PIRACICABA, SP, BRAZIL
ESALQ – Universidade de São Paulo

Immunodetection of methylcytosine in maize chromatin by a denaturating protocol

--Andrade, LM; Fernandes, R; Mondin, M

DNA methylation is an important process in the silencing of genes and mobile genetic elements. Visualization of cytosine methylation at different chromatin states is possible using antibodies against DNA modifications. Lysak et al. (Methods in Molecular Biology, vol. 323: Arabidopsis Protocols) report that the accessibility of the antibodies to modified DNA is improved when the chromatin is denaturated. We have found that using native material, antibodies do not access the heterochromatic GC-rich blocks near centromeres of *Crotalaria juncea* (unpublished). We have applied the protocol described by Lysak et al., with minor modifications, using a Piranão variety of maize with a high content of heterochromatic knobs.

Seeds were germinated on water and transferred to *Sphagnum*. The root tips were harvested when 1 to 2 cm long, treated with 8-hydroxyquinoline for 2 hours 40 minutes, fixed with Carnoy (3 parts of ethanol and 1 part of acetic acid) overnight, and stored at -20°C until use. For slide preparations, the root tips were water-washed, equilibrated in citrate buffer and digested for 1 hour in a mixture of cellulase [9.2 units mL^{-1}] and pectinase [14.7 units mL^{-1}]. Root tips were rinsed in cold citrate buffer to stop the reaction. Single root tips were squashed in 60% acetic acid. For best results, the coverslip was removed, followed by liquid nitrogen and air-drying.

Prior to immuno-detection, the slides were baked at 60°C for 30 minutes, washed 2x5 minutes in 1x PBS, post-fixed 5 minutes in 4% paraformaldehyde in 1xPBS, followed by washing twice, for 5 min each, in 1x PBS, then an alcohol series (70%, 90% and 100%), and finally air-dried. The hybridization mixture was applied as 50 µl to each slide, covered with a glass coverslip and denaturated using a hot plate at 75°C for 10 minutes. Slides were immediately chilled 5 minutes using 1x PBS at 0°C or less, and the process repeated one time. The slides were blocked with 1% BSA in 1x PBS, and incubated1 hour in a moist 37°C chamber. The primary antibody, sheep anti-5' methylcytosine (Fitzgerald), diluted 1:500 in 1x PBST, was applied to each slide, and incubated 1 hour in a moist 37°C chamber. Excess primary antibody was removed with two 5 min washes in 1x PBST, then the second antibody, rabbit anti-sheep-FITC, was diluted 1:250 in 1% BSA, applied and incubated 1 hour in a moist 37°C chamber. The washes were applied as described for the primary antibody. Then, the tertiary antibody, goat anti-rabbit-FITC, diluted 1:125 in 1% BSA, was applied and incubated 1 hour at 37°C in a moist chamber. Slides were finally washed in 1x PBST, air-dried and mounted in Vectashield (Vector) with DAPI. A Zeiss Axiophot-2 epifluorescence microscope with appropriate filter was used for viewing, images were digitalized by a CCD camera, processed using ISIS Metasystems software and Adobe Photoshop.

In the diploid interphase nucleus depicted in Figure 1A, 1B, and 1C, two quenched regions correspond to the nucleus, with heterochromatic blocks appearing as very bright spots. Methylcytosine appears widely distributed and some heterochromatic

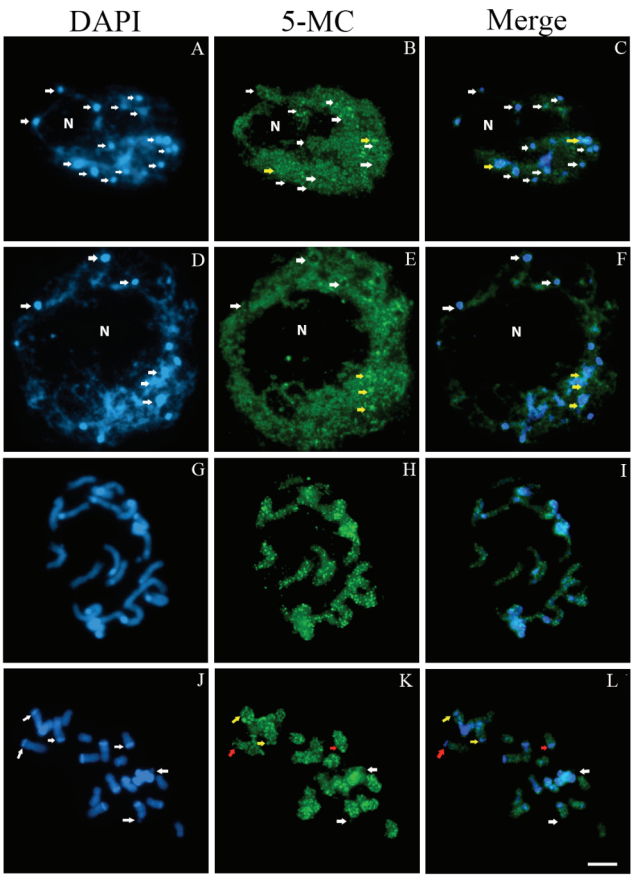

Figure 1. Interphase diploid nucleus (**A, B, C**), endomitotic nucleus (**D, E, F**), prophase (**G, H, I**), and metaphase (**J, K, L**) viewed as DAPI, anti-body treated (5-MC) and merged. **A, D**: Arrows indicate heterochromatic knobs as brighter spots with the nucleolus (N) quenched. **B:** arrows indicate knobs, with yellow arrows for brighter signals. **E**: white arrows indicate quenched regions and yellow arrows brighter regions. **J**: white arrows indicate satellites and heterochromatic knobs. **K**: white arrows indicate satellite methylation, yellow arrows the brighter signals, and red arrows the quenched knob. Bar = 5µm.

blocks appear homogeneously labeled, others as negative regions, and others with brighter spots (Fig. 1B). In the merged view (Fig. 1C), the heterochromatic knobs are not clearly labeled, except for a few, observable as small bright spots.

In the interphase endomitotic nucleus, the results are similar to those for the diploid interphase nucleus, except that some quenched regions do not correspond to heterochromatic blocks (Fig. 1D, 1E and 1F). Only the biggest knobs showed some small, brighter regions.

Prophase (Fig. 1G, 1H and 1I) and metaphase (Fig. 1J, 1K and 1L) chromosomes showed similar labeling patterns: anti-5MC is distributed over the chromosome arms, with small, brighter regions in interstitial positions. The hetechromatic knobs are either not distinguishable, or, in the central regions, appear as negative or quenched regions. Centromeric heterochromatin is not seen as enriched, in comparison to other heterochromatic or euchromatic regions. The NOR-heterochromatin does not appear differentially brighter, although the satellite of chromosome 6 is labeled. In some cells both satellites were labeled, and in some, only one pair was labeled (data not shown).

We conclude that the detection of methylcytosine by this protocol does not differentiate the heterochromatic blocks of maize

chromatin. There are many possible reasons: heterochromatin has low levels of accessible methylcytosine residues, so that the antibody is unable to distinguish differences; or, the denaturation process was not adequate for the antibody to access all the methylcytosine residues

REGENSBURG, GERMANY
University of Regensburg
COLD SPRING HARBOR, NEW YORK
Cold Spring Harbor Laboratory

α-tubulin-YFP labeled sperm cells for live cell imaging of the fertilization process in maize and relatives such as *Tripsacum dactyloides*

--Kliwer, I; Jackson, D; Dresselhaus, T

In vivo imaging of double fertilization processes is a prerequisite to studying and understanding the underlying molecular mechanisms involved in cross-talk between the male (pollen tube) and female gametophyte (embryo sac), as well as sperm cell release, migration and fusion with the two female gametes (egg and central cell). Tremendous progress is currently being made in understanding these fundamental biological processes using *Arabidopsis* as a model system because a large number of tools have been developed over the last couple of years (Berger et al., Trends Plant Sci. 13:437-443, 2008). Comparable tools are missing for maize. In order to visualize these fertilization processes in maize also, we have screened a number of fluorescent protein tagged maize lines generated via the NSF project #0501862 (for review see http://maize.jcvi.org/cellgenomics/) to identify sperm cell marker lines.

One line could be identified displaying YFP-tagged α-tubulin in many cell types of roots, leaves, inflorescences, etc., but exclusive labeling of sperm cells inside mature pollen grains. During microsporogenesis, the YFP signal could be detected earliest after the first mitotic division in the generative nucleus. The generative nucleus then divided to form two sperm cells with similar YFP signal intensity. In the mature pollen grain, only the two crescent-shaped sperm cells displayed YFP signals. This marker line is superior even to *Arabidopsis* marker lines, as the entire sperm cell boundaries are labeled by cortical tubulin strands. Furthermore, the accumulation of thick tubulin bundles at both sides surrounding the sperm cell nucleus allows simultaneous visualization of the nuclei (Fig. 1A). There is no detectable signal in the vegetative cell of the developing pollen grain at any stage of microsporogenesis nor at a later stage during pollen tube growth. Due to its specific expression pattern during microgametogenesis, the endogenous promoter of this *α-tubulin* gene family member, which drives expression of the fusion protein, provides a tool to specifically deposit other proteins or RNAs inside maize sperm cells. Moreover, this marker line enables studies on sperm migration dynamics during pollen tube growth and fertilization. In pollen grains of maize and many other plant species, the two sperm cells lie in immediate proximity to each other and are connected by an unknown mechanism. During tube growth, the two sperm cells leave the pollen grain through the germ pore shortly after germination and migrate back and forth inside the growing pollen tube

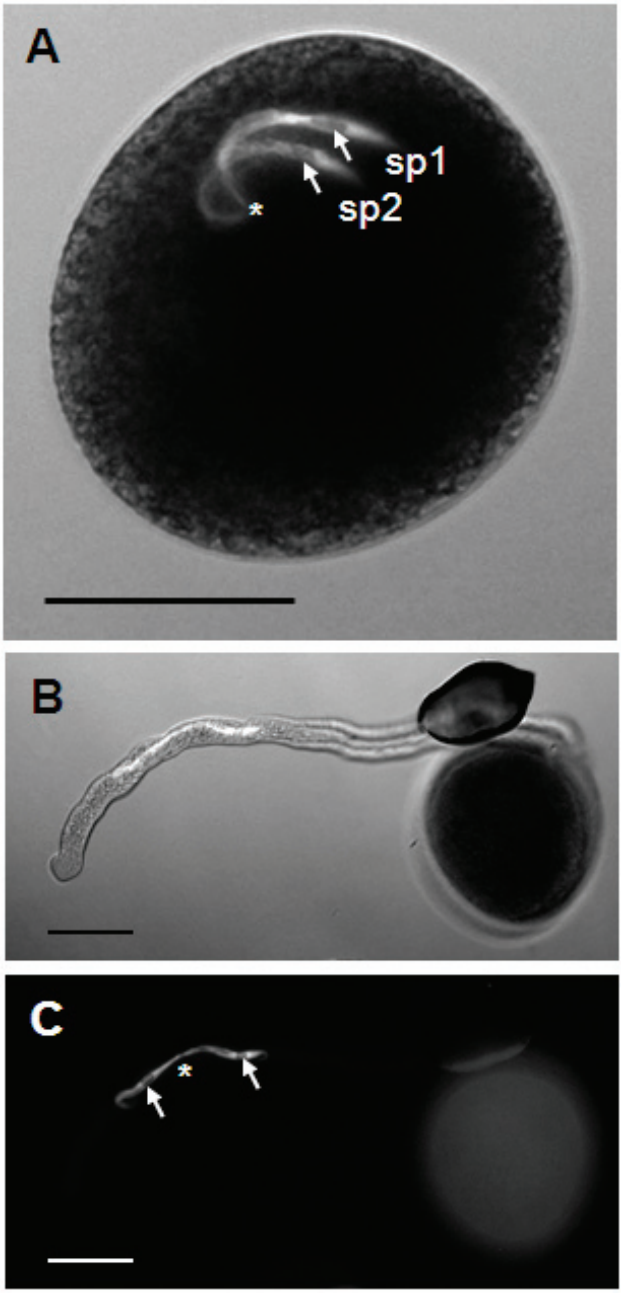

Figure 1. α-tubulin-YFP labeled sperm cells. **A**: Mature pollen grain containing two sickle-shaped sperm cells (sp1 and sp2). Arrows point at sperm nuclei and an asterisk marks the connection between both cells. **B**: Sperm cells migrating towards the tip of a growing pollen tube (merged bright field and fluorescence image). **C**: Same as B but only YFP signal is visible. Arrows and asterisk as in A. Scale bars 50µm.

while still attached to each other (Fig. 1B and C). As soon as the pollen tube reaches the embryo sac, it discharges its contents though interaction with the synergids. These processes occur inside the ovary, which is deeply embedded in maternal tissues, and have never been observed in maize and other grasses. However, a number of labs have already developed methodologies for in vitro fertilization and separation of the female gametophyte from maternal tissues, which will enable study of these fundamental processes using the marker line described above.