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# Cytogenetic characterization of telomeres in the holocentric chromosomes of the lepidopteran *Mamestra brassicae*

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**Abstract**

Telomeres of the *Mamestra brassica* holocentric chromosomes were studied by Southern blotting, *in-situ* hybridization and *Bal*31 assay evidencing the presence of the telomeric $(TTAGG)_n$ repeat. Successively, molecular analysis of telomeres showed that TRAS1 transposable elements were present at the subtelomeric regions of autosomes but not in the NOR-bearing telomeres of the Z and W sex chromosomes. TRAS1 appeared to be transcriptionally active and non-methylated, as evaluated by RT-PCR and digestion with *Msp*I and *Hpa*II. Finally, dot-blotting experiments showed that the 2.8±0.5% of the *M. brassicae* genome consists of TRAS1.

## Introduction

Telomeres, the physical chromosomal termini, are essential chromosomal structures. They are involved both in preventing end-to-end chromosome fusion and in the protection of the chromosomal ends from the gradual erosion occurring during successive rounds of replication (Blackburn 1991, Biessmann & Mason 1994).

Telomeric DNA consists frequently of a simple sequence: one strand is G-rich and its 3′ end points to the chromosome end (Sahara *et al.* 1999). Although different telomeric sequences were reported in eukaryotes, some are more frequently represented. The $(TTAGGG)_n$ sequence, for example, constitutes telomeres of all vertebrates (Meyne *et al.* 1989) and the $(TTTAGGGA)_n$ repeat is conserved in the plant kingdom (Cox *et al.* 1993), with few exceptions (Pich *et al.* 1996).

Insects represent the most numerous group of organisms in the animal kingdom. Three different telomere types were reported in this group: the pentanucleotide $(TTAGGA)_n$ repeat in *Bombyx mori* (Okazaki *et al.* 1993, Sahara *et al.* 1999), the Het-A and TART transposable elements in *Drosophila melanogaster* (Biessmann *et al.* 1990) and the complex tandem repeat in the *Chiranomus* genus (Zhang *et al.* 1994).

In this paper, the organization of telomere in the holocentric chromosomes of the lepidopteran *Mamestra brassicae* is described. In view of the suitability for both *in-vivo* and *in-vitro* analysis, *M. brassicae* could represent an important model for the study of the architecture of holocentric chromosomes.

## Materials and methods

Chromosome preparations were made by spreading CRL-8003 hemocyte cells of *Mamestra brassicae* following a modified protocol of the method described by Odierna *et al.* (1993). In brief, cells were kept in a 0.8% hypotonic solution of sodium citrate for about 45 min and then transferred to minitubes and centrifuged at 350*g* for 3 min. Methano–acetic acid 3 : 1 was successively added to the pellet, which was made to flow up and down for 1 min through a needle of a 1-ml hypodermic syringe. Finally, the pellet was resuspended in 200 $\mu$l of fresh fixative and 20 $\mu$l of cellular suspension was dropped onto clean slides and air dried.

C-banding treatment was performed according to Sumner's technique (1972). After the treatments, slides were stained with chrornomycin $A_3$ ($CMA_3$), as reported by Schweizer (1976), and with 4′-6′-diamidino-2-phenylindole (DAPI), as described by al Donlon & Magenis (1983).

The $(TTAGG)_n$, $(TTAGGG)_n$ and $(TTAGGGG)_n$ telomeric probes were obtained by PCR amplification according to Ijdo *et al.* (1991). The 197-bp *Chironomus* telomeric probe has been obtained by PCR using the primers F (5′-TGAGGAAATTGAAGGGCAAA) and R (5′-CTTCCTCATCGGCTGGAGTT) designed on the basis of the GenBank sequence AF026061 from *Chironomus pallividittatus* larvae DNA. TRAS1 probe has been obtained by PCR using the primers F (5′-ATGAAGGGTGGGTGAGAGTG) and R (AGGGTTACGGGCACTCTTT) designed on the TRAS1 sequence from *B. mori* (BMOTRAS1). The 28S rDNA probe has been amplified according to Mandrioli *et al.* (1999).

Probes for digoxigenin- and biotin-labeling were performed by random priming following Roche protocols. Fluorescent *in-situ* hybridization (FISH) was carried out as described by Mandrioli *et al.* (1999) making two stringency washes in 0.1×SSC for 10 min at room temperature and two in 0.1×SSC at 45°C for 10 min after hybridization.

DNA extraction from cultured hemocytes has been performed using a standard protocol as described in Mandrioli *et al.* (1999), whereas Southern blotting, restriction enzyme digestion and *Ba*l31 assay have been carried out following Okazaki *et al.* (1993).

Finally, RNA extraction and RT-PCR have been performed with the 'SV Total RNA Isolation System' (Promega) and with the 'Access RT-PCR System' (Promega), respectively, according to supplier's suggestions.

Pheromone-binding protein genes (PBP) were amplified using the primers $F_{PBP}$ (5′-ACTAGCATAGCAATTCGG) and $R_{PBP}$ (5′-GCCTTGACCATTACGGACG). This amplification has been used as control of the RNA samples in RT-PCR experiments since these genes are expressed in the antennal tissues only and not in the *M. brassicae* cell line used for our experiments (Maibeche-Coisne *et al.* 1998).

## Results

As, in insects, three different telomeric organizations have been observed, the presence of each telomere type was looked for in *M. brassicae.*

Southern blotting with the $(TTAGG)_n$ probe on *M. brassicae* DNA showed a diffuse smear of the hybridization (Figure 1). The hybridization of the same filter with the $(TTAGGG)_n$ and $(TTAGGGG)_n$ probes did not show any hybridization signal even if weak stringency conditions were used. Analogous negative results have been obtained after Southern blotting with the Het-A, TART and the 197-bp *Chironomus* telomeric repeat.

These results, as a whole, suggested that the $(TTAGG)_n$ repeat could represent *M. brassicae* telomeres.

FISH experiments with the telomeric $(TTAGG)_n$ probe revealed bright fluorescent spots on telomeres of all chromosomes (Figure 2b, c, e), indicating that such sequences constituted *M. brassicae* telomeres.

In *M. brassicae* interphase nuclei, telomeric sequences were not clustered into a few foci but they appeared to be located near the nuclear periphery (Figure 2a).

To determine whether the $(TTAGGA)_n$ repeats were located at the ends of *M. brassicae* chromosomes or below a true telomere, *Mamestra* DNA was digested with *Bal*31 for increasing amounts of time and then it has been digested with

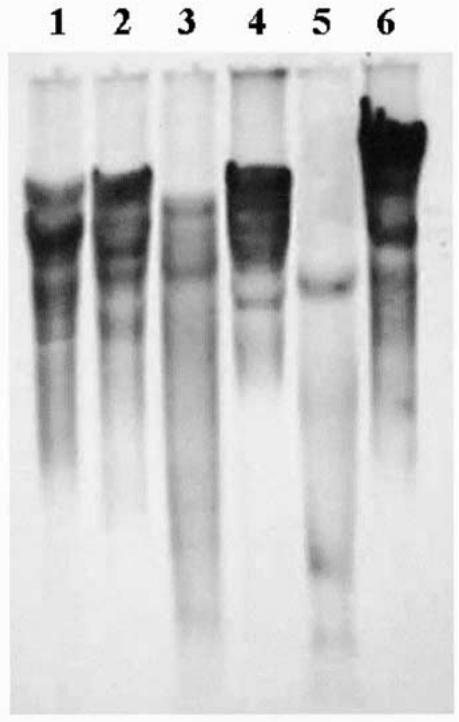


*Figure 1.* Southern blotting with the $(TTAGG)_n$ probe on *M. brassicae* DNA after digestion with *Eco*RI (lane 1), *Sca*I (lane 2), *Hind*III (lane 3), *Bam*HI (lane 4), *Xba*I (lane 5) and *Taq*I (lane 6).

*Hind*III (Figure 3a). After increasing time of *Bal*31 digestion, the diffuse smear of hybridization detected by the $(TTAGG)_n$ probe became progressively shorter and almost disappeared within 60 min of digestion. At high molecular weight, a strong-labeled band has been demonstrated even at 60 min digestion (Figure 3a). This band did not appear to be heavily digested by *Bal*31, suggesting that it could represent non-telomeric $(TTAGG)_n$ sequence as previously reported in other organisms (Salvadori *et al.* 1995).

These results, as a whole, suggest that most of the $(TAGGG)_n$ repeats are located at telomeres.

The effects of the digestion with the exonuclease *Bal*31 have been quantified by a densitometric analysis and they have been compared to the effects of *Bal*31 digestion on a $\lambda$ DNA, according to Okasaki *et al.* (1993). In particular, the length of $\lambda$ DNA at each digestion time has been plotted against the results on the *M. brassicae* genome. In the *Bal*31 experiments, almost all the *M. brassicae* telomeric signals disappeared at the 2,3 rate of $\lambda$ DNA digestion (Figure 4). Since the digestion rate of the telomeric DNA with *Bal*31 is thought to be three to four times faster than that of non-telomeric DNA (Okazaki *et al.* 1993), the TTAGG terminal array of *M. brassicae* should be considered to be about 7–9 kb long.

In order to better analyze the structure of the *M. brassicae* telomeres, attention has been paid to the subtelorneric regions. In particular, the presence of the transposable element TRAS1 has been assessed by PCR (Figure 6). The 300-bp TRAS1 fragment obtained by PCR has been labeled and was used as probes for both *in-situ* (Figure 2d) and filter hybridization (Figure 7). Southern blotting on *M. brassicae* DNA showed several positive bands indicating the presence of numerous TRAS1 copies. In particular, the comparison of the restriction patterns of *Msp*I and *Hpa*II did not reveal any difference in the hybridization signals, suggesting the absence of methylation in TRAS1.

FISH experiments with a digoxigenin-labeled TRAS1 (Figure 2d) probe and a biotinylated $(TTAGG)_n$ probe (Figure 2e) showed bright fluorescent signals at both ends of all *M. brassicae* chromosomes with the $(TTAGG)_n$ probe, whereas just autosomes evidenced both fluorescent telomeres with TRAS1 probe. Only one telomere of each sex chromosome was in fact labeled with the TRAS1 probe. Silver staining of the same metaphases indicated that TRAS1 was absent from the NOR-bearing telomeres of the Z and W chromosomes (Figure 2f).

Identification of *M. brassicae* sex chromosomes has been performed after karyotype reconstruction of DAPI-stained chromosomes (Figure 5). In particular, the *M. brassicae* complement consists of 11 chromosome pairs, including a couple of heteromorphic sex chromosomes that were recognized as Z and W chromosomes on the basis of the data published by Traut & Marec (1996), indicating the presence of female heterogamety in *M. brassicae*.

DAPI staining of C-banded chromosomes demonstrated that most *M. brassicae* heterochromatin possesses AT-rich DNA, as evident after DAPI staining (Figure 2g), whereas only the C-band located on one telomere of each sex chromosome contained $CMA_3$-positive GC-rich DNA

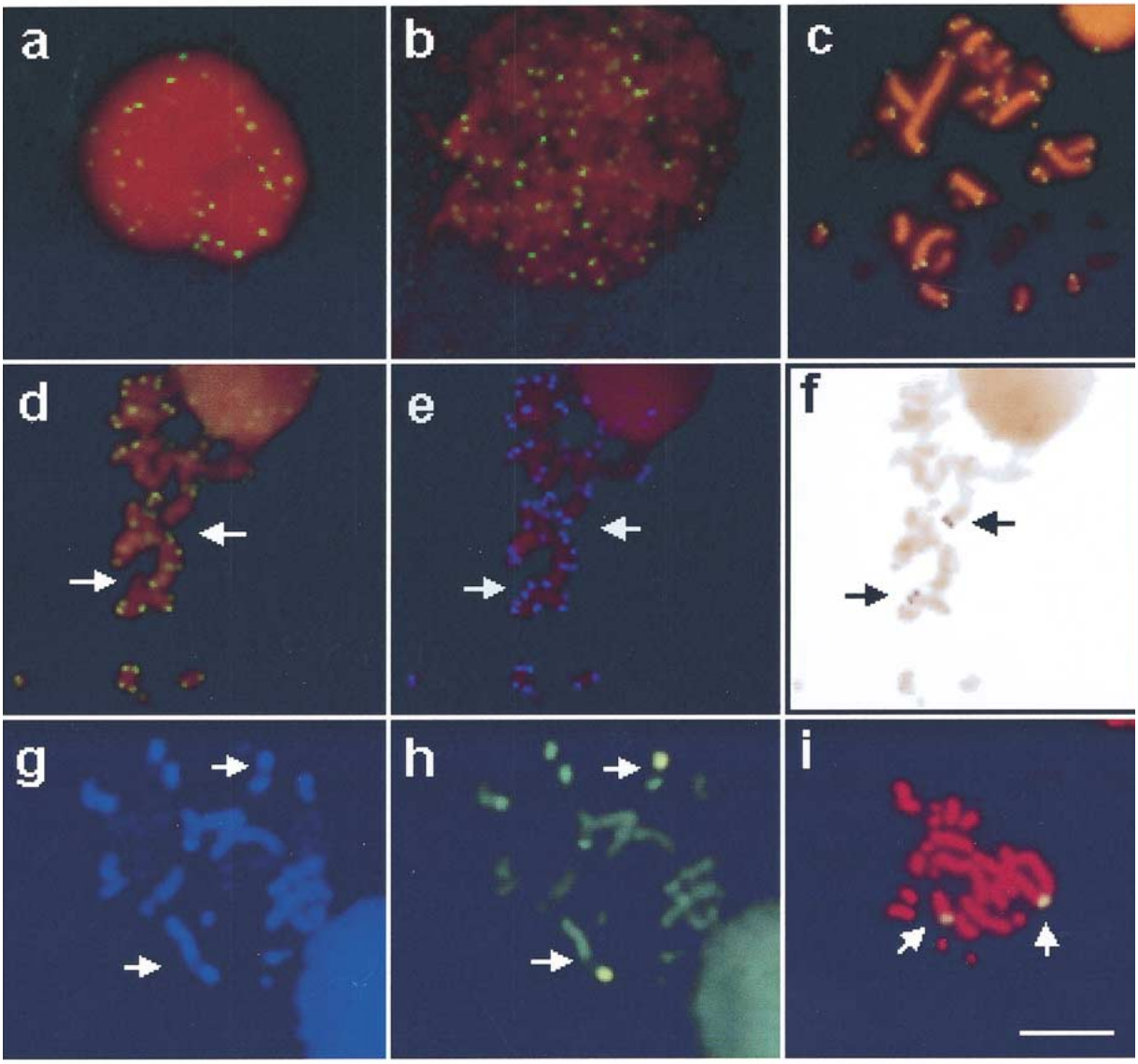


*Figure 2.* Interphase nuclei (**a**) and metaphase chromosomes of *M. brassicae* (**b–i**) after FISH with the $(TTAGG)_n$ probe (**a–c**) showed that this repeat constitutes *M. brassicae* telomere. FISH on the same metaphase plate with the TRAS1 probe (**d**) and with the $(TTAGG)_n$ probe (**e**) indicated that TRAS1 is absent from NORs as deduced by silver staining (**f**). DAPI (**g**) and $CMA_3$ (**h**) staining of C-banded chromosomes indicated that most heterochromatin is AT rich with the exception of NORs that appeared GC rich and fluorescent after FISH with the 28S probe. Arrows indicate NORs. Bar corresponds to 10 $\mu$m.

(Figure 2h). The bright $CMA_3$ fluorescence of one telomere of each sex chromosome was due to rDNA genes, as demonstrated by FISH with a 28S rDNA probe (Figure 2i).

Moreover, C banding (Figure 2g, h) showed that heterochromatin was not present on all chromosomes of the *M. brassicae* complement, but that it was located on the two sex chromosomes and on chromosomes 7 and 11 only. In particular, Z and W chromosomes appeared to be essentially heterochromatic with the exception of a central portion of both chromosomes. In view of these data, there was not any equilocal distribution of heterochromatin in the *M. brassicae* complement.

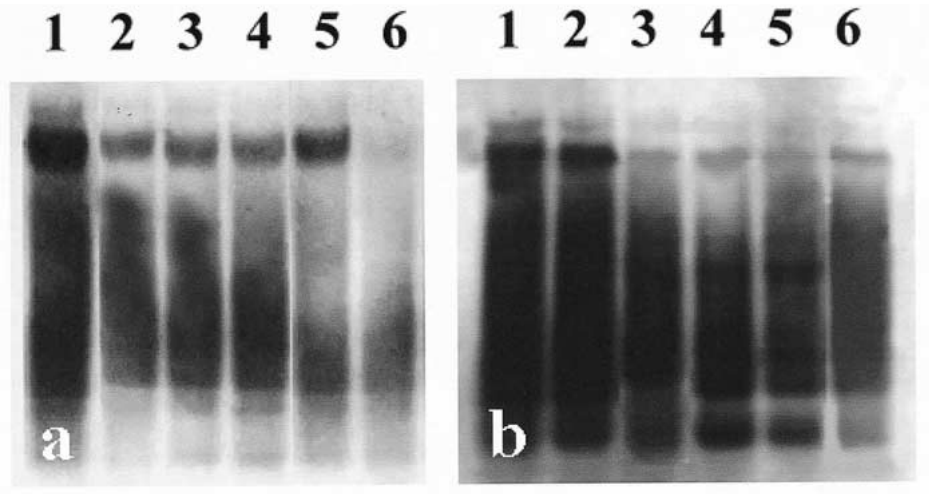


*Figure 3.* (**a**) *M. brassicae* DNA after 0 (lane 1), 5 (lane 2), 10 (lane 3), 20 (lane 4), 40 (lane 5) and 60 min (lane 6) *Ba1*31 digestion respectively and hybridization with the telomeric probe. (**b**) *M. brassicae* genome after 0 (lane 1), 5 (lane 2), 10 (lane 3), 20 (lane 4), 40 (lane 5) and 60 min (lane 6) *Bal*31 digestion and hybridization with the TRAS1 probe.

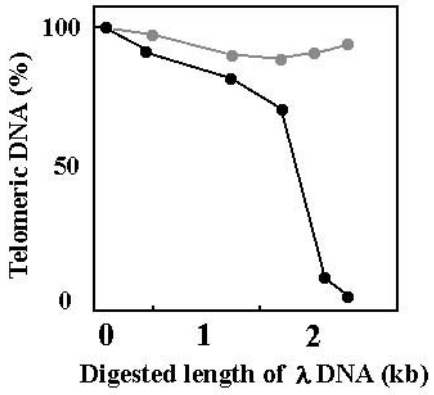


*Figure 4.* Telomere length has been evaluated by plotting the results obtained with *Bal*31 on *M. brassicae* against those obtained on a λDNA.

Finally, only the two sex chromosomes and chromosomes 7 and 11 showed heterochromatic telomeres.

In view of such data, the presence of TRAS1 was not limited to heterochromatin since TRAS1 was present in almost all the telomeres whilst onlya few chromosomal ends were heterochromatic.

In order to evaluate if TRAS1 was located at telomeres or within the subtelomeric regions, *Bal*31 assays were performed (Figure 3b). Filter hybridization on *Bal*31-digested DNA showed that *M. brassicae* TRAS1 elements were partially digested only after long digestion times. TRAS1 was therefore located in the subtelomeric regions of *M. brassicae* chromosomes.

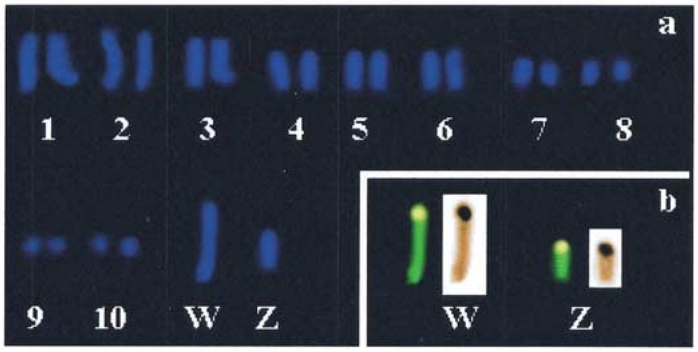


*Figure 5.* (**a**) Karyotype of *M. brassicae* after DAPI staining showed the presence of 11 chromosome pairs including a couple of heteromorphic sex chromosomes (Z and W) that are NOR-bearing as deduced by $CMA_3$ and silver staining (**b**).

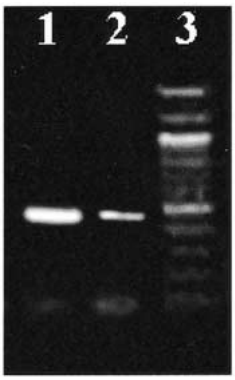


*Figure 6.* PCR (lane 1) and RT-PCR (lane 2) amplification of TRAS1. The molecular weight of the amplified fragments has been evaluated using a 100-bp DNA ladder (lane 3).

Furthermore, RT-PCR experiments were performed with the previously described primers evidencing a 300-bp portion of TRAS1 (Figure 6). RT-PCR experiments suggested, therefore, that TRAS1 was transcribed and therefore it could be actively transposing in the *M. brassicae* genome. The presence of DNA contaminants in the RNA samples used for RT-PCR has been evaluated using the PBP primers. PBP genes are intronless coding sequences that are not expressed in the *M. brassicae* cell line used for these experiments. The absence of a PBP band after PCR strongly suggested that no DNA contaminants were present in the RNA sample. Therefore RT-PCR results were due exclusively to TRASl RNA.

Finally, dot-blotting experiments showed that 2.8±0.5% of the *M. brassicae* genome consists of TRAS1.

## Discussion

As three different telomere structures have been observed in insects (Biessmann *et al.* 1990, Okazaki *et al.* 1993, Zhang *et al.* 1994, Sahara *et al.* 1999), the presence of each telomeric type was investigated in *M. brassicae*.

Southern blotting indicated that the $(TTAGG)_n$ telomeric sequence was present in the *M. brassicae* genome, whereas no signal has ever been observed after hybridization with the $(TTAGGG)_n$ and $(TTAGGGG)_n$ probes, with the Het-A, TART or *Chironomus* telomeric repeat probes.

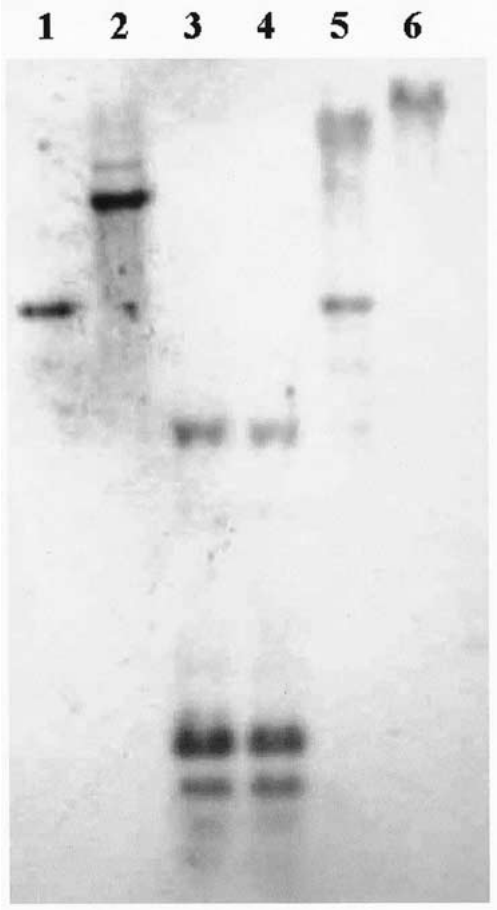


*Figure 7.* Southern blotting with the TRAS1 probe on *M. brassicae* DNA digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Msp*I (lane 3), *Hpa*II (lane 4), *Sca*I (lane 5) and *Taq*I (lane 6). Particularly interesting is the absence of difference in the restriction pattern of *Msp*I and *Hpa*II, suggesting that TRAS1 is not methylated.

FISH results with the $(TTAGG)_n$ repeat are consistent with those reported in numerous insects (Okazaki *et al.*, 1993, Sahara *et al.* 1999), suggesting that the $(TTAGG)_n$ sequence is a component of the *M. brassicae* telomeres. The absence of interstitial hybridization signals might indicate that the $(TTAGG)_n$ repeat is present in a very low copy number in interstitial locations that are not detectable by FISH or, alternatively, that no chromosomal rearrangements (such as chromosome fusions) have occurred recently in the *M. brassicae* complement.

The present data, as a whole, suggested therefore that most of the TTAGG repeats are restric-

ted to the terminal regions of *M. brassicae* chromosomes.

This hypothesis has been confirmed by *Bal*31 digestion that allowed us not only to definitively locate the telomeric repeats, but also to estimate the telomere length (about 7–9 kb).

In the interphase nuclei of most organisms, the telomeric regions are situated in an ordered fashion with an association to the nuclear matrix and clustering at least at some stage of cell life (Palladino *et al.* 1993, Luderus *et al.* 1996, Pryde *et al.* 1997). In *M. brassicae* interphase nuclei, telomeres were not clustered into a few foci but they appeared to be located near the nuclear periphery as reported in *D. melanogaster* (Hochstrasser *et al.* 1986). In view of such data, it appears evident that, even if telomeric structure and function are conserved in eukaryotes, the positioning of telomeres in the interphase nuclei seems to vary among species.

Southern blotting, FISH and *Bal*31 assay performed on *M. brassicae* indicated the presence of the retrotransposon TRAS1. Whereas most retroelements are dispersed through the chromosome complement, TRAS1 appeared preferentially inserted into the telomeric regions of *M. brassicae* chromosomes. This peculiar chromosomal location, already reported in *B. mori* (Okazaki *et al.* 1995), strongly indicated that TRAS1 has a high specificity of insertion.

However, TRAS1 was not present in all *M. brassicae* telomeres since it was absent from the NOR-bearing telomeres of both sex chromosomes. Small subtelomeric regions in the NOR-bearing telomeres of sex chromosomes have already been reported in other insects having holocentric chromosomes but its functional meaning is still unclear (Spence *et al.* 1998). As a possible function of the subtelomeric repetitive DNA is to facilitate chromosome pairing in meiosis (Loidl 1990), the presence of a large subtelomeric region could make pairing between sex chromosomes more difficult. This hypothesis would be particularly plausible if *M. brassicae* rDNA were involved in sex chromosome pairing as already reported in other insects (Mckee & Karpen 1990, Mandrioli *et al.* 2000).

Moreover, *Bal*31 digestion revealed that TRAS1 was located in the subtelomeric region of all the chromosomes and not at the telomeres, as reported for the mobile elements Het-A and TART in *D. melanogaster* (Pardue & DeBaryshe 1999).

The RT-PCR experiments and the analysis of the methylation status indicated that TRAS1 may be actively transcribed and unmethylated. In this regard, several authors have reported that transposable elements are frequently methylated in order to prevent transposition that would otherwise be expected to damage the genome (Arnaud *et al.* 2000). The absence of methylation in TRAS1 could suggest a high frequency of transposition of this mobile DNA into the lepidopteran genome or that methylation is not involved in transposon control in Lepidoptera.

C-banding applied to *M. brassicae* mitotic metaphases revealed that heterochromatin is essentially linked to the sex chromosomes, whereas autosomes contain only faint bands. The tendency to accumulate heterochromatin on the sex chromosomes has been repeatedly described in insects with holocentric chromosomes (e.g. Mandrioli *et al.* 1999). This fact substantially differs from what is observed in monocentric chromosomes, where heterochromatic regions typically occupy specific zones of all chromosomes, corresponding to centromeres and sometimes telomeres (Schweizer & Loidl 1987).

Finally, only telomeres of chromosomes Z, W, 7, and 11 appeared to be heterochromatic. These data suggest therefore that TRAS1 is associated with the *M. brassicae* telomere independently of the presence of heterochromatin at telomeres.

The interest in telomeres of holocentric chromosomes is not limited to the comparison of their architecture with that of monocentric ones, but it is emphasized by their peculiar structure which allows karyotype rearrangements due to chromosomal fragmentation (Okazaki *et al*. 1993). All fragments produced by X-ray irradiation will be in fact conserved in the chromosomal set in view of a centromeric activity in each fragment (Hughes-Schrader & Schrader 1961, White 1973). Therefore, on the basis of such properties and in view of the presence of both *in-vivo* and *in-vitro* analysis tools, *M. brassicae* holocentric chromosomes appear to be an intriguing model for the study of telomere dynamics and in particular of *de-novo* telomere formation at breakage sites.

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