Alterations in core histone variant ratios during maize root differentiation, callus formation and in response to plant hormone treatment

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ABSTRACT

Although several 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 evaluated the ratio of each histone variant in each of the four core histone classes in the three developmental zones of maize (*Zea mays* L.) root and in callus cultures derived from them, in order to define possible alterations either during plant cell differentiation or dedifferentiation. We also evaluated core histone variant ratios in the developmental zones of roots treated with auxin and gibberellin in order to examine the effects of exogenously applied plant hormones to histone variant distribution. Finally, immunohistochemical detection was used to identify the root tissues containing modified forms of core histones and correlates them with the physiological status of the plant cells. According to the results presented in this study, histone variant ratios are altered in all the cases examined, i.e. in the developmental zones of maize root, in callus cultures derived from them and in the developmental zones of roots treated either with auxin or gibberellin. We propose that the alterations in linker histone variant ratios are correlated with plant cell differentiation and physiological status in each case.

Key terms: callus culture, histone variant, 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 core 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 and it has been proposed that they could be necessary in distinct chromatin functions (Gabrielli, 1989, Brown, 2001). On the other hand, histone covalent modifications, such

as acetylation, phosphorylation, methylation and ubiquitination, provide another level of heterogeneity to these proteins and it is well established that they also play important roles in all chromatin functions (Strahl and Allis, 2000, Berger, 2001). Although histone variants and modifications have been identified and studied in plants (Waterborg, 1993, Jermanowski et al., 2000, Loild, 2004), little is known about their spatial distribution during plant cell differentiation and dedifferentiation, processes that involve several alterations in chromatin structure and function. However, alterations in chromatin composition between meristematic and differentiated plant cells and during callus formation have been observed in previous studies (Burkhanova et al., 1975, Koleva et al., 1982, Williams et al. 2003).

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Even less is known about the effect of exogenously applied hormones on histone variant distribution, although there is evidence about alterations in histone pattern after hormone treatment of animal cells (Wurtz, 1985). Expression of distinct histone variants induced by plant hormone treatment, as well as alterations in histone modification level have been also reported in plants (van der Knaap and Kende, 1995, van den Heuvel et al., 1999, Sokol et al., 2007, Chinnusamy et al., 2008). Plant hormones are compounds known to regulate almost all aspects of plant development (Berleth et al., 2004, Chow and McCourt, 2004). Auxin was the first plant hormone discovered and is known to play a significant role in plant growth, apex domination, root and shoot branching, xylem and phloem differentiation and plant response to environmental signals, such as gravity and light (Friml, 2003). Auxin regulates cell division, elongation, polarity and differentiation through controlling the expression of several response genes (Guilfoyle and Hagen, 2001, Leyser, 2001). Gibberellins, a large family of over one hundred diterpenoid compounds, have also been associated with several developmental processes. It is well established that gibberellins are necessary in seed germination, root and shoot elongation, flowering and fruit development, acting through a complicated signaling pathway and the regulation of several response genes (Gomi and Matsuoka, 2003, Fleet and Sun, 2005).

In order to define possible alterations in histone variant distribution, primarily during plant cell differentiation and subsequently during dedifferentiation, we estimated the ratio of each histone variant in each histone class in 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 either with auxin or gibberellin in order to define the effect of exogenous applied plant hormones on histone variant distribution. Finally, we used immunohistochemical analysis to directly define root tissues containing the modified forms of core

histones in order to correlate them with the physiological status of plant cells.

METHODS

Plant material, plant hormone treatment and in vitro callus culture

Maize seeds (*Zea mays* L., cv. Polaris) were germinated in darkness at 28°C on paper sheets soaked with distilled water, until root length reached approximately 3-5 cm. At that point, the three developmental zones (meristematic zone, 2 mm; elongation zone, 2-6 mm; differentiation zone, the rest of the root; 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 either with 0.01mM indole-acetic acid (auxin-treated roots) or 0.01 mM gibberellic acid (gibberellin-treated roots) 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 darkness at 28°C for approximately two months and were then collected separately and stored at -70°C, while part of each callus was placed in new tubes containing MS medium to generate the second or third subculture.

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) gum arabic, 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 gum arabic), placed over Buffer B (50 mM Tris-HCl pH 8.0, 4 mM $(\text{CH}_3\text{COOH})_2\text{Mg} \cdot 4\text{H}_2\text{O}$, 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 gum arabic and Triton X-100), centrifuged at 900g and afterwards 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 H_2SO_4 , 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 acetic acid-urea-containing polyacrylamide gels. Approximately 10 μg of total protein, diluted in loading buffer consisting of 1 M acetic acid, 6 M urea, 45 mM NH_4OH and 15 mM β -mercaptoethanol, was loaded on the 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 electrophoresis was performed at 210 V for approximately 1 h. Subsequently, histone variants and modified forms were separated in two-dimensional AUT/AUC-PAGE

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

(acetic acid-urea-Triton X-100/acetic acid-urea-cetyltrimethylammonium bromide [CTAB]) as described by Bonner et al. (1980). Approximately 50 µg of total protein was loaded on the first dimension 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'-tetramethylethylenediamine (TEMED) and 0.027% (v/v) riboflavin, the running buffer was 1 M acetic acid and 0.1 M glycine and electrophoresis was performed at 210 V for approximately 1 h. Afterwards, 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 NH₄OH 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 NH₄OH, 0.5% (v/v) TEMED and 0.027% (v/v) riboflavin and 0.15% (w/v) CTAB was added to upper 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 immunodetection was performed after electrophoresis and western blotting according to Towbin et al. (1979). Proteins from acetic acid-urea containing gels and two dimensional 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 antibodies employed were against histone H3 (monoclonal, a generous gift by Jockers-Wretou and co-workers, diluted 1: 5000) (Muller et al., 1985), histone H2B (polyclonal, a generous gift by Sekeri-Pataryas and co-workers, diluted 1:

100) (Sourlingas et al., 2003), histone H4 (polyclonal, Cell Signaling Technology, diluted 1: 2000), the phosphorylated and the acetylated forms of histone H3 (Cell Signaling Technology and Upstate Biotechnology respectively, both diluted 1: 2000) and the acetylated form of histone H4 (Serotek, diluted 1: 2000). Immunodetection of histone H2A and histone H1 have been described in detail elsewhere (Alatzas & Foundouli, 2006, Alatzas et al., 2008). 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 washings as above, the immunoblots were visualized using enhanced chemiluminescence.

Densitometry and statistical analysis

After identifying histone variants by western blotting, the Coomassie- or silver nitrate-stained gels were scanned and analyzed by means of Gel Pro Analyzer 3.1 software (Media Cybernetics) as described elsewhere (Alatzas et al., 2008). The optical density of each spot representing histone variant was measured and its portion of total histone class in each sample was evaluated. Each sample was derived from an individual extraction and three independent experiments (i.e. plant material collection, 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 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, the vial contents (roots and paraffin) were 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 stored at -20°C. Immunodetection of modified histone forms was performed in the same way as with 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. The antibodies employed were against the phosphorylated and the acetylated form of histone H3 (Cell

Signaling Technology and Upstate Biotechnology, respectively) and the acetylated form of histone H4 (Serotek), all diluted 1: 2000. 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

Histones were extracted from each of the three developmental zones of maize root in order to define possible alterations in histone variant distribution during plant cell differentiation. They were separated in acetic acid-urea containing polyacrylamide gels, where protein mobility is based on the mass/charge ratio and western blotting was performed with polyclonal antibodies against each histone class (Fig. 2). By means of specific antibodies, we identified two variants and two modified forms of histone H3, two variants of histone H2B, histone H4 and the acetylated form of H4. Linker histones were identified as two bands, one of them also recognized by the H1(A+B) antibody and the other by the H1⁰ antibody, while two bands were recognized by the H2A antibody. Two-dimensional AUT/AUC-PAGE (acetic acid-urea-Triton X-100/acetic acid-urea-CTAB), as described by Bonner et al. (1980), was performed in order to separate histone variants and modified forms (Fig.

Figure 2: A representative acetic acid-urea polyacrylamide gel and immunoblotting detection of the five histone classes and the modified forms of histone H3 and histone H4. The sample presented was derived from meristematic zone.

3A) and immunodetection was performed with antibodies recognizing the core histones H2B, H3 and H4. By means of these antibodies, we identified two variants of histone H2B (Fig. 3D), two variants of histone H3 and the modified forms in variant H3.2 (Fig. 3E) and histone H4 and the acetylated form of H4 (Fig. 3F). Immunodetection of linker histone H1 (Fig. 3B), histone H2A and the ubiquitinated form of H2A (Fig. 3C) has been described in detail elsewhere (Alatzas

& Foundouli, 2006, Alatzas et al., 2008). A more detailed and representative two-dimensional separation of each core histone variant in samples derived from either meristematic or differentiation zones is presented in Figure 4.

Gel densitometry and quantification analysis

After identifying histone classes, two-dimensional gels were scanned and

Figure 3: A representative two-dimensional acetic acid-urea-Triton X-100/acetic acid-urea-CTAB polyacrylamide gel (A) and immunoblotting detection of histone H1 (B), H2A (C), H2B (D), H3 (E) and H4 (F). The sample presented was derived from the differentiation zone of the root.

analyzed by means of Gel Pro Analyzer 3.1 software, in order to indirectly estimate the ratio of each histone variant in maize root developmental zones. The optical density of each spot representing a histone variant or modified form was measured and its portion of total histone class 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 among the variant ratios.

According to gel analysis, the distribution of histone H2A was altered in the developmental zones of maize root (see Fig. 5). A slight increase in the H2A.1 ratio and a significant decrease in the ubiquitinated H2A ratio were observed in samples from the differentiation zone, compared to samples from the two other zones. Histone H2A distribution pattern in callus cultures, with the exception of first callus culture, resembled the pattern of the meristematic

zone; i.e. the H2A.1 and H2A.2 variants and the uH2A represented approximately 48%, 33% and 19%, respectively. In samples from first callus cultures derived from the differentiation zone, an increase in uH2A to above 27% was observed, which decreased to 19% of total H2A in the second and third callus subcultures, an observation discussed in detail elsewhere (Alatzas & Foundouli, 2006). The distribution of histone H2A was also altered in samples from roots treated with plant hormones. A significant increase, compared to samples from controls, was observed in main variant H2A.1 ratio to almost 65% in roots treated with auxin and to above 60% in roots treated with gibberellin, while the other variant H2A.2 ratio decreased to below 25% in both cases. The ubiquitinated H2A ratio decreased to approximately 13% regardless of the zone from which samples were derived, similar to the percentage of samples from the differentiation zone of untreated roots, with the exception of the meristematic zone of gibberellin-treated roots, where it remained at the level of controls i.e. approximately 20% of total H2A.

Figure 4: Representative two-dimensional separation of each core histone variant in samples derived from either the meristematic (upper panel) or differentiation zone (lower panel). Histones H2A and H2B have two variants each (H2A.1, H2A.2 and H2B.1, H2B.2, respectively). Histone H3 also has two variants (H3.1 and H3.2) and two modified forms in H3.2 variant (unmodified, phosphorylated and phospho-acetylated forms of H3 are indicated by 0, 1 and 2, respectively). Finally, histone H4 has the unmodified and the acetylated form (indicated as acH4).

Histone variants H2B.1 and H2B.2 represented approximately 60% and 40% of total H2B in samples from the meristematic and elongation zones, but these ratios changed to 53% and 47%, respectively, in the differentiation zone. In callus cultures, the H2B variant ratios were similar to the ratios observed in samples from the meristematic zone. Histone variant H2B.1 represented approximately 53% of total H2B in samples derived from the meristematic zone of roots treated with auxin and 60% in samples from the two other zones. In samples derived from the meristematic and differentiation zones of roots treated with gibberellin, the histone variant H2B.1 ratio also increased, compared to controls, while in samples derived from the elongation zone, each of

H2B variants represented approximately 50% of total H2B (Fig. 6).

According to gel analysis, histone H3 variant distribution is also altered during plant cell differentiation. A dramatic decrease in the H3.1 ratio was observed in the differentiation zone, where it represented only 18% of total H3, while in the meristematic and elongation zones, the ratio was approximately 30%. On the other hand, an increase in the H3.2 ratio, accompanied by a slight increase in the modified form ratio was observed in samples from the differentiation zone, compared to samples from the meristematic and elongation zones. Histone H3 variant distribution in callus cultures, in contrast to the other histone classes, was considerably different from that of the meristematic zone

Figure 5: Histone H2A variants and ubiquitinated H2A ratio to total H2A in samples derived from the developmental zones of maize root (M: meristematic zone; E: elongation zone; D: differentiation zone), callus cultures derived from each zone (average of second and third callus subcultures) and from the developmental zones of roots treated with auxin and gibberellin. Each value, expressed as a percentage of total H2A, 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.

of controls. In samples derived from callus cultures, histone variant H3.1 represented approximately 25% of total H3, while in the meristematic and differentiation zones of the root the above ratio was 30% and 20%, respectively. On the other hand, the histone H3 modification levels in samples from callus cultures were similar to the levels obtained in the meristematic zone of controls. The histone variant H3.1 ratio increased in samples from roots treated with auxin compared to samples from control roots. According to Fig. 7, H3.1 variant represented 37% of total H3 in meristematic and differentiation zones, and more than 40% in the elongation zone, while this ratio was only 30% in the meristematic and elongation zones and below 20% in the differentiation zone of

controls. A significant increase in the H3.1 ratio to approximately 33% was also observed in samples from the differentiation zone of roots treated with gibberellin compared to the differentiation zone of control roots, where it represented only 18% of total H3. In samples from the two other zones, The H3.1 variant ratio was similar to that of controls. On the other hand, the histone H3 modification level was also altered in samples derived from auxin- and gibberellin-treated roots, compared to controls. In particular, the phosphorylated H3 ratio increased slightly to approximately 40%, while the phosphoacetylated H3 ratio decreased significantly to approximately 10% in both cases, compared to almost 20% in samples from control roots.

Figure 6: Histone H2B variant ratios to total H2B in samples derived from the developmental zones of maize root (M: meristematic zone; E: elongation zone; D: differentiation zone), callus cultures derived from each zone (average of first, second and third callus subcultures) and from the developmental zones of roots treated with auxin and gibberellin. Each value, expressed as a percentage of total H2B, 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.

Finally, the acetylated form of histone H4 represented approximately 42% of total H4 in samples derived from the meristematic zone and the portion decreased to 37% in samples from the two other zones. In samples from callus cultures, the acetylated H4 ratio was similar to the meristematic zone, regardless of the developmental zone calli were derived from. The distribution of acetylated histone H4 also changed during plant cell differentiation after both auxin and gibberellins treatment. According to gel analysis, in roots treated with auxin, acetylated, H4 ratio was approximately 37%, regardless of the developmental zone from which samples were derived, a pattern similar to the elongation and differentiation

zone of control roots. In samples derived from gibberellin-treated roots, the acetylated H4 ratio decreased even more, to approximately 33% of total histone H4 (Fig. 8).

Immunohistochemical detection of histone modified forms in maize root sections

We used immunohistochemical detection in maize root sections in order to localize the cells containing the modified forms of core histones. In contrast to the meristematic and elongation zones, the cells within differentiation zone are known to form distinct tissues. Vascular cylinder, containing xylem, phloem and pith, are surrounded by pericycle, endodermis and

Figure 7: Histone H3 variants and modified form ratios to total H3 in samples derived from the developmental zones of maize root (M: meristematic zone; E: elongation zone; D: differentiation zone), callus cultures derived from each zone (average of first, second and third callus subcultures) and from the developmental zones of roots treated with auxin and gibberellin. Each value, expressed as a percentage of total H3, 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.

cortex, while exodermis and epidermis are the exterior root tissues (Hochholdinger et al., 2004). We observed that histone modified forms examined in this study, the phosphorylated and the acetylated form of histone H3 and the acetylated form of histone H4, were present in every cell within the meristematic and elongation zones, while they were located only in pericycle and epidermis cells in the differentiation zone. These cells are known to retain their meristematic properties, contrary to the parenchymatic, non-proliferating cells of the cortex and pith (Hochholdinger et al., 2004), where almost no modified forms were detected (Fig. 9 E-L). The same pattern was also observed after immunodetection of the ubiquitinated

H2A, as presented and discussed elsewhere (Alatzas and Foundouli, 2006). Detection without primary antibody was also performed in maize root sections in order to define any non-specific staining (Fig. 9 A, E and I).

DISCUSSION

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

Figure 8: Histone H4 and acetylated H4 ratio to total H4 in samples derived from the developmental zones of maize root (M: meristematic zone; E: elongation zone; D: differentiation zone), callus cultures derived from each zone (average of first, second and third callus subcultures) and from the developmental zones of roots treated with auxin and gibberellin. Each value, expressed as a percentage of total H4, 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.

(Hochholdinger et al., 2004). On the other hand, it is well established that during callus formation, differentiated plant cells are transformed into callus culture cells that regain the ability for rapid proliferation (Koleva et al., 1982, Williams et al., 2003). It is also known that plant hormones like auxin and gibberellin play significant roles in root growth by promoting or inhibiting root differentiation. Apart from morphological differences observed between control roots and roots treated with

auxin or gibberellin (see Figure 10), physiological and biochemical differences also exist, induced by exogenous applied plant hormones (Raghavan, 1999).

According to the results presented in this study and elsewhere (Alatzas and Foundouli, 2006, Alatzas et al., 2008), histone variant distribution is altered among developmental root zones, particularly between the meristematic and differentiation zones. There are variants like H1⁰ (Alatzas et al., 2008), H2A.1 (Fig. 5), H2B.2 (Fig. 6) and

Figure 9: Immunohistochemical detection of core histone modified forms in maize root sections from the meristematic (sections A-D) and differentiation zones (sections E-L). Detection without primary antibody was performed in maize root sections as negative controls (sections A, E and I). The phosphorylated (sections B, F and J) and the acetylated forms of histone H3 (sections C, G and K) and the acetylated form of histone H4 (sections D, H and L) are present in every cell of the meristematic zone, while in the differentiation zone they are only located in pericycle and epidermis cells and absent in parenchymatic cells, e.g. cortex and pith. The sections from the meristematic zone presented were taken at approximately at 1mm from the root tip, while the sections from the differentiation zone were taken at approximately 1cm from the root tip (ep: epidermis; co: cortex; pe: pericycle; pi: pith).

H3.2 (Fig. 7), whose ratios were increased in samples from the differentiation zone compared to samples from the other two zones, while the ratios of ubiquitinated H2A and acetylated H4 (Figures 5 and 8 respectively), were decreased in the differentiation zone. Similar differences have been studied in various plant and animal organisms and correlated with the physiological stage of the cells. For example, it is well established that histone variant H3.2 is related to differentiated plant cells (Waterborg, 1993), while histone ubiquitination, histone H3 phosphorylation and histone H4 acetylation have been positively correlated with cell division in both animal and plant cells (Rozbyk et al., 2000, Nowak & Corces, 2004, Jasencakova et al., 2000). The increased histone modified form ratios observed in samples from meristematic the zone concur with their

immunohistochemical detection in every cell of this zone, while they were only detected in pericycle and epidermis cells in the differentiation zone (see Figure 9). This could be additional evidence correlating the modifications examined in this study to cell division, considering that pericycle and epidermis cells are the only ones that retain their meristematic properties among the cells present in this zone (Hochholdinger et al., 2004). The fact that the differentiation zone consists mainly of parenchymatic, non-proliferating cells, like cortex and pith, where almost no modified forms were detected, might provide the explanation for the decreased modified forms' ratios within this zone.

It is known that during callus formation, previously differentiated plant cells restore their meristematic properties, especially the ability for rapid proliferation (Koleva et al.

Figure 10: Morphological differences between a representative root developed in darkness at 28 °C on paper sheets soaked with 0.01mM indole-acetic acid, a root developed in darkness at 28 °C on paper sheets soaked with 0.01mM gibberellic acid and a root developed under the same conditions on paper sheets soaked with distilled water.

1982, Williams et al. 2003). According to gel analysis, the core histone variants' ratios during plant cell dedifferentiation in callus culture were similar to meristematic zone, regardless the developmental zone from which calli were derived. An exception to this observation were the ratio of histone variant H3.2 (Fig. 7), which was increased compared to meristematic zone and the increased ratio of uH2A in first callus culture derived from differentiation zone. These data concur with the fact that, despite morphological similarity observed in calli derived from divergent developmental stages, differences in biochemical properties continue to exist during plant cell dedifferentiation (Koleva et al. 1982). The increased H3.2 ratio 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 histone H3 variants, a process that is possible to alter chromatin structures in specific regions. In the case of uH2A, it was proposed by Alatzas and Foundouli (2006) that the increased ratio in first callus culture derived from differentiation zone could be related to DNA damage due the sudden change in proliferation rate during plant cell dedifferentiation. This ratio is leveled in second and third callus subcultures, where cells are already "habituated" to the new physiological state.

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 10) and physiological differences between auxin treated and control plants (Raghavan, 1999). Auxin is a plant hormone known to regulate root growth and differentiation, by promoting phloem and xylem formation and also induce the patterned differentiation of cells in meristems (Friml, 2003, Berleth et al., 2004). On the other hand, evidence from several plant species indicates that gibberellin is necessary for normal root development. However, exogenous applied gibberellin was unable to promote root elongation in garden pea (*Pisum sativum* L.), unless roots were pretreated with a

gibberellin biosynthesis inhibitor (Yaxley et al., 2001), an observation suggesting an inhibition of root development by exogenous applied gibberellin (see also Figure 10). In samples derived from roots treated with auxin, we found alterations in histone variants' ratios to the level similar to that obtained in differentiation zone of control roots, regardless the developmental zone the samples derived from. In particular, histone H1⁰ variant was increased to above 30% of total H1 in all of the three developmental zones (Alatzas et al., 2008), while the ratios of ubiquitinated H2A and acetylated H4 (Figures 5 and 8 respectively) were decreased to 13% and 37% respectively, a pattern observed in samples from differentiation zone of controls. An exception to these observations was the increase in histone variant H3.1 (Fig. 7) ratio in all samples to above 37%, whereas in differentiation zone of control roots it represented less than 20% of total H3. Histone pattern in gibberellin-treated roots is even more complicated. In this case, we found alterations in histone variants' ratios to the level similar to that obtained either in differentiation or in meristematic zone of control roots. In general, a decrease in histone modified forms' ratios (Figures 5, 7 and 8) and in H1⁰ variant ratio (Alatzas et al., 2008) was observed in all samples, while H3.1 variant ratio (Fig. 7) was similar to meristematic zone of controls. There is several line of evidence from previous studies that suggest that histone pattern can be influenced by hormone treatment in both animal and plant cells. For example, alterations in linker histone H1 variants' ratios have been observed during hormone-induced cell proliferation in mouse mammary glands as well as during hormone-induced differentiation of mouse myeloid leukemia cells (Wurtz, 1985). Gibberellin treatment has been shown to enhance the expression of a histone H3 variant in rice and histone H1 and three histone H2B variants in tomato (van der Knaap and Kende, 1995, van den Heuvel et al., 1999), while abscisic acid treatment caused changes in histone modification level in tobacco and Arabidopsis cells (Sokol et al., 2007).

These alterations observed in chromatin during hormone stimulation render the cell capable of responding to the applied hormone, a mechanism which may be common in both animal and plant cells (Sokol et al., 2007).

The remarkable heterogeneity of histones suggests an important role in chromatin function, considering that individual variants and covalent modifications might participate in distinct processes (Brown, 2001). If this is the case, the relative amounts of histone variants will be altered during processes that imply changes in chromatin function, such as cell differentiation, either programmed or regulated by plant hormones, and dedifferentiation. These processes result in alterations in the physiological state of the cell and consequently in chromatin function that probably necessitate distinct variants' ratios during each process. Future studies in this field will probably enrich our knowledge about the precise role of histone variants in these processes.

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