DNA methylation patterns on plant chromosomes

M. RUFFINI CASTIGLIONE1, *, R. CREMONINI1 and M. FREDIANI2
1 Department of Botanical Sciences, University of Pisa, Via L.Ghini 5, 56126 Pisa, Italy.
2 Department of Agrobiology and Agrochemistry, Tuscia University, Viterbo, Italy.

Abstract - DNA methylation patterns of chromosomes belonging to several plant species have been investigated. We have reported previous data obtained by means of different indirect immunocytochemical approaches with specific antibodies against 5-methyl cytosine. The processed results have been comparatively discussed with the aim to provide further insight into certain aspects of the structure and organization on the chromosome level of the plant genome.

Key Words: chromosome banding, 5-methyl cytosine, plant chromosome structure, polyclonal and monoclonal antibodies.

INTRODUCTION

DNA methylation represents the main covalent modification occurring at position 5 of cytosine belonging to the DNA of most organisms, including vertebrates, plants and many lower eukaryotes. DNA methylation has been linked with stability of the chromatin structure (LI et al. 2002), with the control of gene expression (ATTWOOD et al. 2002) and, more in general terms, with epigenetic inheritance (MARTIENSSEN and COLOT 2001). Although gene promoters often contain methylation sites, the main regions of genomic methylation are located in heterochromatic domains, rich in repetitive elements and transposons (ADAMS and BURDON 1985; KUBIS et al. 1999; RABINONICZ et al. 1999). From an evolutive point of view DNA methylation may be considered a part of a host defence mechanism, able to direct a response to the invasion of the genome against parasitic DNA, both in unicellular and in multi-cellular organisms.

This type of host defence mechanism provided the eukaryotic cell with a remarkable evolutive opportunity to develop an epigenetic tool, symbol of its independence and contemporary of its role within complex organisms.

Certainly the processes and signals specifically required to methylate DNA are heterogeneous and involve in plants at least three classes of different enzymes, many of these not completely characterised. The Met class, structurally associated with the mammalian DNAmethyltransferase1 (Dnmt1); the DRM class (Domain Rearranged Methyltransferase) resembling Dnmt3 and the CMT class (ChromoMethylase), unique to plants and hypothesised to be related to RNA-directed DNA methylation mainly in CpXpG asymmetric sites (MARTIENSSEN and COLOT 2001 and references therein).

Substantial data supports a relationship between variations in DNA methylation and several biological events such as, differentiation and development (DURANTE et al. 1990; FREDIANI et al. 1992; FINNEGAN et al. 1996), cancer (GOODMAN and WATSON 2002), environmental adaption and stress resistance (KOVARIK et al. 1997; FINNEGAN et al. 1998; STEWARD et al. 2000), plant flowering time (BURN et al. 1993), senescence (COONEY 1993), tumour induction during plant tissue culture (DURANTE et al. 1989) even if it is...
debateable whether methylation is a primary signal for these phenomena or a maintenance signal for patterns established by other mechanisms.

Due to the increasing interest in the DNA methylation and its structural and functional roles, in recent years, researchers have been able to set up several different methods to identify 5 methyl-cytosine (5-mC) and related covalently modified bases (REIN et al. 1998; THOMASSIN et al. 1999). The occurrence of 5-mC may be quantified by means of HPLC or mass spectrometry with high sensitivity, but entirely lack sequence information. Other approaches including bisulfite, hydrazine, permanganate and MRSE (modification sensitive restriction endonucleases) methods, enable mapping of methylated bases at specific DNA sites in complex DNA genomes.

Since modern biochemistry and biotechnology need in situ approaches, in parallel to biochemical and molecular studies, cytological and cytogenetic techniques have also given significant support in understanding some aspects linked to epigenetic patterns and DNA methylation. Investigations on chromosome levels on genomic methylation have mainly been developed using both methylation-sensitive restriction endonucleases with the in situ detection of the cleaved sites (PRANTERA and FERRARO 1990; MANICARDI et al. 1994; OLSZEWSKA et al. 1999) and immunological approaches.

The first paper characterised by in situ immunological determination of DNA methylation pattern on chromosomes was published in 1974 by Miller and collaborators (MILLER et al. 1974). This paper described the distribution of methylated regions by means of polyclonal antibodies against 5-methyl cytosine (anti 5-mC) located in constitutive heterochromatin blocks of mammalian chromosomes. The following works describing in situ localisation of highly methylated chromosome regions focussed mainly on the animal kingdom (EASTMAN et al. 1980; SCHNEDL et al. 1975, 1976).

In parallel, after these first reports, other polyclonal (ACHWAL and CHANDRA 1982; FREDIANI et al. 1986; ANGELIER et al. 1986) and monoclonal antibodies (SANO et al. 1988; REYNAUD et al. 1991; PODESTÀ et al. 1993; Mizugaki et al. 1996) were produced as more and more specific tools to investigate DNA methylation patterns.

The first investigation on the chromosomal level in the plant kingdom comes from the work of Frediani and collaborators (FREDIANI et al. 1986) on polytene chromosomes of the Phaseolus coccineus embryo suspensor. In light of the interest in this problem and in order to obtain more information regarding 5-mC in plant chromosomes, over the last years we have studied, with different purposes, the DNA methylation patterns of metaphase chromosomes of a number of different species (RUFINI CASTIGLIONE et al. 1995; FREDIANI et al. 1996; ŠRÖKY et al. 1998; CASTILHO et al. 1999; CREMONINI et al. 2002) by using a monoclonal antibody anti 5-mC (PODESTà et al. 1993).

This paper aims at providing a general overview of plant chromosomes and their cytological methylation pattern characterised by the immunological approach.

**CONSTANT HIGHLY METHYLATED RICH-REGIONS**

In plant metaphase chromosomes it was at times possible to determine a lengthwise differentiation concerning the distribution of highly methylated regions, at least for specific chromosome sites.

In polytene chromosomes of Phaseolus coccineus embryo suspensor the immunoperoxidase approach by means of specific polyclonal anti 5-mC (FREDIANI et al. 1986) shows preferential binding to centromeric heterochromatic blocks. However certain heterochromatic bands do not appear to bear 5-mC, although they hybridise both with highly repeated (HR) DNA and with a DNA satellite (b. d: 1.702 g/ml).

In Allium cepa metaphase chromosomes (RUFINI CASTIGLIONE et al. 1995), after treatment with specific monoclonal antibodies (PODESTà et al. 1993) and immunogold detection of the highly methylated regions, a preferential binding of anti 5-mC occurs on telomeric sites but it is also evident in the proximal regions of almost all chromosome arms and in some intercalary bands. Chromosomal localisation of methylated regions does not overlap that of HR DNA as already demonstrated in P. coccineus (FREDIANI et al. 1986) polytene chromosomes. Minor differences result if we compare the cytological localisation of 5mC rich regions and of GC satellite DNA (BARNES et al. 1985)

In Vicia faba preparations, processed using the immunogold technique (FREDIANI et al.
1996), subtelocentric chromosomes contain a detectable amount of 5-mC in the short arms, in telomeric and subtelomeric regions and in several intercalary bands of long arms. In the long submetacentric pair I, anti 5-mC binding is appreciable mainly in the satellite and in the primary constrictions. In this plant system the binding of anti 5-mC does not apparently involve specific classes of sequences or particular chromosome locations, as occurs in *A. cepa* (Ruffini Castiglione et al. 1995). Indeed since satellite DNA in *V. faba* is A+T rich (b.d.: 1.677 g/ml) and the fast renaturing DNA sequences are scattered all over the length of metaphase chromosomes (Cionini et al. 1985) we could reasonably suppose that DNA methylation process in *V. faba* may preferentially involve particular DNA sequences of the HR fraction scattered over the chromosomes.

Furthermore, during the study of cytological methylation pattern of chromosomes belonging to plant species with a low chromosome number, *Zingeria biebersteniana* and *Haplopappus gracilis* (Ruffini Castiglione et al. 1998; Cremonini et al. 2002) we demonstrated the constant presence of positive immunogold signal to anti 5-mC binding mainly on telomeric regions and on ribosomal DNA localisation.

After indirect immunofluorescence *Melandrium album* (Široky et al. 1998) shows a very random variation concerning the cytological methylation pattern in autosomal chromosomes. It should be noted that, due to the high compactness of *M. album* chromosomes, it was not possible to distinguish individual heterochromatin, 5-mC rich bands or smaller chromosome domains.

*Triticale* plants, after treatment with monoclonal antibodies against 5-mC and detection with secondary biotinylated antibody-streptavidine method, show a strong response which is not uniform along the chromosomes but features a punctuate pattern of binding (Castilho et al. 1999), as in *Melandrium*. Moreover, *Triticale* chromosomes do not show preferential distribution on particular chromosome regions or chromosome arms, as partly occurs in *V. faba*.

The presence of highly methylated regions which constantly characterise the chromosome complement of the analysed plant species may assume a particular significance due to the establishment and maintaining of a genomic order, especially in metaphasic structures, despite the occurrence of a gradual accumulation of transposable elements (Martienssen and Colot 2001). In fact in cultured human lymphocytes, after several different treatments with 5-azadeoxycitidine and immunocytochemical approaches, a connection can be shown between DNA methylation and chromatid/chromosome compaction, even if such a phenomenon seems to be only partially related to DNA methylation (Flagiello et al. 2002). Within this context, it is also reasonable in the case of plants to maintain that DNA methylation may represent one of the various mechanisms involved in the stable packaging of repeats and transposons in a silenced, invisible and harmless genetic form.

**VARIABLE METHYLATED LOCATIONS**

If the frequencies and intensity of labelling in each chromosome region are taken into account, the analysis of cytological DNA methylation patterns in plants highlight a range of variations. Indeed, in line with chromosome regions showing a constant response to anti 5-mC binding, our analysis has revealed an irregular occurrence of immunolabelling in specific chromosome locations. The uneven distribution of the highly methylated rich-regions concerns either a qualitative (intensity of labelling) or quantitative (percentage of labelling) variability. In some cases these variations prevented us from proposing a general model describing a precise distribution of highly-methylated rich regions (e.g. *Triticale, Melandrium*). However, in other plant systems it was possible to identify and partly quantify as a percentage the occurrence of such heterogeneity, a clear example of this shown by polytene chromosomes of *P. coccinens* where, even if most euchromatic regions are methylated, the frequency of methylation is highly variable and at times low. Also in the *A. cepa* metaphase chromosome system, in which telomeric regions were always methylated, a generally lower percentage of labelling at centromeric and intercalary bands was detected (Ruffini Castiglione et al. 1995).

Inconstant and/or heteromorphic methylated regions were often detectable even along the same chromosome in *V. faba*. Also, in some cases, a close association between the presence of an appreciable amount of 5-mC in a chromosome region and the absence of antibody binding in another of the same chromosome was detected (Frediani et al. 1996).
Unfortunately these results do not offer a clear means for interpretation, especially as the chromosomal regions in question belong to intercalary sites often inadequately defined by other standard banding techniques. It can be excluded that such variations and heterogeneity can arise from mechanical distortion involved in the preparation of the metaphase spread and/or the various treatments, with inconstant bands occurring constantly in parallel with the constant ones. Also in human lymphocytes (Barbin et al. 1994) the occurrence of regional variations of methylation pattern and a wide range of polymorphic signal intensity were typically detectable after the immunological treatment. However, such phenomenon is confined and limited to the binding site of the short arms of acrocentric chromosomes. Another similar example comes from a study conducted on metaphase chromosomes of Piaractus mesopotamicus (Pisces, Characiformes), in which, after indirect immunofluorescence with anti 5-mC, heterogeneous labelling was shown along the chromatids in correspondence to euchromatic sites (Almeida-Toledo et al. 1998). It is worth noting that while in the specified examples the observed heterogeneity is not notably represented, it seems to stand out more in plant systems analysed.

It is reasonable to maintain that DNA modification patterns are tissue specific and that specific gene expression are connected to variable and dynamic methylation patterns. Indeed in plants epigenetic changes linked to DNA methylation are strongly involved both in vegetative (Zluvova et al. 2001) and reproductive development (Vyskot 1999).

In fact the potential role of DNA methylation remains widely debated, as on the one hand a large amount of methylated sites belong to transposable elements, making the changes in the tissue-specific methylation patterns difficult to construct, while on the other some specific genes assume variable methylation pattern in differentiated cells (Jones and Takai 2001 and references therein).

It is worth noting that the limited and uneven distribution of 5-mC sites detected in human chromosomes may be related to the specific biological system. Human lymphocytes cultures constitute a more homogeneous system in comparison to plant samples. Indeed our data are obtained starting from meristematic cells of root tips, in which the commitment to the differentiation is progressively occurs in parallel with epigenetic changes and heterogeneous DNA methylation pattern may accompany such differentiation processes.

**DIFFERENCES IN CORRESPONDING REGIONS OF HOMOLOGOUS CHROMOSOMES**

The study of cytological methylation patterns along metaphase chromosomes have offered us a starting point to support some interpretations which require much future work to be considered of general significance, at least as regards the plant kingdom. Until now our results seem to note a further kind of heterogeneity. When the behaviour of homologous chromosome regions in different cells was analysed comparatively, it was seen that corresponding regions may or may not display the same state in two chromosomes of the same pair. In several cells, a different labelling intensity after anti 5-mC binding was detectable in homologous regions of some chromosome pairs of A. cepa. This was particularly evident as regards one telomeric region of chromosome V and intercalary band present in the long arm of chromosome VII.

Other examples can be found in V. faba and Z. biebersteniana. In these plant systems it must be underlined that some corresponding regions have never showed differences in anti 5-mC binding between homologous, while other corresponding regions may display all three possible labelling types: both unlabelled, both labelled and one labelled and one unlabelled, allowing us to provide a precise percentage data.

In the past, differences between the homologous chromosomes have been reported in several systems: during the asymmetric puffing in polytene chromosomes of P. coccineus (Cionini et al. 1982), by banding techniques (Sumner 1990) and after cytological hybridisation with specific DNA sequences in animals (Hennen et al. 1975; Nardi et al. 1977) and in plants (Durante et al. 1977; Loiero et al. 1982). In particular the work of Loiero and collaborators, after in situ hybridisation with rDNA in A. cepa, suggested that the observed different accessibility of DNA sequences to the hybridisation might reflect a differential activity of the two chromosomes at times other than during mitotic metaphase.
Also in our experiments it cannot be ruled entirely that the differences observed in the DNA methylation pattern between homologues may be the result of variation in the accessibility of the antibody to two DNAs, even if the homogeneity of the labelling obtained after anti-DNA antibody treatments should confirm the same antibody accessibility to the two homologues (Široký et al. 1998). However, also in the case of an eventual differential accessibility of the antibody to the antigen, a divergent chromatin organization (i.e. an interaction with different proteins, etc.) in corresponding regions of the homologues could be hypothesised: as a consequence, differences between homologous chromosomes, once more, clearly should result.

Such differences between corresponding regions of homologues regarding anti 5-mC binding in our opinion may indicate a different DNA methylation pattern between homologues, which could reflect different transcriptional activities. In a recent paper on mouse differences were detected in the chromatin packaging state of some allelic sites belonging to imprinted genes, indicating a close correlation between imprinted domains and chromatin compaction in corresponding regions of homologues (Watanabe et al. 2000).

Another well-known example of differences in homologous chromosomes is that concerning the facultative heterochromatisation of the one X female chromosome. In animal systems molecular data have well documented a positive correlation between late replication, inactivation and DNA methylation of GpC islands of the X chromosome. On the contrary, the molecular cytogenetic and immunocytochemical approaches have provided different and not unequivocal data on this subject (Miller et al. 1982; Viegas-Pequignot et al. 1988; Adolph and Hameister 1990; Prantera and Ferraro 1990; de la Torre et al. 1992).

More recently data from the work of Bernardino et al. (2000), by immunological approach with specific monoclonal antibodies, and from Andersen et al. (1998), by means of the SPRINS, self-primed in situ labelling, showed that methylation of mammalian sex chromosomes seems to depend on the cell type and on the species considered.

In the plant kingdom, at least in the best described example of model species possessing a pair of heteromorphic sex chromosomes, M. album, the situation seems to be clearer. Indeed, DNA methylation pattern appears significantly different between the two X chromosomes either by in situ restriction enzyme nick translation (Viskot et al. 1993) or by the immunocytochemical approach (Široký et al. 1998). With this second approach it has been shown that methylation pattern of the less methylated X chromosome is superimposable on the pattern belonging to the X chromosome present in male plants; the other female X chromosome is clearly hypermethylated in comparison to the homologous and corresponds to that which is late replicating and inactive.

The inactivation of one of the two Xs accompanied mainly by hypermethylation processes in female plants could appear to clash with the recent results obtained in mammalians, but should be due to the different karyotype evolution in plants belonging to dioecious species, in which X chromosomes represent an uncommon peculiarity not yet completely correlated with sex determination.

**CONCLUDING REMARKS**

The study of DNA methylation and its distribution along chromosome structures enable us to map in situ the locations of clusters strongly positive to specific antibodies against 5mCyt in several plant species. From the reported and discussed data we have drawn a number of conclusions concerning the genomic DNA methylation and the chromosome complement. The presence of constant highly methylated locations detected in plant systems may be one of the components involved in maintaining a defined three-dimensional order within both metaphasic and polytenic structures. In our opinion, the observed polymorphic and variable immunolabelling in specific chromosome locations could be related to defined functional domains depending on the cell commitment to specific differentiation programs. Within this context we can hypothesise that both the specific and variable presence of methylated clusters distributed in karyotypes are likely to be involved in the global genomic functioning, supporting the view that DNA methylation may be related to the structure and the functional activity of the chromosomes. Our results also indicate that differences may exist between corresponding regions of homologues as far as anti 5-mC binding is concerned. From these observations we sug-
gest that the heterogeneous behaviour in the methylation pattern of homologous chromosomes might be a reference point for processes that require the distinction between homologues, including the inactivation connected to equalize X chromosome gene expression between male and female in dioecious Melandrium album. Moreover, the information obtained from the distribution of methylated region may be useful as a further parameter to establish the evolutionary position of a species inside a genus and for a better understanding of the relationships between the species belonging to the same genus.

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