profiles occurring among the 13 transformants. The highest ratio of native to exogenous H1 occurred in two plants (lanes a and d).

a strong band was detected, with electrophoretic mobility on both SDS and AU gels exactly matching that of *Arabidopsis* H1 (Fig. 2 *A* and *B*, *Left*). The faint band seen in this position in the TransH1− lane on the SDS gel does not comigrate with *Arabidopsis* H1 on the AU gel (Fig. 2*B*, *Left*). The anti-wheat H1 antibody stains tobacco H1 more strongly than *Arabidopsis* H1 (Fig. 2 *A* and *B*, *Right*, lanes 1 and 3). The weaker staining by antibody of the extra band present in TransH1+ in comparison to tobacco H1 (Fig. 2 *A* and *B*, *Right*) confirms that it is indeed *Arabidopsis* histone H1.

To determine the cellular location and relative amounts of *Arabidopsis* H1 in TransH1+ cells, we analyzed the electrophoretic patterns on SDS gels of total histones extracted from chromatin of TransH1+ and TransH1− tobacco and wild-type *Arabidopsis* (Fig. 2*C*). In TransH1+ the proportion of native tobacco H1 to transgene-coded *Arabidopsis* H1 was the same as in 5% PCA extract from whole leaf (Fig. 2*C*, lane 2; compare with lane 2 in Fig. 2*A*, *Left*), indicating that all transgene-coded *Arabidopsis* H1 protein in TransH1+ was confined to chromatin. To estimate the linker histone-to-DNA ratio in TransH1+ we assumed that the profile of total *Arabidopsis* histones (Fig. 2*C*, lane 3) was representative of the physiological complement of histones in chromatin. The weaker staining by Coomassie blue dye of *Arabidopsis* H1 compared with *Arabidopsis* core histones and H1 from other sources is characteristic for this protein and has been reported earlier (18). We assumed also that histone H4 from tobacco and *Arabidopsis* stained with comparable intensities and thus could be used as the internal reference to correct for the amount of H1.

Densitometric analysis of the gel in Fig. 2*C* indicated that the TransH1+ plant had about 2.8 times more linker histone than

Table 1. H1 composition of chromatin from control (TransH1−) and transgene-expressing (TransH1+) tobacco plants

	% of total H1*†		% of H1 present in chromatin of wild-type tobacco‡		
Plants*	Tobacco H1	*Arabidopsis* H1	Tobacco H1	*Arabidopsis* H1	Total H1
TransH1−	100	0	100	0	100
TransH1+(I)	11	89	30	250	280
TransH1+(II)a	21	79	55	205	260
TransH1+(II)b	0	100	0	230	230
TransH1+(II)c	11	89	25	210	235
TransH1+(II)d	19	81	51	220	271
TransH1+(II)e	5	95	15	260	275

Data were from densitometric analyses of Coomassie blue-stained AU/polyacrylamide gel electrophorograms of total chromatin histones, like that in Fig. 2.

*(I) indicates plant obtained by transformation of leaf discs, and (II)a–e indicates plants (the same as in Fig. 2*D*) obtained by transformation of young seedlings.

†Determined by the ratio of peak area of the tobacco or *Arabidopsis* H1 to total area of all H1 peaks. The peak areas were adjusted to account for differences in the amount of histone H4.

‡Determined by the ratio of peak area of the indicated H1 to peak area of H1 in electrophorograms of chromatin histones from wild-type tobacco. The peak areas were adjusted to account for differences in the amount of histone H4.

Table 2. Phenotypes of histone H1-overexpressing plants

No. of plants	Description of main phenotypic changes
2	Dwarf appearance caused by extremely short internodes; no flowers formed or flowering delayed by over 12 weeks with only few undeveloped flower buds formed and thrown off within 1 week
9	Plants on average 25% smaller compared with TransH1− controls; flowering delayed 3–4 weeks; flowers formed but all thrown off at different stage of development; no fruits formed
3	Plants on average 25% smaller compared with TransH1− controls; flowering delayed 3–4 weeks; flowers kept; produced fruits (on average 50% of those produced by TransH1− control)

did the control TransH1− plant (Table 1). Almost 90% of this linker histone was the transgene-encoded *Arabidopsis* H1. The amount of native tobacco H1 in TransH1+ dropped to about 30% of the H1 level in TransH1−.

Similar electrophoretic analyses of histones extracted at the 5- to 6-week stage from each of the 13 TransH1+ plants obtained upon transformation of young seedlings revealed that all these plants expressed the transgene-encoded *Arabidopsis* H1 protein. In each of the TransH1+ plants the level of tobacco H1 protein was decreased. Typical profiles of linker histones occurring in these plants are shown in Fig. 2*D*. The extent of decrease varied from 55% to complete elimination of the native histone H1 (Table 1). Estimates based on densitometric analysis similar to that presented in Table 1 showed that the ratio of total linker histone to DNA in 13 TransH1+ plants varied between 2.3 and 2.8.

Phenotypes of Tobacco Plants That Overexpress *Arabidopsis* Histone H1. Phenotypic changes occurred in all TransH1+ plants, but the extent of changes varied (Table 2). The most severe changes occurred in two plants; one was the single TransH1+ plant obtained in the experiment with transformation of leaf discs, the other was one of the 13 TransH1+ plants obtained upon transformation of young seedlings (Fig. 3). It is important to note that the morphological (as well as ultrastructural, see below) appearances of these two plants were identical. The changes could already be noticed 5–6 weeks after the plants had been regenerated from callus. The plants had dwarf appearance with very short internodes and shortened rolled-down leaves. Flowering was strongly affected. One of the two plants (obtained by leaf disc transformation) did not produce inflorescence up to 36 weeks after regeneration (normal flowering occurred in 24–26 weeks), and the other produced unusually small inflorescence after 38 weeks. On this inflorescence occurred a few small flower buds, which, however, did not develop into flowers and were all thrown off within 1 week. However, the total number of leaves as well as the rate of appearance of new leaves in these two plants were not changed compared with control TransH1− plants. The appearance of the remaining TransH1+ plants was more similar to that of TransH1− plants, although the TransH1+ plants were on average 25% smaller and produced inflorescence 3–4 weeks later. The most dramatic difference concerned flowering. In nine TransH1+ plants all flowers that were made were thrown off in different stages of development, so none of these plants produced fruits. This was not due to a defect of pollen, which could be used to fertilize the wild-type tobacco plants (results not shown). Three TransH1+ plants produced fruits, but their number was only 50% of the average fruit number in control TransH1− plants. It should be noted that all TransH1− plants showed very little phenotypic variation. They all developed similarly, had no visible morphological differences, and flowered in the same time.

We did not notice any correlation between the extent of phenotypic change in TransH1+ plants and the variation of total linker histone-to-DNA ratio or the level of native tobacco H1. For example, the plant with native linker histone decreased to 55% and the plant with its own H1 completely eliminated had identical phenotypes, whereas the plants with severely affected phenotypes had the same ratio of *Arabidopsis* to native H1 as some of the plants with milder phenotypic changes.

Anatomical and Ultrastructural Changes in TransH1+ Plants. For microscopical analysis leaf sections were taken from the plants at the 5- to 6-week stage and at the time of flowering (>30 weeks). At the 5- to 6-week stage the most pronounced changes were seen in two TransH1+ plants with severe phenotypes. Photographs in Fig. 4 depict a TransH1+ plant obtained upon transformation of leaf discs. The heterochromatinized nuclei in the second TransH1+ plant with severe phenotype (analyzed at the same stage of growth) had identical appearance and occurred with similar frequency. In comparison to TransH1− plants the most dramatic difference concerned the ultrastructure of nuclei. In the cells of TransH1− plants euchromatin is dispersed, filling the greater part of the nucleus. Constitutive heterochromatin, which makes electron-dense conglomerations, occurs in small amounts (Fig. 4*A*, micrographs 1 and 3). In nuclei of TransH1+ cells large heterochromatin areas replace a considerable part of euchromatin. The extended and extremely condensed areas of heterochromatin with small light holes inside give a characteristic, unusual appearance to nuclei of

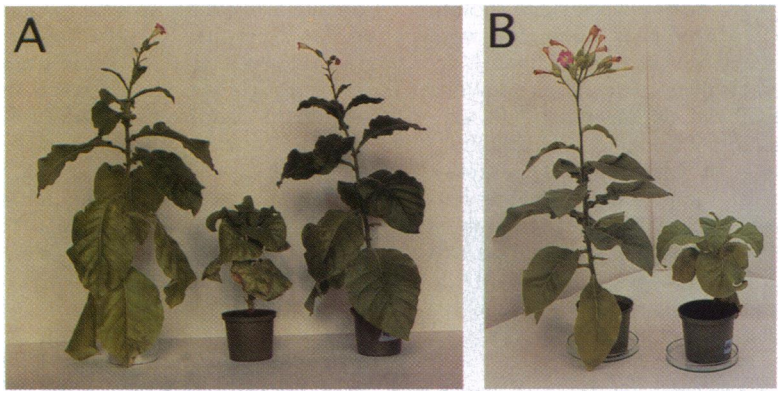

FIG. 3. Severe phenotypic changes in tobacco plants highly overexpressing exogenous histone H1. (*A*) TransH1+ plant obtained by transformation of leaf discs flanked by two TransH1− plants obtained in the same transformation. (*B*) TransH1+ plant (right) obtained by transformation of young seedlings compared with TransH1− plant (left) obtained in the same transformation. The TransH1+ plants overproduce transgenic histone H1 protein. In TransH1− plants the transgene is not expressed. Photographs depict 26-week-old plants.

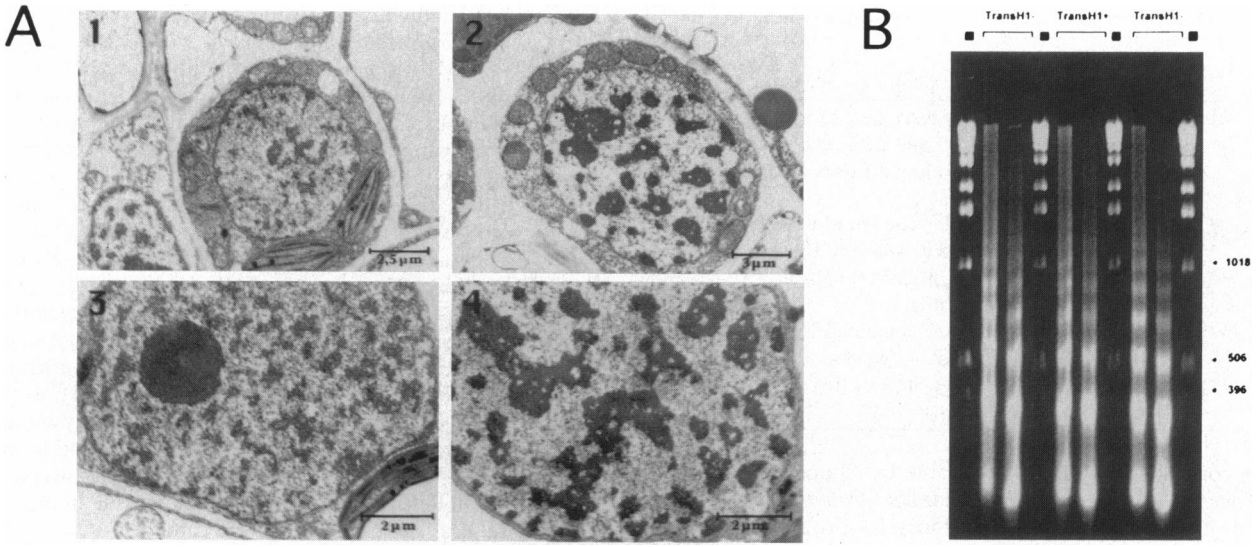

FIG. 4. Ultrastructural morphology of nuclei and the nucleosomal repeats in control TransH1− plant and in one of the two TransH1+ plants with severe phenotype. (*A*) Electron micrographs of leaf parenchymal cells of TransH1− (micrographs 1 and 3) and TransH1+ (micrographs 2 and 4) plants. Fragments of differentiating vascular bundles showing phloem parenchyma cells are illustrated in micrographs 1 and 2. A higher magnification of a fragment of nucleus from a palisade parenchymatic cell is shown in micrographs 3 and 4. (*B*) Electrophoretic analysis of DNA fragments generated upon digestion of nuclei of TransH1+ and two different TransH1− plants with micrococcal nuclease. For each sample the digestion was for 2 and 5 min. ■, DNA size markers.

virtually all parenchymal cells of the TransH1+ plant (Fig. 4*A*, micrographs 2 and 4). The appearance of these nuclei bears no resemblance to that of heterochromatinized nuclei occurring sometimes in aging tobacco cells (results not shown). As a result of the condensation of euchromatin fibers, the euchromatin regions become visibly lighter (compare micrographs 1 and 2 in Fig. 4*A*). The presence of overcondensed chromatin apparently does not interfere with the ability of leaf cells to differentiate. This is illustrated by the comparison of phloem parenchyma cells in TransH1− and TransH1+ plants (Fig. 4*A*, micrographs 1 and 2).

The spacing of nucleosomes in chromatin isolated from leaves of the TransH1+ plants with severe phenotypes and highly heterochromatinized nuclei was not changed compared with that in wild-type tobacco (Fig. 4*B*). No changes in spacing were noticed for other TransH1+ plants (results not shown).

The leaves of TransH1+ plants with severe phenotypes are thinner, but they retain the typical anatomical structure. There is no decrease in the number of cells. However, the cells are on average 33% smaller and contain more chloroplasts with both more numerous and larger starch grains, as compared with TransH1− plants. No differences are observed in mitochondria and other organelles (results not shown).

In TransH1+ plants that at the 5- to 6-week stage showed no distinctive morphological differences compared with TransH1− plants, strongly heterochromatinized nuclei occurred at that stage only sporadically in phloem parenchymal cells. However, the amount of leaf parenchymal cells with distinctive heterochromatinization of nuclei increased dramatically in these plants at the stage of flowering (30–32 weeks). The frequency and ultrastructural appearance of highly heterochromatinized nuclei in leaf tissues at that stage resembled closely those occurring at the 5- to 6-week stage in the two TransH1+ plants with severe phenotypes. In none of the control TransH1− plants examined at the same late stage was there any visible increase of heterochromatinization.

DISCUSSION

So far the effect of overexpression of histone H1 gene has been studied in unicellular organism (yeast) and in cultured mammalian cells. The level of H1 protein overexpressed in these cells was either very low (7) or at best reached slightly over 1 molecule per nucleosome (6). In the present study it was possible to follow the development of complex multicellular organisms in which the stoichiometry of linker histone to DNA in chromatin and the profile of major linker histone variants were drastically perturbed. The overexpression of *Arabidopsis* H1 in tobacco led to 2.5-fold (on average) increase over the physiological level of the total linker histone-to-DNA ratio. The fact that similar amounts of transgenic H1 were extracted from whole tissues and from isolated chromatin indicates that most if not all of the extra H1 was located in chromatin. Chromatin or nuclear location of overexpressed H1 has been reported for yeast and mammalian cells also (6, 5). This is compatible with the existence of secondary H1 binding sites in chromatin (25). The expression of the *Arabidopsis* H1 in tobacco was correlated with gross reduction of the chromatin level of native H1. In *Arabidopsis* H1-overexpressing plants tobacco H1 varied from 55% of the physiological level to undetectable. The latter probably amounts to complete replacement of all major native variants of H1 for heterologous variant derived from an organism of a different family. The overexpressed *Arabidopsis* histone H1 and the native tobacco H1 (26) have similar globular domains, but *Arabidopsis* H1 has about 15% less positive charge in the C-terminal tail. While it is not likely that the observed drop in the native H1 results from down-regulation at the transcriptional level, we cannot distinguish between inhibition at the translational level and the dominant negative mutation type effect caused by the competition of transgenic and native H1s for chromatin binding sites.

The phenotypes of plants that overexpressed heterologous H1 varied. Some plants were only slightly different from controls, some had distinctive defects in middle/late stages of flowering but relatively normal look, and some had visibly changed overall appearance and severe defects at the early stages of flowering. It is important to note that the severe phenotypes that occurred in two independent transformation experiments were strikingly similar. The extent of phenotypic change correlated neither with the minor differences in the ratio of total linker histone to DNA, which occurred among plants that overexpressed H1, nor with the ratio of exogenous to native H1. The only difference between plants with severe

and milder phenotypes was in the stage of development at which the extensive and highly unusual heterochromatinization of nuclei was first noticed. In plants with severe phenotypes the heterochromatinization was already present at the 5- to 6-week stage. In plants with milder phenotypes that contained similar amounts of extra H1 it occurred considerably later. While we have not conducted the systematic ultrastructure examination from the earliest stages of development of TransH1+ plants, the above results are indicative of the positive correlation between the timing of the extensive heterochromatinization of nuclei and the severity of the phenotypic change.

Our observations have several implications concerning the possible role of H1 as a general repressor of cellular transcription.

The fact that cell division, growth, and the early stages of development, as well as differentiation into major organs and tissues, can occur in the presence of a large overdose of linker histones indicates that, at least in plants, the H1-to-DNA ratio is not a critical factor in determination of the state of transcription repression/derepression of the majority of genes. However, the reproducibility of the correlation between the occurrence of the extra H1 and the perturbations of the same selected phenotypic features of the plant (the ability to attain the correct growth pattern and to complete the flowering program) is indicative of the dependence of some specific functions (presumably important later in development) on chromatin structural changes which are influenced by H1-to-DNA ratio. This suggestion is supported by the observation that there is a correlation between the time of the occurrence of the phenotypic changes and the extensive heterochromatinization of nuclei. The lack of any accompanying change in nucleosomal spacing supports the view that the effect of extra H1 is exerted on the level of higher-order chromatin structures.

We think that the observed differences in time of the occurrence of heterochromatinization in individual plants that overexpress similar amounts and type of H1 reflect the developmentally imposed differences in chromatins of the initial cells from which transgenic plants were regenerated. It is probably not a coincidence that all milder phenotypes occurred in plants obtained upon transformation of young seedlings, which, in addition to already differentiated cells, contain many meristematic cells. It is known that, while older plant tissues require redetermination of already differentiated cells to achieve the embryogenic state, the cells of young, meristematic tissues can start embryogenesis directly (27). The differentiated state in both plants and animals has been linked with changes in stable heterochromatinization brought up by the presence of specific condensing factors such as the methylation of DNA (28) or (in animals) the products of the *Polycomb* group genes (29, 30). The chromatin of older differentiated cells (these are the only cells present in discs cut from mature leaf), despite redetermination occurring prior to the beginning of somatic embryogenesis, could retain (perhaps in different configuration) some of the original condensing factors. This in turn could stimulate earlier heterochromatinization in the presence of extra H1.

The complete replacement of native by heterologous H1 has no additional phenotypic effects compared with the situation of partial replacement. This observation supports the view that differences between major linker histones may be of limited functional importance.

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