Distribution of linker histone variants during plant cell differentiation in the developmental zones of the maize root, dedifferentiation in callus culture after auxin treatment

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ABSTRACT

Although several linker histone variants have been studied in both animal and plant organisms, little is known about their distribution during processes that involve alterations in chromatin function, such as differentiation, dedifferentiation and hormone treatment. In this study, we identified linker histone variants by using specific anti-histone H1 antibodies. Each variant's ratio to total H1 in the three developmental zones of maize (*Zea mays* L.) root and in callus cultures derived from them was estimated in order to define possible alterations either during plant cell differentiation or during their dedifferentiation. We also evaluated linker histone variants' ratios in the developmental zones of maize roots treated with auxin in order to examine the effects of exogenous applied auxin to linker histone variant distribution. Finally, immunohistochemical detection was used to identify the root tissues containing each variant and correlate them with the physiological status of the plant cells. According to the results presented in this study, linker histone variants' ratios are altered in the developmental zones of maize root, while they are similar to the meristematic zone in samples from callus cultures and to the differentiation zone in samples from roots treated with auxin. We propose that the alterations in linker histone variants' ratios are correlated with plant cell differentiation and dedifferentiation.

Key terms: callus culture, linker histone, plant cell differentiation, plant hormone; *Zea mays*

INTRODUCTION

The primary level of DNA compaction into the chromatin of eukaryotic cells is the nucleosome, where 146bp of DNA are wrapped around a histone octamer core, comprised of two of each histones, H2A, H2B, H3 and H4, while its structure is completed with the association of H1 linker histone (Wolffe and Guschin, 2000). Each histone class, with the exception of H4, is represented by a family of structurally similar polypeptides called histone variants. Histone variants are synthesized in different relative amounts during the cell cycle, depending on the physiological status of the cell. It has been proposed that they could be

necessary in distinct chromatin functions (Gabrielli, 1989, Brown, 2001).

Linker H1 histones have been found to display greater variability when compared to core histones (Brown, 2001). This heterogeneity is conserved between eukaryotes, suggesting that individual H1 variants may have unique properties, resulting in specific roles in chromatin function (Cole, 1987, Zlatanova et al., 2000). Several H1 variants have been studied in both animal and plant organisms and most of them are considered tissue- or stage-specific, while others are accumulated during cell differentiation or in response to specific environmental signals (Jerzmanowski et al., 2000, Khochbin, 2001). For instance, in

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several liliaceous plants specific histone H1 variants appear during male meiosis (Ueda and Tanaka, 1995), other variants expressed during water deficit have been identified in several monocots and dicots (Scippa et al., 2004) whereas a specific H1 variant has been found to localize in nucleoli of higher plant cells (Tanaka et al., 1999).

Although linker histones have been studied in several plant species (Ivanchenko et al., 1987, Srebrevia et al., 1989, Tanaka et al., 1999, Jerzmanowski et al., 2000, Scippa et al., 2004), little is known about their spacial distribution during plant cell differentiation and dedifferentiation, processes that involve several alterations in chromatin structure and function. Even less is known about the effect of exogenous applied hormones on linker histone distribution, although there is evidence about alterations in histone H1 patterns after hormone treatment of animal cells (Wurtz, 1985). Plant hormones are compounds known to regulate almost all aspects of plant development while auxin was the first plant hormone discovered (Berleth et al., 2004, Chow and McCourt, 2004). Auxin is known to play a significant role in plant growth, apex domination, root and shoot branching, xylem and ploid differentiation and plant response to environmental signals like gravity and light (Went, 1974, Friml, 2003). Auxin regulates cell division, elongation, polarity and differentiation through the control of several response genes' expression (Guilfoyle & Hagen, 2001, Leyser, 2001).

In order to define possible alterations in linker histone H1 distribution primarily during plant cell differentiation and subsequently during dedifferentiation, we estimated the ratios of each histone H1 variant to total linker histone in each of the three developmental zones of maize (*Zea mays* L.) root and in callus cultures derived from them. We also estimated the above ratios in samples derived from roots treated with auxin in order to define alterations during auxin treatment. Finally, we used immunohistochemical analysis to directly define the tissues containing each linker histone variant in order to correlate them with the physiological status of the plant cells.

METHODS

Plant material, auxin treatment and in vitro callus culture

Maize seeds (*Zea mays* L., cv. Polaris) were germinated in the dark at 28 °C on paper sheets soaked with distilled water, until root's length reached approximately 3-5 cm. At this time, the three developmental zones (meristematic zone, 2 mm; elongation zone, 2-6 mm; differentiation zone, 6 mm-2 cm; also see Fig. 1) were separately collected and stored at -70 °C (Burkhanova et al., 1975). Maize seeds were also germinated under the same conditions on paper sheets soaked with 0.01mM indoleacetic acid and were collected the same way. Callus cultures were developed from root segments, representing the three developmental zones, which were placed under aseptic conditions in glass tubes containing sterilized Murashige and Skoog medium (Murashige and Skoog, 1962) that was supplemented with 1 mg.L⁻¹ 2,4-dichlorophenoxyacetic acid (Sigma) and 3% (w/v) sucrose and solidified with 0.5% (w/v) agar. Calli were developed in the dark at 28 °C for approximately two months and then were collected separately and stored at -70 °C.

Nuclei isolation and histone extraction

Nuclei isolation was performed according to Muller et al. (1980). Plant material from each zone or callus culture was homogenized with five volumes of Buffer A (50 mM Tris-HCl pH 8.0, 4 mM (CH₃COOH)₂Mg.4H₂O, 0.25 M sucrose, 2% (w/v) Arabic gum, 0.5% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% (v/v) dimethyl sulfoxide (DMSO) and 5 mM β-mercaptoethanol), filtered through Nylon Screen Mesh 25μ filter and centrifuged at 900g for 30 min. The pellet was suspended in Buffer A (without Arabic gum), placed over Buffer B (50 mM Tris-HCl pH 8.0, 4 mM (CH₃COOH)₂Mg.4H₂O, 1.2 M sucrose, 1 mM PMSF, 0.5% (v/v) DMSO and 5 mM β-mercaptoethanol) and centrifuged at 900g for another 30 min. Nuclei were washed

with Buffer A (without Arabic gum and Triton X-100), centrifuged at 900g and afterwards were suspended in TBS (10 mM Tris-HCl pH 7.5, 150 mM NaCl) and centrifuged at 20,000g for 30 min. Histones were extracted as described by Murray and Key (1978). The pellet was suspended in 0.4 N H₂SO₄, stirred for 1h and centrifuged at 20,000g for 30 min. Four volumes of cold ethanol were added to the supernatant and histones precipitated in approximately 48 h at -20 °C. The precipitant was washed with 70% ethanol, ethanol/ether (3/1, v/v) and acetone. Each step was followed by centrifugation at 20,000g for 30 min and was performed twice. The samples were vacuum dried and stored at -20 °C. The amount of protein was determined with the method described by Lowry et al. (1951).

Electrophoretic analysis of histone preparations

Extracted proteins were initially analyzed on SDS-containing 15% polyacrylamide

gels as described by Laemmli (1970). Approximately 10 µg of total protein was loaded on each lane and prestained protein markers (New England Biolabs) were used to determine their molecular masses. Histone variants and modified forms were separated in two-dimensional AUT/AUC-PAGE (acetic acid-urea-Triton X-100/ acetic acid-urea-cetyltrimethylammonium bromide [CTAB]) as described by Bonner et al. (1980). First dimension was performed in gel consisting of 12% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide, 1 M acetic acid, 6 M urea, 0.625% (v/v) Triton X-100, 45 mM NH₄OH, 0.5% (v/v) N,N,N',N'-tetramethyl-ethylenediamine (TEMED) and 0.027% (v/v) riboflavin, running buffer containing 1 M acetic acid and 0.1 M glycine and the samples were diluted in loading buffer consisting of 1 M acetic acid, 6 M urea, 45 mM NH₄OH and 15 mM β-mercaptoethanol. Approximately 50 µg of total protein were loaded on the gel and electrophoresis was performed at 210 V for approximately 1 h. Afterwards,

Figure 1: A longitudinal view of maize root: meristematic zone (2mm), elongation zone (2mm-6mm) and differentiation zone (6mm-2cm). The regions of sectioning are indicated, while (M), (E) and (D) are representative transversal sections of meristematic, elongation and differentiation zone respectively.

strips containing the samples were cut, stained with 0.1% Coomassie Brilliant Blue R-250 in 40% ethanol, 5% acetic acid and 0.1% (w/v) cysteamine for 5 min, destained in 20% ethanol, 5% acetic acid and 0.1% (w/v) cysteamine for 10 min and soaked in 1 M acetic acid, 5 mM NH_4OH and 0.5% (w/v) cysteamine for 20 min. Each strip was placed on the top of a second dimension gel, consisting of 16.5% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide, 1 M acetic acid, 6 M urea, 45 mM NH_4OH , 0.5% (v/v) TEMED and 0.027% (v/v) riboflavin and 0.15% (w/v) CTAB was added in upper tank's running buffer. The electrophoresis was performed at 210 V for approximately 2 h. Two-dimensional gels were stained with Coomassie Brilliant Blue R-250 or alternatively with silver nitrate (Wray et al., 1981).

Western blotting

Histone H1 immunodetection was performed after electrophoresis and western blotting according to Towbin et al. (1979). Proteins from SDS-PAGE gels were transferred onto PVDF membranes using buffer containing 25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol at 100 V for 1 h, while proteins from AUT/AUC-PAGE gels were transferred using buffer containing 0.7% (v/v) acetic acid, 20% methanol at 60 V for 1 h. Afterwards, membranes were washed twice in PBST (0.1% (v/v) Tween20 in PBS) and then incubated for 2 h in blocking solution (5% (w/v) non-fat dry milk in PBST). The polyclonal antibodies used in this study were raised against mouse total histone H1, the H1 main variants H1(A+B) and H1⁰ variant and were diluted 1:1000. The specificity of the antibodies and their immunoreactivity with plant linker histones have been described in detail elsewhere (Ivanchenko et al., 1987, Srebrevia et al., 1989). The reaction was carried out at room temperature for 2 h. Following removal of excess antibody by washing three times with PBST, the membranes were incubated with the second antibody (horseradish peroxidase labeled goat anti-rabbit, diluted 1:2000). After the washes as above, the

immunoblots were visualized using enhanced chemiluminescence.

Densitometry and statistical analysis

After identifying linker histone variants, the stained gels were scanned and analyzed by means of Gel Pro Analyzer 3.1 software (Media Cybernetics). The optical density of each spot representing histone H1 variant was measured and its portion of total linker histone in each sample was evaluated. Each sample was derived from an individual extraction and three independent experiments (i.e. histone extraction, electrophoresis and gel analysis) were performed for each of the developmental zones and callus cultures in order to examine the reproducibility of the results and to estimate the average and the standard deviation of the quantification analysis. A Student's *t*-test, suitable for groups of data having different averages and variations, with an error probability of less than 5%, was applied in order to assess the statistical significance of the differences among the results.

Immunohistochemistry

Maize roots were embedded in paraffin according to Yang et al. (1991). Firstly, roots were fixed in phosphate solution pH=6.8 containing 100 mM NaCl, 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde. Afterwards, they were dehydrated and cleared in increasing concentration solutions of ethanol and xylene respectively and stained with 5% (w/v) safranin in 50% ethanol. Xylene was saturated with paraffin at RT, then at 42 °C and finally the vials were transferred at 60 °C and the mixture (xylene and paraffin) was replaced by melted and filtered paraffin. Paraffin was refreshed twice a day for the next ten days and after this, vials' content (roots and paraffin) was transferred onto cold templates and stored at 4 °C. Ten-micrometer sections were taken by means of a microtome and placed onto glass slides which had previously been washed extensively, sterilized and coated with poly-L-lysine solution (0.01% (w/v) poly-L-

lysine (Mol. Wt. 30,000-70,000, Sigma) in 10 mM Tris-HCl, pH=8). They were placed overnight onto a 42 °C surface and then in xylene, xylene/ethanol (1/1, v/v) and ethanol and were stored at -20 °C. Immunodetection of linker histones was performed in the same way as PVDF membranes, except that the second antibody was alkaline phosphatase labeled and that nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solutions diluted in alkaline phosphatase buffer (10 mM Tris-HCl pH 9.2, 100 mM NaCl, 50 mM MgCl₂) were used for detection. After detection, sections were dehydrated in increasing concentration solutions of ethanol, photographed by means of a digital camera and stored at RT.

RESULTS

Histone separation and immunodetection

Maize root is an appropriate biological system for developmental studies since it consists of three zones, which can be easily separated (Fig. 1). Meristematic and elongation zones contain proliferating cells and the primary root tissues respectively, whereas differentiation zone consists mainly of parenchymatic, non-proliferating

cells (Burkhanova et al., 1975). Histones were extracted from each of the three developmental zones in order to define possible alterations in linker histone variants distribution during plant cell differentiation. They were separated in 15% SDS-PAGE gels and western blotting was performed with polyclonal antibodies against total histone H1, the H1 main variants H1(A+B) and H1⁰ variant (Ivanchenko et al., 1987, Srebrevia et al., 1989). Linker histones were identified as three bands (Fig. 2B), with molecular masses of approximately 43-, 39- and 37kDa. The 43- and 39kDa bands were also recognized by the H1(A+B) antibody (Fig. 2C) and the 37kDa band was recognized by the H1⁰ antibody (Fig. 2D). At the same time no reaction was observed with any other protein in the preparation, suggesting that the fractions that gave a positive reaction represent molecules belonging to the H1 family. Two-dimensional AUT/AUC-PAGE was used in order to separate histone variants and their posttranslational modified forms (Fig. 3A) and immunodetection was performed with the anti-histone H1 antiserum. As shown in Figure 3B, the antiserum only stained the H1 histones, which were identified in the central region of the gel, above the core histones.

Figure 2: A representative SDS-PAGE gel (A) and immunoblotting detection of total H1 (B), H1(A+B) (C) and H1⁰ (D). Prestained protein markers were used to determine the molecular mass of each histone protein. The sample presented was derived from meristematic zone.

Figure 3: A representative two-dimensional AUT/AUC-PAGE gel (A) and immunoblotting detection of histone H1 (B). The sample presented was derived from differentiation zone.

Linker histone variant distribution in the developmental zones of maize root

After identifying linker histones, two-dimensional gels were scanned and analyzed by means of Gel Pro Analyzer 3.1 software, in order to indirectly estimate each histone H1 variant's ratio in maize root developmental zones. The optical density of each spot representing the histone H1 variants was measured and each variant's portion of total histone H1 in each sample was evaluated. Each sample was derived from an independent experiment and three samples from each of the developmental zones and callus cultures were analyzed in order to estimate the average and the standard deviation of the measurement. A Student *t*-test was applied to assess the statistical significance of the differences between the variants' ratios. According to gel analysis, in samples derived from meristematic and elongation zone, the two main histone H1 variants represented approximately 41% and 34% of total histone H1, while H1⁰ variant represented approximately 25%. This ratio was altered in samples derived from differentiation zone, where H1⁰ ratio was increased up to 34% and the ratios of the main variants were decreased to 35% and 31% respectively (Fig. 4A).

Linker histone variants distribution in callus culture cells

In order to study linker histone variants distribution during plant cell dedifferentiation, callus cultures were developed from root segments representing the three developmental zones. Histones were extracted from each culture, separated in two-dimensional gels and linker histones were identified. Two-dimensional gels were scanned and each variant's ratio to total histone H1 was estimated in three independent experiments as described above. According to gel analysis, the linker histone variants ratios in callus cultures were similar to those derived from meristematic zone (as is shown in Fig. 4A), i.e. the two main histone H1 variants represented approximately 41% and 34% while H1⁰ variant represented approximately 25% of total histone H1, regardless of the developmental zone from which calli were derived (Fig. 4B).

Linker histone variants distribution in maize root zones after auxin treatment

We also wished to probe any possible deviation in linker histone variant distribution in each of the three developmental zones upon exogenous

Figure 4: Histone H1 variants (H1A; H1B; H1⁰) ratio to total H1 in samples derived from the developmental zones (M: meristematic zone; E: elongation zone; D: differentiation zone) of maize root (A), callus cultures derived from each zone (B) and from the developmental zones of roots treated with auxin (C). Each value is the average of three independent experiments and bars indicate the standard deviation of the measurement. Values significantly different at $p < 0.05$ from corresponding values measured in samples derived from the meristematic zone of control roots are indicated by asterisks.

applied auxin. As shown in Figure 6, roots developed on paper sheets soaked with 0.01mM indole-acetic acid are significantly different in length and diameter from roots developed on paper sheets soaked with distilled water. Histones were extracted from each of the three developmental zones from roots treated with auxin and each variant's ratio to total histone H1 was estimated in three independent experiments as described above. According to gel analysis (Fig. 4C) after auxin treatment the H1⁰ ratio in samples derived from meristematic and elongation zone was increased to above 30%, and the main variants ratios were decreased by comparison with untreated roots (see Figure 4A). In contrast, there were no significant differences between samples derived from the differentiation zone of controls and roots treated with auxin (Fig. 4C).

Immunohistochemical detection of histone H1 in maize root sections

Finally, we used immunohistochemical detection in maize root sections in order to identify the tissues containing the linker histone variants. In contrast to meristematic and elongation zones, the cells within differentiation zone are known to form distinct structures. Vascular cylinder, containing xylem, phloem and pith is surrounded by pericycle, endodermis and cortex, while exodermis and epidermis are the exterior root tissues (Hochholdinger et al. 2004). After staining with anti-histone H1 antibodies, histone H1 immunoreactivity was always nuclear and the staining intensity was usually high and uniform. We observed that the main histone H1 variants were present in every cell of the root, regardless of their tissue-type, physiological state or proliferation rate (Fig. 5C, G, K and O). In contrast to main histone H1 variants, H1⁰ was localized mainly in differentiated cells, like the parenchymatic cells of cortex and pith or epidermal cells, displaying strong immunostaining, while it was absent in pericycle cells (Fig. 5L and P). However, a faint immunoreaction was obtained in sections from meristematic and elongation

zones treated with anti-H1⁰ antibodies (Fig. 5D and H) compared to those treated with total H1 and H1(A+B) antibodies (Fig. 5B, C, F and G). Detection without primary

antibody (Fig. 5A, E, I and M) and with total H1 antibody (Fig. 5B, F, J and N) was also performed in maize root sections as negative and positive controls.

Figure 5: Immunohistochemical detection of linker histones in maize root sections from meristematic (sections A-D), elongation (sections E-H) and differentiation zone (sections I-P). Detection without primary antibody and with total H1 antibody were performed in maize root sections as negative and positive controls (sections A, E, I, M and B, F, J, N respectively). The main histone H1 variants are present in every cell of the root (sections C, G, K and O) while H1⁰ is detected mainly in differentiated cells, like the parenchymatic cells of cortex and pith (sections L and P). However, a less intense H1⁰ signal is also observed in sections from the meristematic and elongation zone (sections D and H). The sections from the meristematic zone presented were taken at approximately at 1mm from the root tip while the sections from elongation and differentiation zone were taken at approximately 3mm and 1cm from the root tip, respectively (ep: epidermis; co: cortex; pc: pericycle; pi: pith).

DISCUSSION

The biochemical and immunological analysis of the distribution of linker histones in the developmental zones of maize root was performed by two types of electrophoresis and by using specific anti-histone H1 antibodies. An important result of these experiments was the occurrence of histone H1⁰ variant in samples isolated from meristematic zone (Fig. 2D), a fact that was also supported by the immunohistochemical analysis that showed positive H1⁰-immunoreactivity of the meristematic cells (Fig. 5D). The relative amounts of histone H1 variants in the maize root's developmental zones were determined by densitometry of the stained gels and found to be altered during plant cell differentiation. The quantitative estimations presented in Figure 4A show that the H1⁰ variant ratio was 25% in meristematic and elongation zone and increased in samples derived from differentiation zone up to 34%. At the same time, the ratios of the main histone variants (H1A and H1B) were decreased during plant cell differentiation. The H1⁰ linker histone variant was originally discovered in tissues of low proliferation rate in mammals and shown to accumulate in terminally differentiated cells. The protein was later found in all classes of animals as well as in plant organisms and it is now widely accepted that its accumulation is correlated with the transition of the cells to the state of terminal differentiation (Zlatanova and Doenecke, 1994).

It is known that during callus formation, differentiated plant cells can be transformed into callus culture cells, which restore their meristematic properties, especially the ability for rapid proliferation (Koleva et al., 1982, Williams et al., 2003). According to the results presented in Figure 4B, the linker histone variants' ratios during plant cell dedifferentiation in callus culture were similar to meristematic zone, regardless of the developmental zone from which calli were derived. The main H1 variants, H1A and H1B, represent approximately 41% and 34% while H1⁰ variant represent 25%

of total histone H1. The occurrence of the differentiation-related H1⁰ variant in the proliferating cells of callus cultures might be necessary in the proper function of cells due to its cell cycle-independent expression and ability to replace other linker histone variants, a process that is possible to alter chromatin structures in specific regions.

In contrast to callus formation, auxin treatment is known to promote the opposite process, i.e. root differentiation, which results in both morphological (e.g. longer and thicker roots, as shown in Figure 6) and physiological differences among auxin treated and control plants (Raghavan, 1999). Auxin is known to play a significant role in phloem and xylem differentiation and also in patterned differentiation of cells in meristems (Berleth et al., 2004). According to the results presented in Figure 4C, the linker histone variants' ratios in samples from roots treated with auxin were similar to that obtained in differentiation zone. In particular, the H1⁰ linker histone variant's levels were increased to above 30% of the total H1, regardless of the developmental zone from which the samples were derived. Alterations in histone H1 pattern have been observed during hormone-induced cell proliferation in mouse mammary glands as well as during hormone-induced differentiation of mouse myeloid leukemia cells. Histone H1 variants' ratios exhibited differences in samples from hormone-treated and non-treated cells and it has been suggested that the alterations observed could be due to rearrangements of chromatin that take place during hormone stimulation (Wurtz, 1985).

Linker histones are known to play an important role in chromatin structure by stabilising the higher order structure of chromatin fiber (Wolffe and Guschin, 2000) and it has been proposed that they also participate in transcriptional regulation of specific genes by allowing or preventing the access of transcription factors to DNA template (Zlatanova et al., 2000). The remarkable heterogeneity of linker histones suggests an important role in chromatin

function, considering that individual variants might participate in distinct processes, although evidence for the opposite has been also reported (Khochbin, 2001). If this is the case, the relative amounts of H1 variants would be altered during processes that imply changes in chromatin function, such as cell differentiation and/or dedifferentiation. Both plant cell differentiation, either programmed or induced by plant hormones, and dedifferentiation result in alterations in the physiological state of the cell and consequently in chromatin function that probably necessitate distinct variants' ratios during each process.

Figure 6: Morphological differences between a representative root developed in the dark at 28 C° on paper sheets soaked with 0.01mM indole-acetic acid and a representative root developed under the same conditions on paper sheets soaked with distilled water.

The results presented in this study indicate that linker histone variant distribution is altered during plant cell differentiation and dedifferentiation. The variants' ratios in each case probably depend on the physiological state of plant cells and are similar to meristematic cells during callus formation while they resemble differentiated cells during auxin treatment. Future studies in this field will probably enrich our knowledge about the precise role of linker histone variants in these processes.

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