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Chromomeric pattern of maize pachytene chromosomes after trypsin treatment

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The bivalent chromomeres of maize were analyzed by means of enzymatic treatment with trypsin. The aim was to verify if the effect of swelling observed in human meiotic chromosomes also occurs in these bivalents, and if this morphological change allows a greater visual resolution of the chromomeric pattern. Bivalents treated with trypsin showed a structure with sequential and linear distribution of small rings. Comparisons between bivalent morphology, treated and non-treated, indicated a collinearity among the sequences of rings and the chromomeres. The results obtained were considered useful to analyze and to make a chromomere map. This treatment can also provide cytological evidence that may help to understand the pairing process. It was concluded that the ring-shape was caused by trypsin-induced "disjoining" just in the chromomeric but not in the interchromomeric regions.

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The chromomeric pattern analysis has been useful not only to identify meiotic chromosomes but also to clarify different aspects of the genetics of higher organisms, such as: (1) understanding the genetic organization of the eukaryotic chromosomes (LIMA-DE-FARIA et al. 1973); (2) studying the banding mechanism (LUCIANI et al. 1975); (3) localizing the break points involved in chromosomal rearrangement (FANG and JAGIELLO 1981; SHEN and WU 1992; SYBENGA 1992); (4) making genetic maps (FANG and JAGIELLO 1981; SHEN et al. 1987; SHEN and WU 1992); (5) analyzing chiasmata (FANG and JAGIELLO 1988); and (6) studying chromatin structure and function (FANG and JAGIELLO 1991).

In this research, the bivalent chromomeres of maize were analyzed by means of enzymatic treatment with trypsin. The aim was to verify if the effect of swelling observed in human meiotic chromosomes also occurs in these bivalents, and if this morphological change allows a greater visual resolution of the chromomeric pattern. Trypsin was used as a standard method for G-banding in humans and in some other animal cytogenetics. This treatment causes a chromosome swelling as a secondary effect due to a proteolytic action of this enzyme (BURKHOLDER 1988; MUSIO et al. 1997).

This methodology is a powerful tool for chromomeric pattern analysis. The result obtained with trypsin treatment showed also a regionalized separating or disjoining of the bivalents, which occurred only in the chromomeric regions, while the interchromomeric regions remain paired. It could present cytological evidence that provides new insights into the pairing process of chromosomes on maize pachytene.

MATERIALS AND METHODS

Preparation of cytological material

Maize anthers (*Zea mays* L.), lines L-869, were fixed in methanol: acetic acid solution (3:1), changed three times and stored in freezer (−20°C) for one to several days. The anthers were transferred to a 1.0 ml Eppendorf™ tube with a nylon screen (100 μm) attached to the bottom, and washed with distilled water three times for 15 minutes. The anthers were covered with a Flaxzyme™ enzymatic solution (Bioindustrial Group NOVO Nordisk), without dilution, and the tube was placed into an incubator at 35°C for 50 minutes. Subsequently the anthers were washed with distilled water for 15 minutes. They were chopped in distilled water using a stiletto and filtered through a screen into a 1.5 Eppendorf™ tube. The suspension of cells was centrifuged at 300 rpm for 5 minutes. The supernatant was discarded and 30 μl of the same enzymatic solution was added to the pellet (0.5 ml). After maceration (at 35°C, for 1 hour), the enzyme was removed with distilled water, changed four times. A fixative methanol:acetic acid (3:1) solution was gently dropped into the pellet. After changing this solution three times, the supernatant was discarded, leaving about 0.3 ml in the tube. Three to four drops of cell suspension were dropped onto very clean and frozen glass slides. These slides were air-dried and placed on to a hot-plate at 50°C for 10 minutes. Some of them were aged in an incubator at 35°C for 7 days. Others were immediately stained with a 5% Giemsa solution in phosphate buffer at pH 6.8 for 7 minutes, washed twice in distilled water

and air-dried. After analysis, these slides were destained with a solution of methanol: acetic acid (3:1), three times, for 20 minutes each, and aged for 7 days at 35°C.

Trypsin treatment

Unstained aged slides were immersed for five seconds in a 5% trypsin solution (DIFC™ 1:250) in a phosphate-buffered salt solution (PBS) pH 7.4 at room temperature (25–26°C). For destained and aged slides, a digestion period of 20 to 22 seconds was used. The slides were immediately washed three times in distilled water (for 10 seconds each), air-dried on a hot plate at 50°C for 10 minutes, and Giemsa stained. They were observed under bright field köehler illumination microscopy.

Some trypsin-treated slides were stained in a solution of 2.0 μ/ml DAPI (4′, 6-diamidino-2-phenylindole) in McIlvaine buffer pH 7,0. After staining in the dark for five minutes at room temperature, the slides were briefly rinsed with buffer and air-dried. The preparation was then mounted in the same buffer, sealed with rubber and observed under an epifluorescent microscope equipped with UV filter cassette.

Image analysis

Figures of pachytenes and individual bivalents were captured with a CCD video camera and digitalized by image analysis system attached to the microscope with immersion objective (100 ×). Analyses were made using the public domain Image SXM software (RASBAND 1997). The images were converted to a 256 gray value and the scale was standardized to the range from 0 (white) to 255 (black). Using the software, a 3-D image was generated to enhance the chromomeric structures and the trypsin treatment effect.

RESULTS AND DISCUSSION

After the pachytene treatment with trypsin solution, the 10 bivalents showed small rings or bubbles sequentially distributed (Fig. 1a and 1b). More than 100 trypsin-treated slides were analyzed and all of them showed this pattern. This effect could be compared to the pachytene with normal morphology (Fig. 1c) and after trypsin treatment (Fig. 1d).

Comparisons among bivalent morphologies indicated a collinearity between the rings obtained after trypsin treatment and the chromomeres of non-treated bivalents. This collinearity could be seen in the bivalent 10, before and after the trypsin treatment (Fig. 2a).

The consistency of the trypsin treatment effect was shown in Fig. 2b, which corresponds to four bivalents 7 (I–IV) analyzed at different times of digestion. Different bivalents on the same slide showed different levels of ring formations. Fig. 2b shows the non-treated (I) and treated (II) bivalent. A partial ring formation in the latter was observed after destaining, trypsin digestion, and re-staining with the Giemsa. In III and IV two other bivalents 7 were progressively digested. The chromomeres (10 to 17) were mapped in the median region of this bivalent. It was noticed that chromomere 12 (Fig. 2b II) was partially opened whereas the others remained closed. In Fig. 2b (III), all the chromomeres of the mapped region were completely opened. In Fig. 2b (IV), besides the completely opened ones, some rings showed enlargement resulting from the fusion of small adjacent chromomeres (10–11 and 16–17). Furthermore, it could be observed that the ring order formation is constant and characteristic for each chromosome. In fact, a slight variation in the number of rings in the analyzed bivalents was detected. This variation could either be a result of the chromosomes condensation level, as described for chromomeres (COMINGS 1978; LUCIANI et al. 1984), or a result of the progressive digestion, which could lead to the fusion of very close rings. A trypsin over-treatment showed a loss of the above mentioned pattern.

Digitalized images helped in the visualization of the trypsin digestion effect on the fixed chromosomes. In Fig. 3, the 3-D image of the trypsin-treated bivalent 8 can be observed. This bivalent was previously stained with Giemsa. In the long arm, the two largest rings of the distal region correspond to the trypsin-induced, "disjoining" in the region of the two knobs (k). The other rings are related to the chromomeres.

To verify if dark borders around the trypsin-treated chromomere indicate concentration of DNA, the slides were stained with DNA specific fluorochromes chromomycin (not shown) and DAPI (Fig. 1b). Both dyes exhibited bright fluorescence on the ring border of the trypsin-treated chromomeres and knobs. This evidence and the same pixel value in the ring center and in the background of the image analysis indicated that the chromatin swelling is unlikely to be the cause of the observed phenomenon.

The results obtained were considered useful to analyze and to make a chromomere map. Moreover, this methodology has a potential use to study the homologous chromosomes pairing process.

The ring formation caused by the trypsin action on the bivalents provided additional evidence of probable regional differences that maintain bivalent pairing. It was concluded that the ring shape rising by trypsin-induced "disjoining" occurred just in the

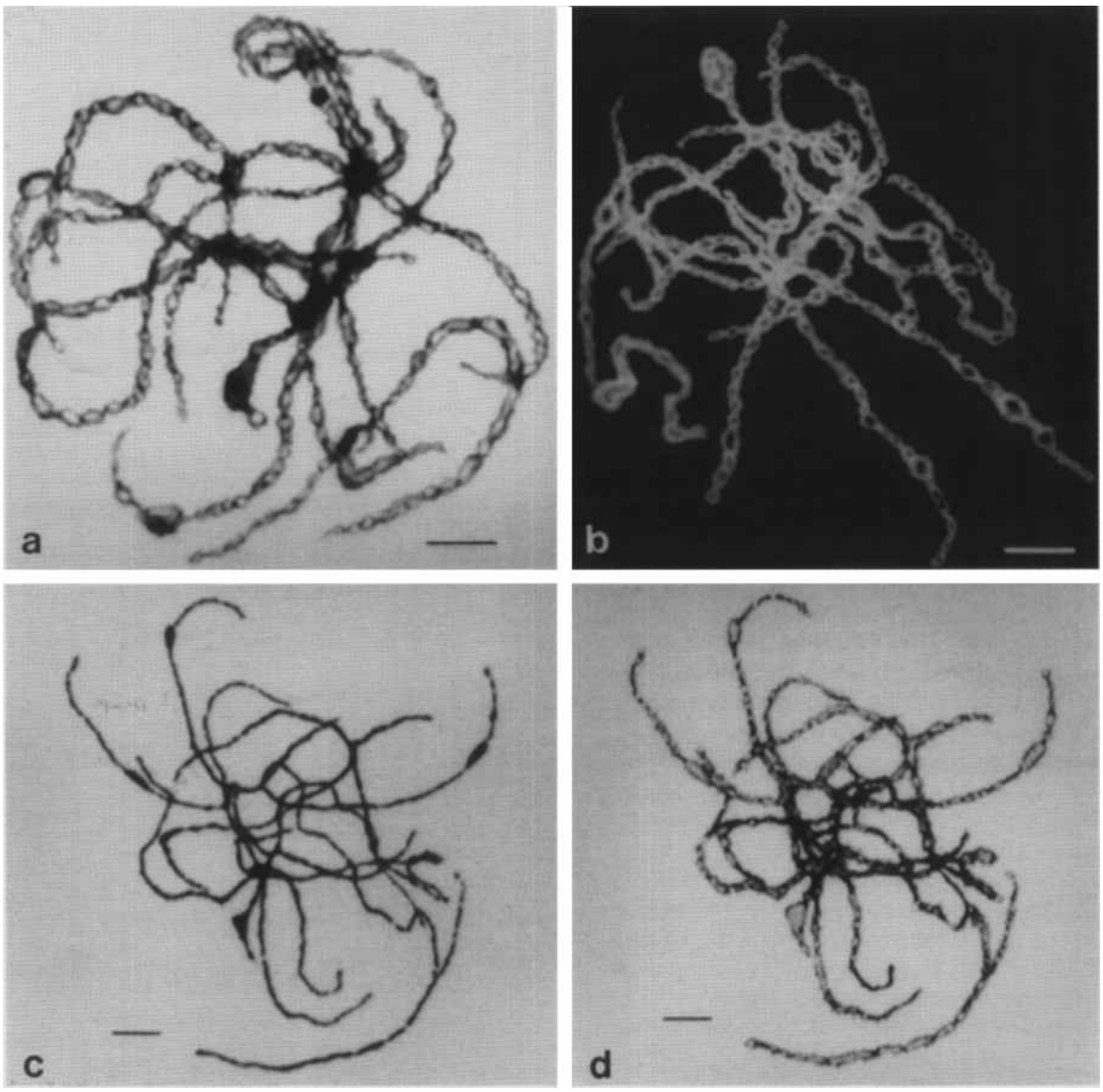

Fig. 1a–d. Maize pachytene after trypsin treatment stained with Giemsa **(a)** and DAPI **(b)**. All bivalents showing a longitudinal small rings pattern. Another pachytene before **(c)** and after **(d)** trypsin-treatment. Bar = 5 μm.

chromomeric and not in the interchromomeric regions. This regional difference related to the resistance or not to trypsin induced "disjoining", may be considered cytological evidence which leads us to suggest that the chromomeres and interchromomeres may have differential involvement in the pairing process.

According to McKee (1996) and Bass et al. (1997), the recognition process of homologous, the pairing of chromosomes, and the formation of the synaptonemal complex (SC) have not yet been clarified. Nevertheless, the cytological evidence presented in this study suggests the possibility of the interchromomeres having a greater commitment to the bivalents pairing process; hence they could be candidates of pairing sites. This interpretation is in accordance with the multiple sites hypothesis of pairing and formation of SC in interstitial regions, longitudinally distributed and positioned in a non-random pattern in the chromosomes (Burnham et al. 1972; Gillies 1975, 1984, 1985; Stack and Anderson 1986; Loidl 1990; Vincent and Jones 1993; Wagner et al. 1993).

This differentiated "disjoining" effect of the chromomeric regions was also observed in the knob regions (Fig. 3). The low resistance to trypsin treatment of the knob plays a similar role to the chromomere in

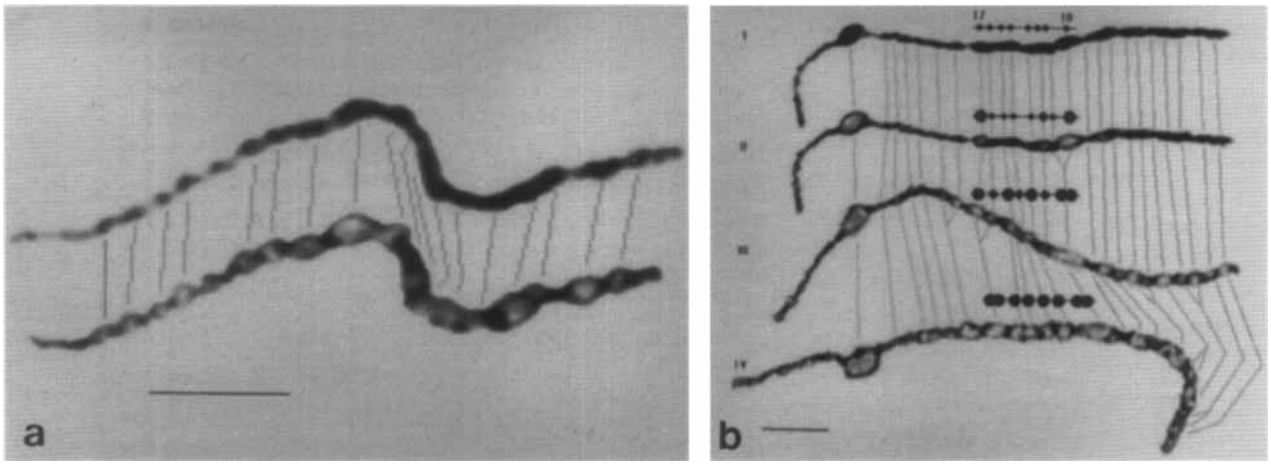

Fig. 2a and b. **a** Bivalent 10 from maize pachytene. The non-treated (top) bivalent and the same after trypsin treatment (bottom). **b** Chromomere-ring patterns after sequential trypsin treatment of four bivalent 7. The same bivalent before (I) and after (II) treatment. The other bivalents (III and IV) after progressive treatment. The dot-map (10 to 17) shows the sequential opening rings process. The lines point to chromomere ring correspondence. Bar = 5 μm.

the pairing process. On the other hand, the centromere was resistant to the digestion like the interchromomeric region (Fig. 3). Therefore, this data is not enough to conclude that this site is a pairing point. Such resistance could be due to a more particular and complex structural organization of the chromatin in this region. The interpretation of this data is in accordance with GILLIES (1975), related to knobs, and BURNHAM et al. (1972) and with GILLIES (1975), related to centromeres. As stated by these authors, the two regions are neither considered initial points of pairing, nor do they enable the formation of the synaptonemal complex.

Concerning the nucleolus organizer region (NOR), the trypsin-induced "disjoining" of the homologous

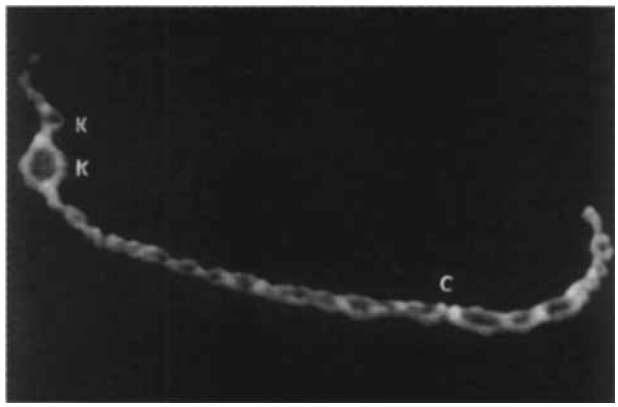

Fig. 3. 3D image of bivalent 8 after trypsin treatment. Longitudinal pattern visualized as opened rings at the chromomere regions. The two sub terminal rings (large and small) are shown "disjoining" at two knob regions. Centromere (C) and knob (K).

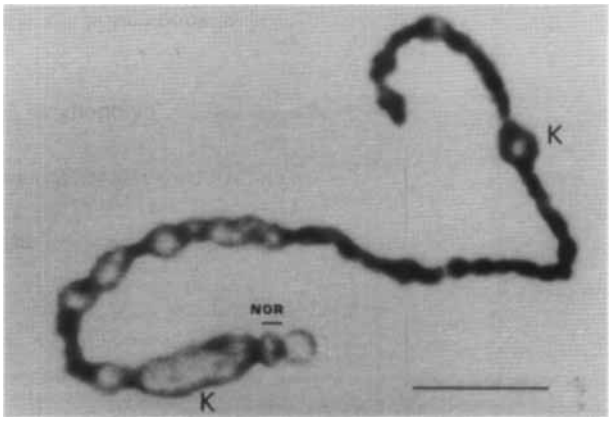

Fig. 4. The 6-bivalent from maize pachytene visualized after trypsin treatment. The NOR region is open like the same ring-shape of the chromomere and knob regions after treatment. The two well known knobs (K) of the 6-bivalent appear separate. Bar = 5 μm.

observed in this region (Fig. 4) also supports the hypothesis that this region is not one of the points of chromosome pairing, as has been suggested by GILLIES (1975).

These cytological results strengthen the argument that the synapsis is a regionalized process and that the chromomeres and interchromomeres are engaged in a differentiated manner in this process. The next questions are why and how it happens.

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