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Review

DNA methylation patterns on plant chromosomes

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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 adaptation 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

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debatable 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 (RUFFINI CASTIGLIONE *et al.* 1995; FREDIANI *et al.* 1996; ŠIROKÝ *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 (RUFFINI 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* (ŠIROKÝ *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. coccineus* where, even if most eterochromatic 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; ADOLFH 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 methaphasic 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.

REFERENCES

ADAMS R.L.P., BURDON R.H., 1985 – *Molecular biology of DNA methylation.* Springer Verlag, New York.

ADOLPH S., HAMEISTER H., 1990 – *In situ nick translation of human methaphase chromosomes with the restriction enzymes MspI and HpaII reveals an R-band pattern*. Cytogenet. Cell Genet., 54: 132-136.

ACHWAL C.W., CHANDRA H.S., 1982 – *A sensitive immunochemical method for detecting 5mC in DNA fragments.* FEBS Lett., 150: 469-472.

ANDERSEN S.J., KOCH J., KJELDSEN E., 1998 – *GpC islands detected by self-primed in situ labelling (SPRINS).* Chromosoma, 107: 260-266.

ALMEIDA-TOLEDO L.F., VIEGAS-PEQUIGNOT E., COUTINHO-BARBOSA A.C., FORESTI F., NJIVELAU A., ALMEIDA TOLEDO-FILHO S., 1998 – *Localization of 5-methylcytosine in methaphase chromosomes of diploid and triploid pacu fish, Piaractus mesopotamicus (pisces, characiformes).* Cytogenet. Cell Genet., 83: 21-24.

ANGELIER N., BONNANFANT-JAIS M., MOREAU N., GOUNON P., LAVAUD A., 1986 – *DNA methylation and RNA transcriptional activity in amphibian lampbrush chromosomes.* Chromosoma, 94: 169-182.

ATTWOOD J.T., YUNG R.L., RICHARDSON B.C., 2002 – *DNA methylation and the regulation of gene transcription.* Cell. Mol. Life Sci., 59: 241-257.

BARBIN A., MONTPELLIER C., KOKALJ-VOKAC N., GIBAUD A., NIVELEAU A., MALFOY B., DUTRILLAUX B., BUORGEOIS C.A., 1994 – *New sites of methylcytosine-rich DNA detected on metaphase chromosomes.* Hum. Genet., 94: 684-692.

BARNES S.R., JAMES A.M., JAMIESON G., 1985 – *The organization, nucleotide sequences and chromosomal distribution of a satelite from Allium cepa*. Chromosoma, 92: 185-192.

BERNARDINO J., LOMBARD M., NIVELAU A., DUTRILLAUX B., 2000 – *Common methylation characteristics of sex chromosomes in somatic and germ cells from muose, lemur and human.* Chromosome Research, 8: 513-525.

BURN J.E., BAGNALL D.J., METZGER J.D., DENNIS E.S., PEACOCK W.J., 1993 – *DNA methylation, vernalization and the initiation of flowering*. Proc. Natl. Acad. Sc., 90: 287-291.

CASTILHO A., NEVES N., RUFFINI CASTIGLIONE M., VIEGAS W., HESLOP-HARRISON J.S., 1999 – *5-Methylcytosine distribution and genome organization in Triticale before and after treatment with 5-azacytidine.* Journal of Cell Science, 112: 4397-4404.

CIONINI P.G., BASSI P., CREMONINI R., CAVALLINI A., 1985 – *Cytological localization of fast renaturing and satellite DNA sequences in Vicia faba*. Protoplasma, 124: 106-111.

CIONINI P.G., CAVALLINI A., CORSI R., FOGLI M., 1982 – *Comparison of homologous polytene chromosomes in Phaseolus coccineus embryo suspensor cells: morphological, autoradiographic and cytophotometric analyses.* Chromosoma, 86: 383-396.

COONEY C.A., 1993 – *Are somatic cells inherently deficient in methylation metabolism? A proposed mechanism for DNA methylation loss, senescence and aging.* Growth Dev. Aging, 57: 261-273.

CREMONINI R., RUFFINI CASTIGLIONE M., GRIF V.G., KOTSERUBA V.V.M PUNINA E.O., RODIONOV A.V., MURAVENKO O.V., POPOV K.V., SAMATADZE T.E.; ZELENIN A.V., 2002 – *Chromosome banding and DNA methylation patterns, chromatin organisation and nuclear DNA content in Zingeria biebersteniana (Claus) P. Smirnov.* Submitted to Biologia Plantarum.

DE LA TORRE J., SUMNER A.T., GONSALVEZ J., STUPPIA L., 1992 – *The distribution of genes on human chromosomes as studied by in situ nick translation*. Genome, 35: 349-354.

DURANTE M., CREMONINI R., BRUNORI A., AVANZI S. AND INNOCENTI A.M., 1977 – *Differentiation of metaxylem cell line in the root of Allium cepa. I. DNA heterogeneity and ribosomal cistrons of two different stages of differentiation.* Protoplasma, 93: 289-303.

DURANTE M., CECCHINI E., NATALI L., CITTI L., GERI C., PARENTI R., NUTI RONCHI V., 1989 – *5-Azacytidine-induced tumorous transformation and DNA hypomethylation in Nicotiana tissue cultur*. Devel. Genet., 10: 298-303.

DURANTE M., FREDIANI M., MARIANI L., CITTI L., GERI C., CREMONINI R., 1990 – *Differentiation of metaxylem cell line in the root of Allium cepa L. III. Levels of endogenous DNA methylation.* Protoplasma, 158: 149-154.

EASTMAN E.M., GOODMAN R.M., ERLANGER B.F., MILLER O.J., 1980 – *5-methyl cytosine in the DNA of the polytene chromosomes of the Diptera sciara coprophila, Drosophyla melanogaster and Drosophila persimils.* Chromosoma, 79: 225-229.

FINNEGAN E.J., GENGER R.K., KOVAC K., PEACOCK W.J., DENNIS E.S., 1998 – *DNA methylation and the promotion of flowering by vernalization*. Proc. Natl. Acad. Sc., 95: 5824-5829

FINNEGAN E.J., PEACOCK W.J., DENNIS E.S., 1996 – *Reduced DNA methylation in Arabidopsis thaliana results in abnormal plant development.* Proc. Natl. Acad. Sc., 93: 8449-8454.

FLAGIELLO D., BERNARDINO-SGHERRI J., DUTRILLAUX B., 2002 - *Complex relationships between 5-aza-dC induced DNA methylation and chromosome compaction at mitosis*. Chromosoma, 111: 37-44.

FREDIANI M., CREMONINI R., SASSOLI O., CIONINI P.G., 1992 – *Changes in DNA methylation with cell differentiation in Vicia Faba.* Chromatin, 1: 79-88.

FREDIANI M., GIRALDI E., RUFFINI CASTIGLIONE M., 1996 – *Distribution of 5-methylcytosine-rich regions in the metaphase chromosome of Vicia faba.* Chromosome Research, 4: 141-146.

FREDIANI M., TAGLIASACCHI A.M., DURAANTE M., AVANZI S., 1986 – *Distribution of polytene chromosomes of Phaseolus coccineus embryo suspensor as shown by the immunoperoxidase technique.* Exp. Cell Res., 167: 337-342.

HENNEN W., MIZUNO S., MACGREGOR H.C., 1975 – *In situ hybridization of ribosomal DNA labelled with 125Iodine to metaphase and lampbrush chromosomes from newts*. Chromosoma, 50: 349-369.

GOODMAN J.I., WATSON R.E., 2002 – *Altered DNA methylation: A secondary mechanism involved in carcinogenesis*. Ann. Rev. Pharm. Toxic., 42: 501-525 2002

KOVARIK A., KOUKALOVÁ B., BEZDEK M., OPATRNY Z., 1997 – *Hypermethylation of tobacco heterochromatric loci in response to osmotic stress*. Theor. Appl. Genet., 95: 301-306.

KUBIS S., SCHIMDT T., HESLOP-HARRISON J.S., 1999 – *Repetitive DNA elements as a major component of plant genomes.* Ann. Bot., 82: 45-55.

JONES P.A., TAKAI D., 2001 – *The role of DNA methylation in mammalian epigenetics.* Science, 293: 1068-1070

LI G.F., HALL T.C., HOLMES-DAVIS R., 2002 – *Plant chromatin: development and gene control.* Bioessays, 24: 234-243.

MANICARDI G.C., BIZZARRO D., AZZONI P., BIANCHI U., 1994 – *Cytological and electrophoretic analysis of DNA methylation in the olocentric chromosomes of Megoura viciae* (*Homoptera, Aphidae*). Genome, 37: 625-630.

MARTIENSSEN R.A. COLOT V., 2001 – *DNA methylation and epigenetic inheritance in plants and filamentous fungi*. Science, 203: 1070-1074.

MILLER D.A., OKAMOTO E., ERLANGER B.F., MILLER O.J., 1982 – *Is DNA methylation responsible for mammalian X chromosome inactivation*. Cytogenet. Cell Genet., 33: 345-349.

MILLER O.J., SCHNEDL W., ALLEN J., ERLANGER B.F., 1974 – *5 methylcytosine localized in mammalian constitutive heterochromatin.* Nature, 251: 636-637.

MIZUGAKI M., ITOH K., YAMAGUCHI T., ISHIWATA S., HISHINUMA T., NOZAKI S., ISHIDA N., 1996 – *Preparation of a monoclonal antibody specific for 5-methyl-2'- deoxycitidine and its application for the detection of DNA methylation levels in human peripheral blood cells*. Biological and pharmaceutical. Bulletin, 119: 1537-11540.

NARDI I., BARSACCHI-PILONE G., BATISTONI R., ANDRONICO F., 1977 – *Chromosome location of the ribosomal RNA genes in Triturus vulgaris meridionalis (Amphibia, Urodela). II. Intraspecific variability in number and position of chromosome loci for 18S+28S ribosomal RNA.* Chromosoma, 64: 67-84.

OLSZEWSKA M.J., GERNAND D., SAKOWICZ T., 1999 – *Methylation-sensitive restriction endonuclease digestion patterns revealed in Vicia faba L. chromosomes by in situ nick-translation.* Folia Histochem. Cytobiol., 37: 267-274.

PODESTÀ A., RUFFINI CASTIGLIONE M., AVANZI S., MONTAGNOLI G., 1993 – *Molecular geometry of antigen binding by a monoclonal antibody against 5-methyl cytidine*. International Journal of Biochemistry, 25: 929-933.

PRANTERA G., FERRARO M., 1990 – *Analysis of DNA methylation and distribution of CpG sequences on human active and inactive X chromosomes by in situ nick translation.* Chromosoma, 99: 18-23.

RABINOWICZ P.D., SCHUTZ K, DEDHIA N., 1999 – *Differential methylation of genes and retrotransposons facilitates shotgun sequencing of the maize genome*. Nature Genetics, 23: p. 305-308.

REIN T., DEPAMPHILIS L., ZORBAS H., 1998 – *Identifying 5-methylcytosine and related modifications in DNA genomes.* Nucl. Ac. Res., 26: 2255-2264.

REYNAUD C., BRUNO C., BOULLANGER P., GRANGE J., BARBESTI S., NIVELAU A., 1991 – *Monitoring of urinary excretion of modified nucleotides in cancer patients using a set of six monoclonal antibodies*. Cancer lett., 61: 255-262.

RUFFINI CASTIGLIONE M., FREDIANI M., PUNINA E., CREMONINI R., 1998 – *The DNA methylation pattern in two angiosperms with low chromo-*

some number in their basic aploid complement (n=2). Cytogen. Cell Genet., 81: 113.

RUFFINI CASTIGLIONE M., GIRALDI E. AND FREDIANI M., 1995 – *The DNA methylation pattern of Allium cepa metaphase chromosomes.* Biologisches Zentralblatt, 114: 57-66.

SANO H., IMOKAWA M., SAGER R., 1988 – *Detection of heavy methylation in human repetitive DNA subsets by a monoclonal antibody against 5-methyl cytosine.* BBA, 951: 157-165.

SCHNEDL W., DEV V.G., TANTRAVAHI R., MILLER D.A., ERLANGER B.F., MILLER O.J., 1975 – *5-methyl cytosine in heterochromatic regions of chromosomes: chimpanzee and gorilla compared to human.* Chromosoma, 52: 59-66.

SCHNEDL W., ERLANGER B.F., MILLER O.J., 1976 – *5-methylcytosine in heterochromatic regions of chromosomes in Bovidae.* Hum Genet., 31: 21-26.

ŠIROKÝ J., RUFFINI CASTIGLIONE M., VYSKOT B., 1998 – *DNA methylation patterns of Melandrium album chromosomes.* Chromosome Res., 6: 441-446.

STEWARD N., KUSANO T., SANO H., 2000 – *Expression of ZmMet1, a gene encoding a DNA methylatransferase from maize, is associated not only with DNA replication in actively proliferating cells, but also with altered DNA methylation status in cold stressed quiescent cells.* Nucl. Ac. Res., 28: 3250-3259.

SUMNER A.T., 1990 – *Chromosome banding.* Unwin Hyman, London.

THOMASSIN H., OAKELEY E.J., GRANGE T., 1999 – *Identification of 5-methylcytosine in complex genomes.* Methods, 19: 465-475.

VIEGAS-PEQUIGNOT E., DUTRILLAUX B., THOMAS G., 1988 – *Inactive X chromosome has the highest concentration of unmethylated HhaI sites.* Proc. Natl. Acad. Sc., 85: 7657-7660.

VYSKOT B., ARAYA A., VEUSKENS J., NEGRUTIU I., MOURAS A., 1993 – *DNA methylation of sex chromosomes in a dioecious plant, Melandrium album.* Mol. Gen. Genet., 239: 219-224.

VYSKOT B., 1999 – *The role of DNA methylation in plant reproductive development.* In: Ainsworth C.C. (ed.) “Sex determination in plants”, pp. 101-120. Bios Scientific Publishers, Oxford.

WATANABE T., YOSHIMURA A., MISHIMA Y., SHIROISHI T., KOIDE T., SASAKI H., ASAKURA H., KOMINAMI R., 2000 – *Differential chromatin packaging of genomic imprinted regions between expressed and non-expressed alleles.* Hum. Mol. Genet., 9: 3029-3035.

ZLUVOVA J., JANOUSEK B., VYSKOT B., 2001 – *Immunohistochemical study of DNA methylation dynamics during plant development.* J. Exp. Bot., 52: 2265-2273.

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