

Methylated DNA sequence extrusion during plant early meiotic prophase

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Abstract — Reproduction in plants has been thoroughly studied and abundant information is currently available in scientific literature. Here we present new data related to a process of DNA modulation along the meiotic steps, first described 50 years ago, but never further pursued. A time course of DNA content/nucleus by cytophotometric analysis, along the reproductive steps from Pollen Mother Cells (PMCs) to seedlings in barley and oil palm displays a significant modulation of nuclear DNA content that reaches its minimum at the zygote stage. Recovery of the DNA content/nucleus during seed germination stages has been demonstrated. Moreover, cytological and immunocytochemical studies indicate that methylated heterochromatic bodies are extruded from PMC nuclei in the progression of the pre-leptotene stage up to the bouquet. The heterochromatic and methylated status of the extruded bodies gives these data a perspective aligned with exciting current data relating to DNA methylation dynamics and chromatin modifications. Our hypothesis is that somatic cells committed to undergoing meiosis need to release the burden of accumulated somatic multicopy DNA sequences and regain totipotency before entering the process of gametogenesis. The event is interpreted as a mechanism for assuring a single copy DNA pairing and therefore avoiding the risk of unequal crossing-over. Methylation represents the evidence of RNA interference machinery at work, leading to heterochromatinization and extrusion of sequences. The recovery of the extruded DNA sequences along embryo development suggests the occurrence of gene amplification mechanisms.

Key words: barley, DNA modulation, meiosis, methylation, oil palm.

INTRODUCTION

Meiosis is the basic fundamental process shared by all organisms undergoing sexual reproduction. It also represents the basis for Mendelian inheritance. It has therefore been widely studied using all possible experimental approaches. Notwithstanding this plethora of researches, many obscure points still need to be addressed. For example, in plants lacking a proper germ line, it is not known which signal triggers somatic cells to undergo meiosis (KLECKNER 1996; DAWE *et al.* 1998).

The mechanisms related to the function of Synaptonemal Complex are not fully understood (GILLIES 1973; STERN *et al.* 1975). Another very interesting point, particularly in the light of recent advances in our knowledge of chromatin remodeling processes, is linked to the totally unknown

meaning of the heterochromatinization processes appearing during early meiotic prophase stages: blocks of heterochromatin, not related to the heterochromatic chromosomal sites of the normal adult plant, are formed in practically all plant species (BENDER 1998) and disappear at zygotene. Very little is known about the fate of these heterochromatin blocks and the implications of their disappearance when chromosomes are arranged in the bundled bouquet configuration.

Moreover, many phenomena are gathered under the umbrella of meiosis, such as the disappearance of transgene copies, or the resetting of epigenetic manifestations (DEPICKER and VAN MONTAGU 1997; HOLTORF *et al.* 1999) and the loss of sequences and recovery of others (LOLLE *et al.* 2005), all of them requiring a genomic surveillance mechanism (YAO *et al.* 2003) of which there are no clues at the moment.

We have been particularly interested since the nineteen-seventies in DNA modulation phenomena during various developmental steps (WALBOT

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and CULLIS 1985; BASSI 1990; BITONTI *et al.* 1993), and specifically during meiotic stages. We have been quite intrigued by the description, more than sixty years ago, of chromatin body extrusions along early prophase stages at meiosis in many plant species (SPARROW and HAMMOND 1947).

KAMRA (1960) in the nineteen-sixties extensively described chromatin extrusion at the early meiotic prophase in barley, and quoted also numerous references describing similar phenomena in many plant genera.

It has been possible to observe similar phenomena also *in vitro*. Since the nineteen-seventies our team has studied DNA modulation (and methylation) of *Nicotiana* species in culture triggered by stress from the cultural conditions and the pressure on cells to differentiate for survival (PARENTI *et al.* 1973; NUTI RONCHI *et al.* 1973; MARTINI and NUTI RONCHI 1977; DURANTE *et al.* 1989). In these reports amplification of a satellite DNA during dedifferentiation processes has been described, and biochemical and cytological data confirm the transfer out of the nuclei into the cytoplasm of nucleolar and perinucleolar chromatin bodies (MARTINI and NUTI RONCHI 1977).

More recently, several papers on genome size reduction at meiosis have been published, indicating different assumed mechanisms, such as illegitimate recombination or expansion/contraction of CAG repeat tracts (JANKOWSKI *et al.* 2000; BUCHOLC *et al.* 2001; DEVOS *et al.* 2002; BENNETZEN 2002). The problem of variation in the amount of non-coding DNA has recently been discussed in the literature (FEUK *et al.* 2006; BENNETZEN *et al.* 2005). Very interestingly, a similar phenomenon has been described also in animals: in *Bubalus bubalis*, a specific sequence is released from meocytes during spermatogenesis, and restored in somatic cells after fertilization (CHATTOPADHYAY *et al.* 2001). But the most compelling example comes from *Tetrahymena thermofila* where double stranded RNA triggers efficient deletion of the targeted genomic regions (YAO *et al.* 2003).

Further information on this point was recently acquired by investigating the process of somatic meiosis occurring whenever plant somatic cells start dividing *in vitro* (NUTI RONCHI *et al.* 1992; NUTI RONCHI 1995). It has been shown in carrot (and in several different cultivars of barley, durum wheat, *Vicia*, etc.) the occurrence of chromosome segregation events as part of a process of general floral reprogramming that, during the first hours of culture, induce the expression of genes related to the reproductive organs in con-

comitance with the presence of meiotic-like divisions (PITTO *et al.* 2001).

In carrot somatic embryogenesis (which is a model system) we showed that during the process of embryogenic competence acquisition, a loss of DNA sequences occurs, and the regenerated embryos show DNA content/cell at its minimum. The DNA loss is composed chiefly of unique sequences, and is recovered during development (GERI *et al.* 1999). In addition, as proof of somatic meiosis we were able to demonstrate that the regenerated plants show segregation of specific markers (GIORGETTI *et al.* 1995).

These *in vitro* data prompted us to undertake a similar investigation *in vivo*. The aim was to demonstrate the process of dismissal of DNA sequences, conspicuous in "*in vitro*" carrot somatic embryogenesis, occurring also from the nuclei during the meiotic prophase till the bouquet stage in plants "*in vivo*". The nuclear nature, the presence of DNA modulation and the methylated state of the discharged bodies were ascertained by immunocytochemical and microdensitometric analysis.

We have chosen the oil palm (*Elaeis guineensis* DuraXTenera) as the phenomenon under investigation was widespread in this species, possibly influenced by its hybrid condition (LANE RAYBURN *et al.* 1993), and barley (*Hordeum vulgare*), since it had been previously the object of an exhaustive investigation (KAMRA 1960), and is a good test system for these studies.

There are two novelties in this paper: this is the first report of a time course of DNA content/nucleus along the vegetative and reproductive stages of the plant life cycle. Secondly, even if sequence extrusion is not new and was extensively described many years ago, only the current immunocytological techniques can demonstrate their methylation status.

The hypothesis of a possible link between these events and plant silencing processes is formulated.

MATERIALS AND METHODS

DNA cytophotometry - DNA cytophotometry was carried out, as previously described (GIORGETTI *et al.* 1995), on tissues or cells from oil palm (*Elaeis guineensis* DuraXPisifera, 309/125) (zygotic embryos and root tip apices) and from barley (*Hordeum vulgare*, cv Aura) (microspores, zygotic embryos and root tip apices). Feulgen/DNA absorption in individual nuclei (minimum 250 nuclei for each sample) was measured by the two-wave-

length method (PATAU 1952) using an MPV Compact Leitz microscope-photometer.

Cytological analysis - Preparations of pollen mother cells were obtained from isolated anthers in immature male flowers of *Hordeum vulgare* and *Elaeis guineensis* fixed in Carnoy (ethanol-acetic acid 3:1 v/v). Cytological analysis of the pre-meiotic and meiotic events was performed: a) after 1 hour hydrolysis in HCl 5N at room temperature and Feulgen staining, b) after staining with 4',6-diamidino-2-phenylindole (DAPI Sigma), 1 µg/ml.

We paid very careful attention to the preparation of the specimens: the samples were slightly squashed, and no enzymatic treatment was performed for the purpose of improving exposure of the chromosomes, as Armstrong and Jones claimed was necessary to study PMCs in *Arabidopsis* (ARMSTRONG *et al.* 2003). Moreover we constantly checked the integrity of the chromosomes and the nucleolus.

Immunological detection - The *in situ* immune-localization of the methylated genome regions was carried out on PMCs of barley and oil palm substantially according to the previously described methods (RUFFINI CASTIGLIONE 1995) using a monoclonal antibody against 5-methylcytidine (5-mCyt) (PODESTÀ *et al.* 1993). The antigen-antibody binding sites were detected by two different indirect approaches. We used both a secondary gold conjugated antibody with a silver enhancing procedure for light microscopy (BioCell Research Laboratories), or a secondary fluorescein conjugated antibody (Vector) combined with a comparative analysis under an epifluorescence microscope.

The binding of anti-5-mCyt was proved specific for 5-mC residues in DNA by the control tests as: i) a treatment with a non-immune goat serum at the same dilution as the primary antibody; ii) further dilution of the primary antibody; iii) the

omission of the secondary antibody; iv) the omission of the DNA denaturation step.

RESULTS

Cytophotometric analysis of nuclear DNA content - The cytophotometric measurements of the nuclear DNA content at different meiotic stages were performed on barley. The histograms in Figure 1

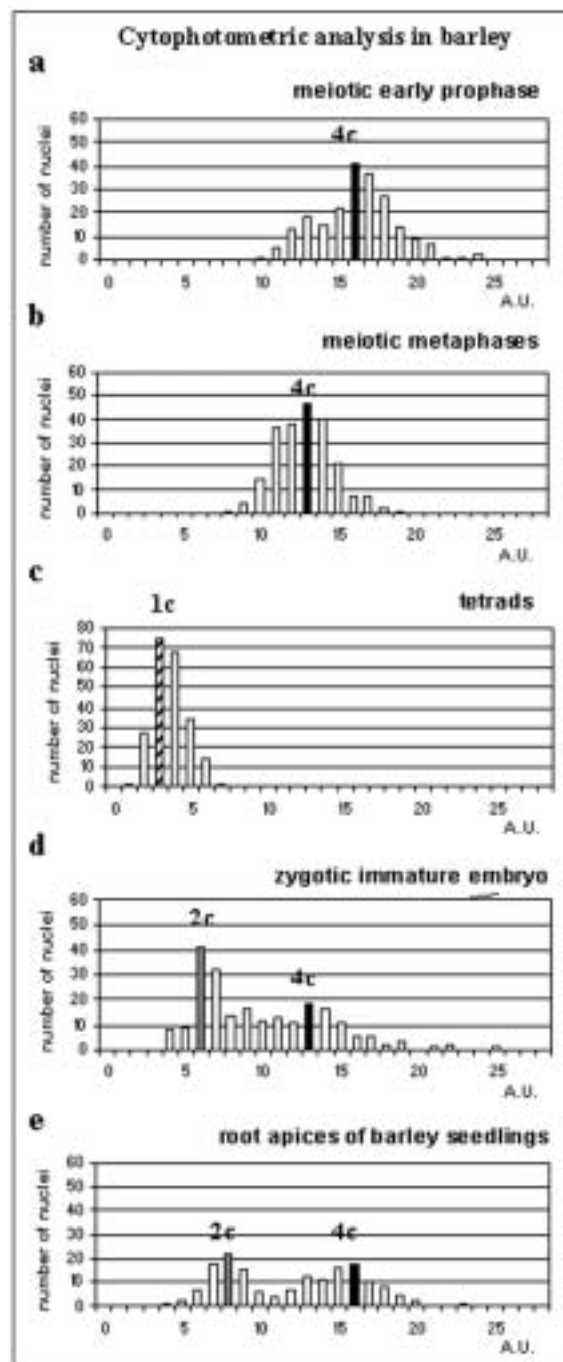


Fig. 1 — Cytophotometric measurements of the nuclear DNA content, expressed in AU (arbitrary units) of different meiotic stages of barley PMCs. A progressive decrease in nuclear DNA content values is evident from **a**, early meiotic prophase, $4C \approx 16.522$ A.U. value, to **b**, meiotic metaphase $4C \approx 13.0177$ AU value. The loss of sequences is confirmed by **c**, tetrad values $1C \approx 3.352$ A.U., and by **d**, $4C \approx 13.509$, values obtained from the zygotic immature barley's embryos. A recovery of DNA sequences, **e**, was again shown by the seedling root apices $4C \approx 15.863$.

show the Feulgen/DNA absorption values in barley individual nuclei of a), meiotic early prophase, b), metaphase I, c), tetrads and d), zygotic embryos compared with e), seedling root tips.

In Table 1 the mean values of $4C \pm$ standard error (S.E.), expressed in Arbitrary Units (A.U.), are calculated from each histogram in Figure 1: comparing the $4C$ values of different stages of meiosis, of zygotic immature embryos, and of seedling root apices, a statistically highly significant decrease of nuclear DNA content is found to be clearly unambiguous in all phases, in comparison with the early meiotic prophase considered as control tissue. The detected difference of 21.21% between the early meiotic prophase and the metaphase I has been ascribed to depletion of DNA sequences occurring during the early prophase stage. A difference of 18.23% was still present in barley immature zygotic embryos, and there was a reduction of 3.98% in seedling root apices.

Analogous results were obtained (Figure 2) by measuring nuclear DNA content in oil palm embryo-cells (pulled off dry seeds). Here the cytophotometric study was focused on zygotic embryo DNA content during germination (see Figure 2 and Table 2). In the reported histograms (Figure 2) $4C$ population appears only after four days of germination and this lag is in accordance with the very long germination time of oil palm seed in nature.

Figures 1 and 2 show also the interesting evidence that an almost complete recovery of the amount of DNA lost occurs gradually, as attested by analyzing seedling root tips of both species. In the time course analysis during the first ten days of growth in oil palm germinating seeds, the nuclear DNA content of ten day old plantlets still showed a difference of 4.1% less than the control plants (Figure 2 and Table 2).

Cytological and immunocytochemical analysis - The cytophotometric results drew attention to the phenomenon of release of heterochromatic bodies before gamete formation.

The possibility of detecting the specific presence of DNA and its methylation pattern in the extruded bodies has afforded fresh insight into the event.

The sequences of events are illustrated for barley meiotic PMCs in Figure 3; the assembly of heterochromatin before the bouquet stage and its extrusion is shown in Feulgen stained cells, (A-E), and in DAPI stained cells, (F-I). In early meiosis, pre-bouquet stage, A, B, F, G, the extrusions are already visible; C, H, represent a bouquet stage and D, E, I, are at the zygotene.

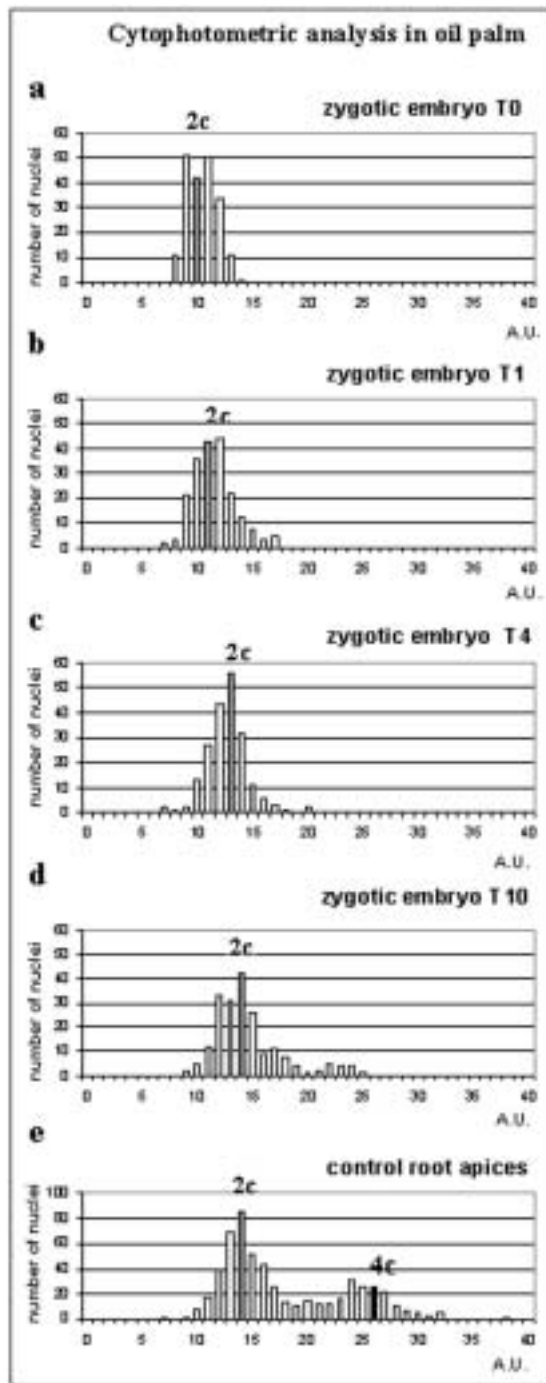
Figure 4 shows the sequence of the ensuing events [A to C, Feulgen staining; D to G, DAPI staining] occurring during the leptotene stage of oil palm PMC's prophase: A, D, early prophase stage shows the heterochromatin gathering in the

Table 1 — $4C$ DNA content during meiosis and in zygotic embryos in barley. Mean values of $4C \pm$ standard error (S.E.) expressed in A.U. in different stages of meiosis, of zygotic immature embryos and of seedling root apices. The $4C$ value of early meiotic prophase (16.522 A.U.) is compared to that of meiotic metaphase (13.0177 A.U.), to the $4C$ values of immature embryos (13.509 A.U.) and seedling root apices (15.863 A.U.). The statistical significance of the observed differences was calculated by t-Test.

Samples	Early meiotic prophase	Meiotic metaphase	Zygotic immature embryos	Seedling root apices
$4C$ (A.U.) \pm S.E.	16.522 \pm 0.0606	13.0177 \pm 0.0785	13.509 \pm 0.139	15.863 \pm 0.115
% difference with early meiotic prophase		21.21%	18.23%	3.98%
t-Test		P<1‰	P<1‰	P<1‰

Table 2 — Zygotic embryos $2C$ DNA content during germination in oil palm. Mean values of $2C \pm$ standard error (S.E.) expressed in A.U. for oil palm zygotic embryos during the first ten days of germination. The $2C$ value (14.21 A.U.) of adult plants root tips (control) is compared to that of the embryos for each germination time and the difference is expressed as a percentage. The statistical significance of the observed differences was calculated by t-Test.

Samples	Control plants	Zygotic embryos T0	Zygotic embryos T1	Zygotic embryos T4	Zygotic embryos T10
$2C$ (A.U.) \pm S.E.	14.21 \pm 0.082	10.37 \pm 0.082	11.06 \pm 0.095	12.54 \pm 0.096	13.65 \pm 0.124
Difference with control	-----	37.03%	28.48%	13.31%	4.10%
t-Test	P<1‰	P<1‰	P<1‰	P<1‰	P<1‰



nucleus; B, E, heterochromatic bodies are formed and detached from the chromosomes to the cytoplasm; C, F, G, the newly formed bodies are being extruded out of the nucleus (bouquet stage).

The figures 3 (J-M) and 5 illustrate the stimulating results obtained by applying the technique of immuno-localisation of methylated sequences by means of a monoclonal antibody against 5-methylcytidine. We could demonstrate that the discharged bodies, inside or outside the nucleus,

Fig. 2 — Cytophotometric measurements of the nuclear DNA content, expressed in AU (arbitrary units), at different days of germination of oil palm zygotic embryos. Modulation of the 2C values from: **a**, dry seed zygotic embryos $2C \approx 10.37$ to **b**, first day $2C \approx 11.06$ and **c**, fourth day showing the shift to higher DNA content values, $2C \approx 12.54$. On day ten, **d**, the recovery is still not complete: $2C \approx 13.65$ compared to the control, **e**, plant root tips $2C \approx 14.2$.

are highly labeled at the time of extrusion in both barley PMCs (Figure 3 J-M) and oil palm (Figure 5).

The figures show results of two different immunological techniques: immunogold silver enhancing method (Figure 5 A-I), and indirect immunofluorescence (Figure 3 J-M and 5 J-L). Figure 3 (J-M) shows a progression of meiotic prophase stages in barley, antibody labelled and evidenced by indirect immunofluorescence (Figure 3 J, K, very early leptotene, L, late leptotene stage, M, zygotene). The dismissed barley chromatin appearance is shaped as droplets of various sizes, dots and Feulgen positive masses, often still hanging from the chromosomes, and is slightly different from that of oil palm, which usually shows one or two micronuclei-like heterochromatic bodies.

In Figure 5 the different stages of meiotic prophase have been sorted into the typical sequence: A, J, oil palm premeiotic PMCs are labeled by the antibody; B, F, C, G, K, cells at the bouquet stage are organizing one or two heterochromatic bodies, respectively. Part of highly methylated regions at this stage are apparently "moving" from the bulk DNA to the new organizing heterochromatic bodies (arrows); D, H, E, I, the process of heterochromatin elimination is evidenced by the presence of micronuclei-like bodies of different size and number which appear to be strongly positive. Figure 5 L, shows the rare appearance of antibody labeled extrusions at diplotene stage (arrow).

A careful analysis shows that all the extruded bodies present antibody labeling, most of them very strongly, others to a lesser extent, but none are heterochromatic and not methylated. Obviously a labeling signal in the nucleus is also detectable, since constitutive heterochromatin also is methylated.

A detailed cytological analysis was performed in barley and in oil palm to estimate the extent of the phenomenon: meiocytes exhibiting this behavior amounted to 40% in barley and 34% in oil

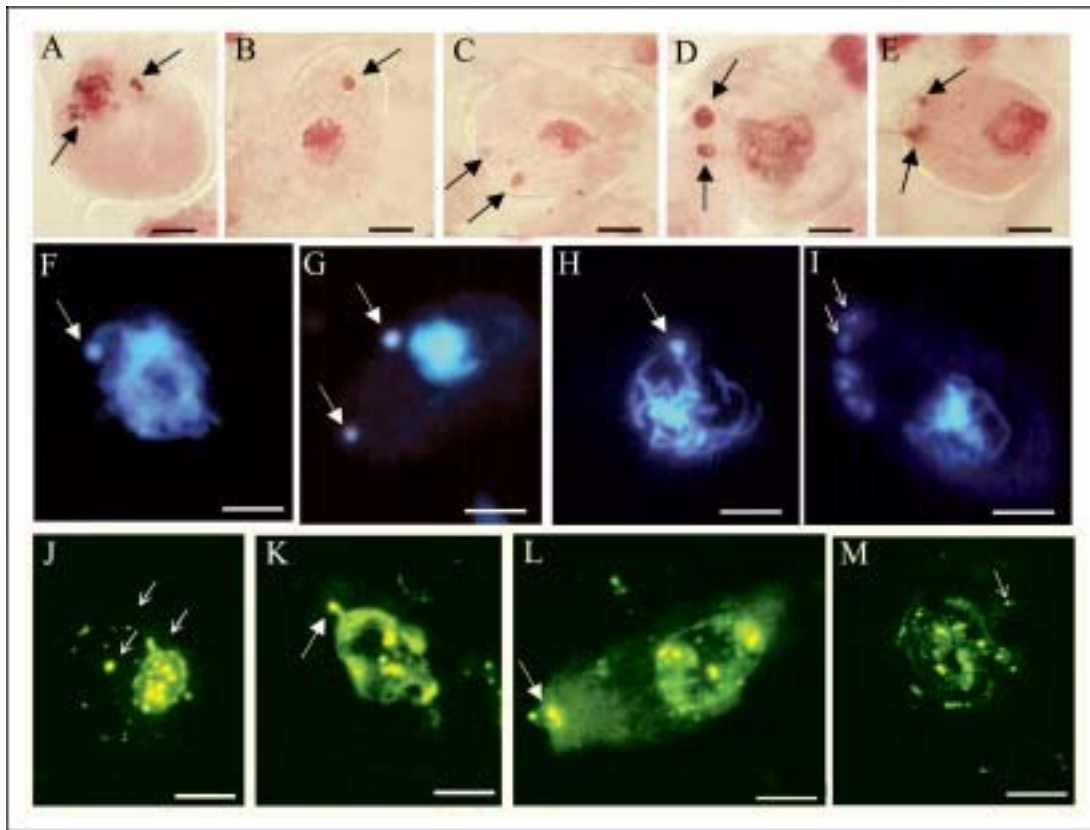


Fig. 3 — Extrusion of heterochromatin during barley PMC early meiotic prophase. **A-E**, Feulgen stained cells; **F-I**, DAPI stained cells; **J-M**, indirect immunofluorescence by the technique of immunolocalization with the antibody against 5-methylcytidine. **A-I**, the assembly of heterochromatin and its extrusion occur in the form of droplets, dots and small masses, often still attached to the chromosomes (arrows); **A, B, F, G**, show a pre-bouquet stage where the extrusions are already apparent; **C, H**, represent a bouquet stage and **D, E, I**, are at the zygotene. **J-M**, succession of events where the extruded bodies appear strongly positive to the antibody against 5-methylcytidine in all the considered stages (**J, K**, very early leptotene, **L**, bouquet stage, **M**, zygotene). Bars = 5 μ m.

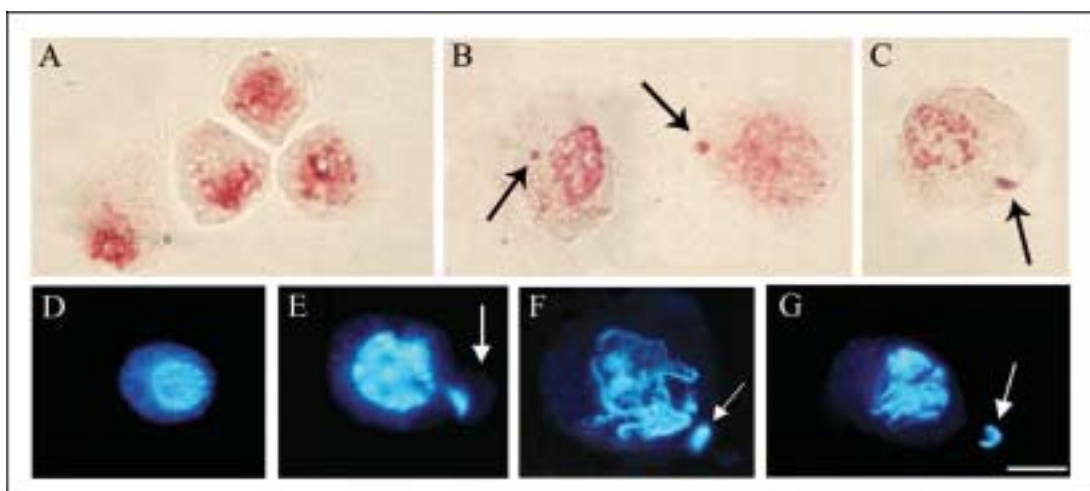


Fig. 4 — Extrusion of heterochromatin during oil palm PMCs early meiotic prophase. **A to C**, Feulgen staining; **D to G**, DAPI staining. **A, D**, very early prophase stage showing heterochromatin assembling in the nuclei; **B, C**, heterochromatic bodies (arrows) are formed and released from the chromosomes into the cytoplasm (early bouquet stage); **E, F**, extrusion of the newly formed bodies out of the cells (late bouquet stage); **G**, heterochromatic body is out of the cell, the chromosomes are at the zygotene stage. Bars = 5 μ m.

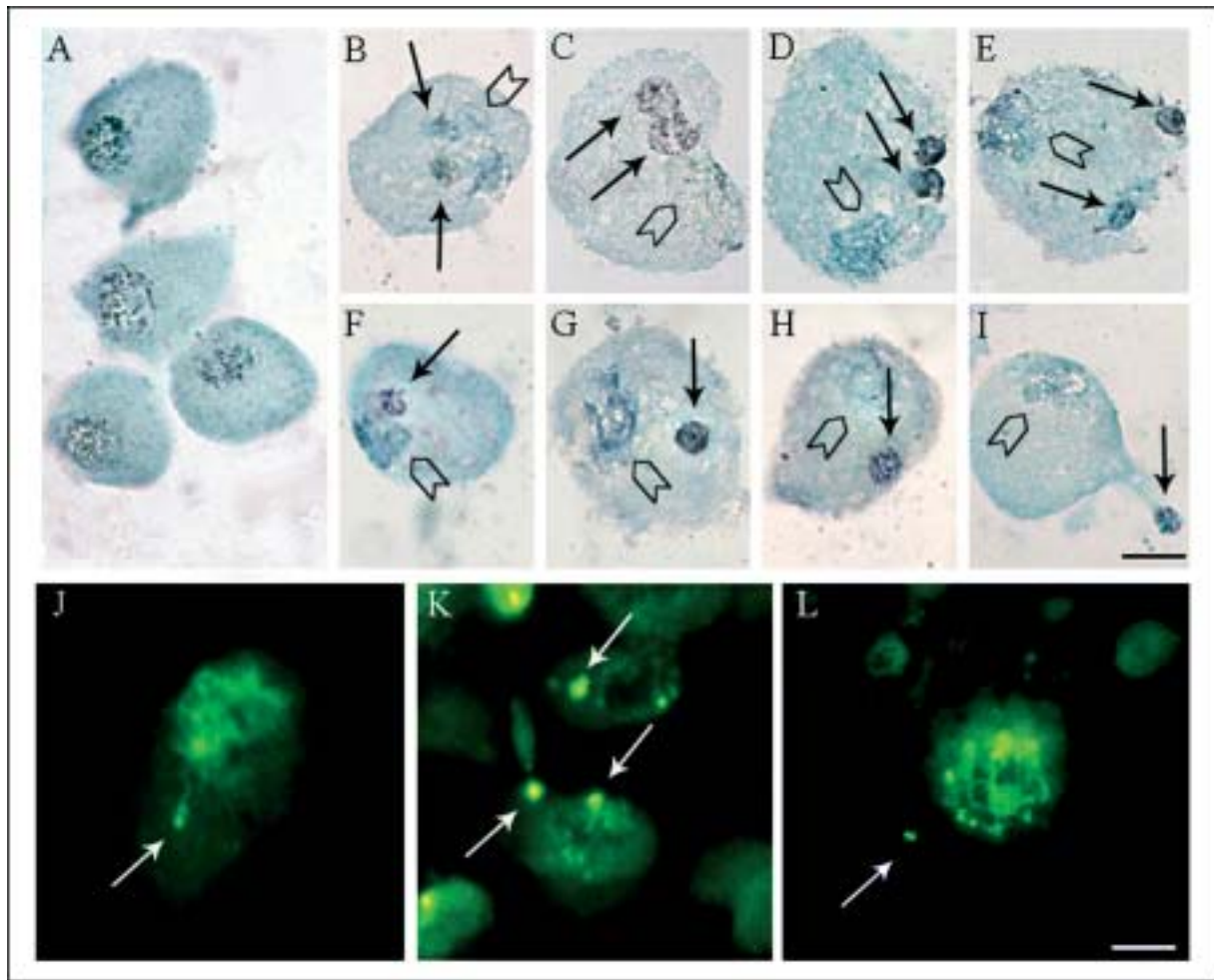


Fig. 5 — Extrusion of heterochromatin in oil palm prophase. Application of the technique of immunolocalization with the antibody against 5-methylcytidine. **A-I**, immunogold silver enhancing method, **J-L**, indirect immunofluorescence. Void arrows point out the bouquet chromosomes. **A-I**: typical sequence of different phases in the early meiotic prophase: **A** and **J**, oil palm PMCs strongly labelled by the antibody at early leptotene; **B, F, C, G, K**, labelled heterochromatic bodies (arrows) are still entangled in the bouquet stage chromosomes; **D, H, E, I**, heterochromatic bodies strongly positive to the antibody are being extruded from the cells; **L**, a labelled body still present at diplotene. Bars = 5 μm .

palm). Notwithstanding meiosis is a fairly synchronous process, a small time discrepancy is always to be expected in biological phenomena, and so the high percentage of extrusion observed allows us to assume that the mechanism involves the whole population.

DISCUSSION

In this paper we have reconsidered and set out the phenomenon of DNA elimination along early male meiosis and, more in general, DNA modulation along basic reproductive processes in plants

as the starting point for some important considerations.

The absolute novelty of the above reported data is represented by the fact that the extruded sequences are all methylated, albeit to varying degrees, and heterochromatic. This is a very interesting result, because it links DNA modulation mechanisms to methylation and meiotic heterochromatinization processes, which are widely known to be related to the phenomena of silencing and epigenesis. Indeed a picture is gradually emerging in current research showing the reciprocal relationships among processes like DNA methylation, the mechanism of silencing, chromatin remodelling and heterochromatin control of

gene expression (MARTIENSEN *et al.* 2001; MATZKE *et al.* 2001; RUFFINI CASTIGLIONE *et al.* 2002; MEINS *et al.* 2005; STRICK and QUESSAVAVIAL 2006).

Our hypothesis assumes that, at the early meiotic prophase stage, a surplus of somatic DNA repeats gathers under heterochromatinization and *de novo* methylation processes. The observed reshaping of chromatin structure and establishing of *de novo* methylation patterns is an extensively described mechanism, also in other systems related to chromatin remodelling factors, as suggested by the studies on DDM *Arabidopsis* mutants (KAKUTANI *et al.* 1999).

The present data offer experimental evidence in support of the hypothesis that plant somatic cells, in order to perform functional gametogenesis, need to get rid of somatic multicopy DNA sequences, which are necessary to the adult plant tissue, but a bar to such a precise process as meiosis.

Heterochromatic somatic DNA may well correspond to the heterochromatic blocks formed on meiotic prophase chromosomes that disappear, as attested previously, exactly at the same meiotic phase (PASZKOWSKI and WITHAM 2001).

We can consider the methylated status of the extruded bodies as a cellular signal of sequences to be made heterochromatic and dismissed. We assume that the purpose of this entire process is to guarantee a functional chromosome pairing and crossing-over in order to produce viable gametes (PAWLOWSKI and CANDE 2005).

Moreover it is reasonable to postulate that, as in carrot (GERI *et al.* 1999) and *Pisum sativum* L. (CECCHINI *et al.* 1992), the modulated sequences may belong mainly to the class of the unique sequences and therefore to the euchromatic sort of DNA: this further suggests that the process of heterochromatinization comes along the new methylation pattern. It is worth noting that the discovery of a requirement for the RNA interference (RNAi) pathway in heterochromatin initiation provides evidence for a linked role for RNAi in controlling both methylation and heterochromatinization by inducing a local alteration of chromosome structure (GREWAL and MOAZED 2003). With regard to this point the *Tetrahymena* example needs a special mention: genome rearrangements occur through an RNAi-related process involved in DNA elimination in somatic macronucleus development (MOCHIZUKI and GOROVSKY 2004). A similar process is implicated in gene silencing in *Arabidopsis thaliana* (and other organisms) (ZILBERMAN *et al.* 2003; HAMILTON *et al.*

2002). Mochizuki and Gorovsky (2004) compared the two organisms' strategies, finding that the only difference was the end point of the process – silencing in the plant and sequence elimination in the ciliate – concluding that the last “can be viewed as an ultimate form of gene silencing”. Taking into account the above-mentioned considerations, loss of sequences (foreign and somatic surplus copies) at meiosis in plants can be likened to the endpoint (sequence elimination) in *Tetrahymena*, depending on the unavoidable assumption of the existence of a general genomic surveillance mechanism (YAO *et al.* 2003).

Cytophotometric measurements provide evidence concerning the modulation of nuclear DNA content along meiosis, immature zygotic embryos of both oil palm and barley demonstrating the lower level of DNA content/nucleus in comparison to the seedling stage, where the 4C content of the vegetative phase is almost recovered.

Oil palm is also a good test system, since we noted a substantial decrement of DNA content/nucleus in regenerated adult plants, in parallel with the appearance of somaclonal variants (data not presented).

These results are a constant find in all the angiosperms so far analyzed in our laboratory. The extent of DNA modulation, detected in plants “*in vivo*” using immunological-cytophotometric techniques, perfectly matches that demonstrated “*in vitro*” by molecular means in carrot somatic embryogenesis (GERI *et al.* 1999). Moreover, the present results are the only data currently available on a time-course of DNA content/nucleus along the meiotic process. One point of discussion is the time interval over which this phenomenon occurs. Our approach has been to perform a sort of cytological time course, starting from the mother pollen cells till the end of the meiotic process. Heterochromatin bodies detached from the chromosomes and extruded in the cytoplasm start appearing in the very early prophase, before the cytological recognition of the bouquet formation. This stage is recognized by the clustering of chromosome ends into a polarized configuration on the nuclear envelope, during the leptotene/zygotene stage, and is considered the first step in homologous chromosome pairing.

Though the bouquet configuration is an almost general occurrence in all eukaryotes, its function in meiosis is not yet completely known. The chromosome movements occurring at the bouquet stage could be involved in the physical removal of the heterochromatic bodies (SCHER-

THAN 2001). A role in releasing the extrusions into the cytoplasm can be played also by the different density of nuclear membrane pores and by a general spatial reorganization of the nuclear envelope contents (ZICKLER and KLECKNER 1998) specific also to this stage. Hence the phenomenon may take on different aspects, frequencies and intensities in different plant species, being strongly influenced by environmental variations (TAKATS 1959) and the meiotic stage involved always being up to the leptotene-bouquet phases (NUTI RONCHI, unpublished results).

The timing of the extrusion phenomenon illustrated above suggests the mechanism underlying this process. The meiotic recombination model comes out from yeast data (CHA *et al.* 2000), but up to date it can be extended to most of the higher eukaryotes, as *Arabidopsis thaliana* (GRELON *et al.* 2001) and Maize (DOONER 2002). It shows that the first step is the Double Strand Breaks (DSBs) formation by the protein SPO11, in the tight interval between the early leptotene and the bouquet stage, followed by the 5'-end resection that is decisive in the creation of recombination products. It has been demonstrated the presence of two separate pathways for the formation of non-crossover versus crossover products (ALLERS and LICHTEN 2001; DOONER 2002), the last ones appearing sensibly later than the others.

We suggest that it is not a coincidence that extruded DNA appears at the same time as non-crossover products. In this hypothesis we are supported by the proposal of PRADO and ANGUILERA (2003), when they suggest that extensive 5'-end resection has the role to avoid ectopic recombination and maintain genome integrity. Our work premise is exactly the same. We propose that these mechanisms are involved in the resection of somatic heterochromatin in the course of a non-crossover event, in order to perform a faultless pairing and crossover products.

The recovery of lost sequences, as described in our previous (GIORGETTI *et al.* 1995; GERI *et al.* 1999) and present papers, is not new in biology: particularly in plant science, amplification and under-replication of sequences have been extensively described since the nineteen-seventies. Sequence amplifications occur in plant cells under many circumstances (PARENTI *et al.* 1973; NUTI RONCHI *et al.* 1973), and can follow the general model of sequence amplification described in two recent erudite reviews (TOWER 2004; CLAYCOMB and ORR-WEAVER 2005). Both reviews report and accurately describe mechanisms of amplification, but there is no mention of the regulatory events

leading to the complete comprehension of this process. In this model multiple replicators and origin elements activate a chromosomal domain, and this initiation zone is subjected to a process of chromatin remodelling.

Our suggestion is that the appearance of epigenetic phenomena, which are particularly evident in the alteration of developmental patterns (in culture or in other stressful environments) such as somaclonal variations in cloned plants (and animals? DYCE *et al.* 2006) is due to defective regulation along the above-mentioned processes.

Our model suggests that the mechanism activated at the early meiotic prophase is the gathering, under methylation and heterochromatinization, of a surplus of somatic sequences that are extruded out of the nucleus. The deletion of the sequences can assure a concatenation of functions such as genomic surveillance, a perfect pairing allowing correct non-crossover (to erase somatic and foreign sequences) and crossover events and the correct regulation of developmental processes. The erased sequences may help solve the problem of the crossover interference, i.e. the systematic difference between the genetic and physical maps of the same chromosome that appears to occur about as universally as meiosis itself (EGEL 1995) and is rationalized, on a molecular basis, by the loss of somatic sequences that reduces the chromosome length available to crossing over (KAGAWA *et al.* 2002). Somatic embryogenesis data (GIORGETTI *et al.* 1995; GERI *et al.* 1999), as well as the results from *in vivo* meiosis presented in this paper, suggest that the phenomenon of chromatin diminution before gamete formation described herein could be likened to a process of germ-line formation; indeed somatic cells are reset before entering meiosis, acquiring a status of typical germ cells.

Regulation processes governing gene amplification or resection, as well as the postulated existence of a (possibly related) genomic surveillance mechanism, and the various facets of epigenetic phenomena are among the most exciting challenges actually facing research in biology.

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