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Epigenetic Regulation of Plant Reproductive Development

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ABSTRACT Epigenetics represents a chromatin-mediated transcriptional repression which plays a control role in both animal and plant development. A number of different mechanisms have been described to be involved in the formation of chromatin structure: especially DNA methylation, nucleosomal histone modification, DNA replication timing, and binding of chromatin remodelling proteins. Epigenetic phenomena include genomic imprinting, dosage compensation of X-chromosome linked genes, mutual allelic interactions, paramutation, transvection, silencing of invasive DNA sequences, etc. They are often unstable and inherited in a non-Mendelian way. A number of epigenetic defects has been preferentially described in floral development. Here, epigenetic phenomena in model angiosperm plants and their corresponding mechanisms are reviewed.

Key words DNA methylation, epigenetic inheritance, histone acetylation, reproductive development, *Silene latifolia*

Introduction

Many recent data demonstrate that DNA hypermethylation and core histone underacetylation are chromatin modifications which characterise a transcriptional inertness in both constitutive and facultative heterochromatin. They do not seem to be causative processes of transcriptional inactivation as demonstrated on the mammalian inactive X chromosome: both DNA hypermethylation and histone H4 underacetylation follow the silencing of X-linked genes. These chromatin modifications rather represent mechanisms by which the "epigenetic" or "chromatin" information on gene expression is passed through mitotic and meiotic cell divisions. The mutual relationship between DNA methylation and histone acetylation is not clear yet. It has been shown that nucleosomes from nonmethylated CpG islands contain highly acetylated histones H3 and H4. Other facts indicate that they may represent alternative ways to transmit an epigenetic information, since some eukaryotes (e.g. some yeasts or *Drosophila*) lack the DNA methylation machinery.

Eukaryotic chromosomes have many thousands replication origins. Genes coding for various RNA types probably number from 5,000 in lower eukaryotes to as many as 100,000 in the most complex species. The DNA amount and structural organisation of coding and noncoding DNA regions in plants varies even among closely related species. Besides two regular constitutive heterochromatic regions on chromosomes (centromeric and telomeric), there are two basic types of genome organisation in plants. In nuclear genomes larger than 2.10^9 pb a majority of unique DNA sequences are present as short stretches (about 2 kbp) surrounded by blocks of repetitive sequences, while in smaller genomes a relative proportion of longer stretches (more than 4 kbp) of single copy sequences, separated by interspersed repeats, is higher. It is possible that interspersed repetitive sequences provide a specific neighbourhood to genes which could influence their expression during development. An estimation of gene distribution along chromosomes has been made in some cereals: long nonmethylated DNA stretches were highly concentrated in distal and subtelomeric regions, while a higher DNA methylation was found in pericentromeric regions. DNA in distal chromosome regions is also less condensed as com-

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pared to the proximal ones and exhibits a higher frequency of recombination.

DNA methylation

It is well established that DNA methylation on cytosine residues negatively controls gene expression in a majority of eukaryotic organisms (for a recent review see Adams 1996). While in vertebrate genomes the methylation of cytosine occurs namely in CG doublets, plant DNA is methylated in CG doublets, CNG triplets, and even in non-palindromic DNA sequences. Cytosine methylation (5-mC; Figure 1) is heritable during cell divisions by means of maintenance DNA methyltransferases which work only on hemimethylated templates. This may represent a mechanism by which an epigenetic information on gene expression is maintained during the individual development. Although demethylation appears to be a necessary step toward transcriptional activation in genes controlled by this mechanism, not all demethylated genes are active. It means that demethylation is not sufficient to initiate transcription; other control steps, such as the binding or release of regulatory proteins, are necessary for transcription to take place. Not all eukaryotic genes are regulated by methylation. These exceptions illustrate that methylation is only one of several possible modifying controls used in genetic regulation. *Drosophila*, nematodes, ciliate protozoans and some fungi lack the DNA methylation system. It suggests that this mechanism appeared relatively late in the evolution of animal and plant kingdoms as a device for transcriptional regulation.

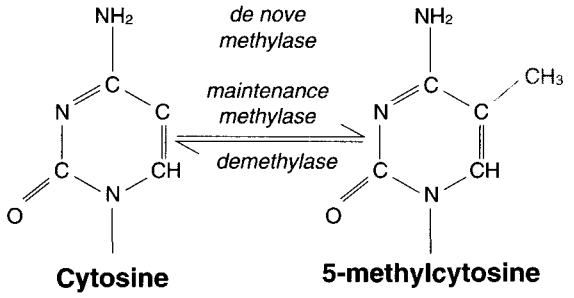

Figure 1. Scheme of chemical modification of cytosine. Cytosine is methylated in 5-C position of its pyrimidine ring; donor of methyl group is S-adenosylmethionine. The reaction is catalysed by DNA methyltransferases, the reverse reaction by putative demethylases.

Many data showing the role of methylation in plant development have been obtained using DNA methylation inhibitors, transgenic plants that express antisense constructs of cytosine-DNA methyltransferase gene or DNA methylation mutants. Some phenotypic effects of undermethylation were similar in different plant species (dwarfing, early flowering, abnormal flowers, reduced fertility) and in some cases these changes as well as a hypomethylation were inherited into sexual progeny (e.g., Vyskot et al. 1995; Janousek et al. 1996; Finnegan et al. 1996). Despite suffering significant developmental defects, undermethylated plants are viable, while comparable hypomethylations lead to the embryonic lethality in mammals. This difference may indicate that plants and mammals could use DNA methylation in different ways (Richards 1997). The development plasticity of plants and their relative tolerance for perturbation of gene expression might allow completion of the plant developmental program, despite gross alteration of genomic modification. While imprinting of parental genomes (realised via different methylation patterns) in mice is strictly required for completion of development, imprinting in plants appears to affect mainly the endosperm, but not plant embryos (Kermicle and Alleman 1990). However, some data show that 5-mC plays a similar role in plant development as in mammals. Drozdenyuk et al. (1976) followed the 5-mC content is wheat seeds during germination. They found that this amount in dry seeds and at day 3 of germination significantly decreased (from 25% to 21%). Changes in DNA methylation may be associated with the regulation of gene activity in differentiating plant cells at various stages of ontogenesis. These data suggest that after fertilisation and during plant development and maturation, embryonic DNA could be subject both to demethylation and to *de novo* methylation (Figure 2).

Many recent data show that although plants highly tolerate changes in DNA methylation patterns (including a substantial degree of undermethylation), genes which play a control role in floral formation are methylation-sensitive. Three examples demonstrating DNA methylation effects on the flower morphology are illustrated in Figure 3. The first example is a class of hypermethylated alleles of an *Arabidopsis* cadastral gene, *SUPERMAN*. Flowers of these epimutants form an increased number of stamens and carpels compared to the standard flower. Seven unstably heritable *superman* epialleles

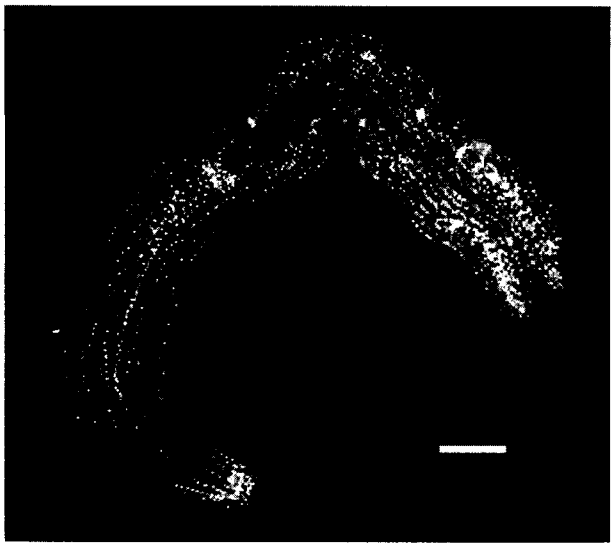

Figure 2. Indirect immunolabelling of a cryosectioned quiescent seed of *Silene latifolia* with a mouse monoclonal antibody raised against 5-methylcytosine and a fluorescein-labeled antimouse secondary antibody (light signals). The highest methylation levels are observed in nuclei of apical and root meristems, and in cotyledons: this pattern is changed during seed germination and plantlet development. Bar represents 100 μm.

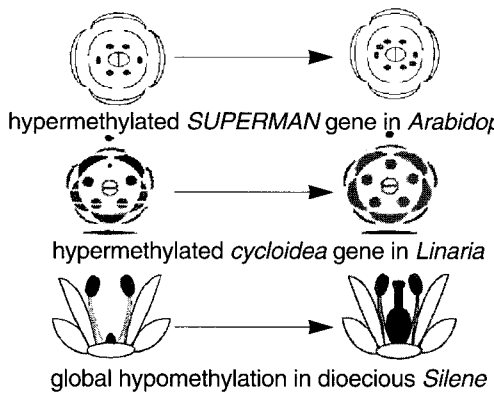

Figure 3. Changes in DNA methylation often lead to a modified floral development. A hypermethylation of the cadastral gene *SUPERMAN* induces an increased number of stamens and carpels in *Arabidopsis* (the upper line), while a hypermethylation of *cycloidea* homologue in *Linaria* leads to a change of floral symmetry, from bilateral to radial (the middle line). A global CpG hypomethylation in dioecious *Silene latifolia* activates the carpel development in male flowers (the bottom line).

were found to be overmethylated and to have a decreased level of the *SUPERMAN* RNA (Jacobsen and Meyerowitz 1997). About 250 years ago, a natural mutant of *Linaria vulgaris* possessing radial flowers (instead of bilateral ones in the wild-type) was described by Carl Linnaeus. It is known that the bilateral symmetry of flowers in a closely related species *Antirrhinum majus* is controlled by the action of *cycloidea* gene. A homologue of *cycloidea* gene was isolated from L. *vulgaris* (now called *Lcyc*) and researchers found that the *Lcyc* gene in the mutants is extensively methylated and transcriptionally silent. The hypermethylation was heritable and co-segregated with the peloric phenotype (Cubas et al. 1999). The third example comes from our laboratory and demonstrates the role of DNA methylation in the control of sex expression. *Silene latifolia* (syn. *Melandrium album*) is a dioecious plant which males possess the Y-chromosome harbouring both female suppressing and male promoting genes. Using a DNA hypomethylation drug (5-azacytidine) applied on seeds, about 20% of male plants reverted to androhermaphroditism (forming bisexual and male flowers). These plants were globally undermethylated and both bisexual and male flowers were able to transmit the epimutation by sexual progeny. Surprisingly, the epimutation was transmitted by sexual progeny only if the androhermaphrodite plants were used as pollen donors (and not seed parents). These data unambiguously show that female sex suppression in *S. latifolia* male plants is dependent on methylation of specific DNA sequences and can be heritably modified by hypomethylating drugs. Since corresponding genes have not been identified yet, we can only speculate about the mechanism involved, namely a 5-azacytidine induced inhibition of Y-linked female-suppressing gene(s) or a heritable activation of autosomal female-determining gene(s) which can be reversed through female meiosis by a genomic imprinting mechanism (Janousek et al. 1996).

The first pollen mitosis results in generative and vegetative cells which are characterised by a striking difference in their chromatin structure. In our study, histone H4 acetylation and DNA methylation were analysed during pollen development in *Lilium longiflorum*. Indirect immunofluorescence procedures followed by laser scanning microscopy enabled a relative quantification of H4 acetylation and DNA methylation in microspores, immature binucleate pollen, mature pollen, and pollen tubes. The results showed that histone H4 of the vegetative nucleus is, in spite of its decondensed chromatin structure, strongly hypoacetylated at lysine positions 5 and 8 in comparison with both the original microspore nucleus and the generative cell nucleus (Janousek et al. 2000). These H4 terminal lysines in the vegetative

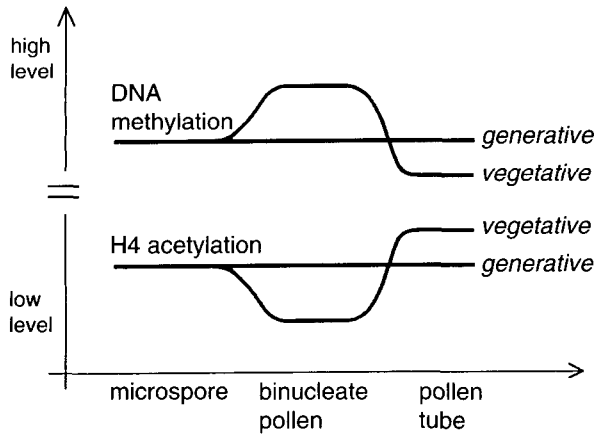

Figure 4. Outline of DNA methylation and histone H4 acetylation dynamics during pollen development in *Lilium longiflorum* (according to Janousek et al. 2000).

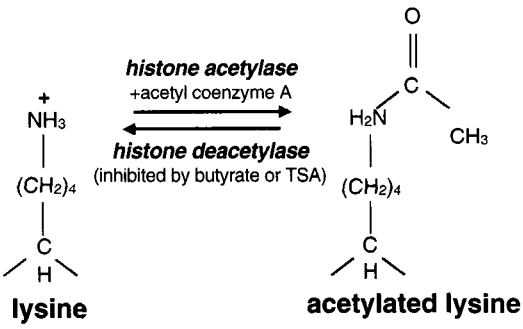

Figure 5. Reversible acetylation of lysine residues in nucleosomal histones. Lysine is modified at its ε-amino group, donor of acetyl group is acetyl coenzyme A. The forward reaction is catalysed by histone acetyltransferases (HAT), the reverse reaction by histone deacetylases (HD; which can be blocked with sodium butyrate or trichostatin)

nucleus were, however, progressively acetylated during the following pollen tube growth. The DNA methylation analysis inversely correlated with the histone acetylation data. The vegetative nucleus in mature pollen grains is heavily methylated, but a dramatic non-replicative demethylation occurs during the pollen tube development (Figure. 4). Any changes neither in H4 acetylation nor in DNA methylation have been found during development of the generative nucleus. The results obtained indicate that the vegetative nucleus enters the quiescent state (accompanied by DNA hypermethylation and H4 underacetylation) during the maturation of pollen grain which enables pollen grains a long term survival without external source of nutrients until they reach the stigma.

Histone acetylation

Histones are most important nuclear structure proteins which pack DNA into chromatin fibres. Histones have been fully characterised in many eukaryotes and their interaction with DNA has been established. The basic charge of histones results from the presence of lysine and arginine residues. H2A, H2B, H3 and H4 are called core histones since they form the beadlike core structure around which DNA wraps to form nucleosomes, while H1 is the linker histone. Histones are often modified by the addition of chemical groups. Simple modifications include the addition of acetyl, methyl, or phosphate groups to amino acids in the charged tails of histone molecules. Addition of acetyl groups to lysines in core histones increases conversion of gene regions from inactive to active form (for a review see Turner 1998; Figure. 5). First studies obtained by immunoprecipitation of chromatin fragments with an antiserum directed against acetylated H4 indicated that transcriptionally active genes carry acetylated core histones. More recently, using a similar experimental approach, it has been shown that H4 histones in coding regions possess a similar level of overall acetylation to bulk chromatin and a similar pattern of acetylation of individual amino-terminal lysines. However, the nucleosomes containing acetylated H4 are essentially absent from centric and telomeric heterochromatin. These data indicate that histone acetylation marks potentially active chromatin domains rather than actually transcribed gene regions and that the absence of acetylation is a characteristic property of constitutive heterochromatin (repetitive DNA sequences that are never transcribed). This conclusion has been experimentally demonstrated by detection of heterochromatic bands depleted in acetylated H4 on *Drosophila* and human chromosomes.

The histone modifications correlated with transcriptional regulation are reversible. Each core histone has from one to four lysines in side chains available for the addition of acetyl groups; in total, an individual nucleosome has 26 sites that can be modified by reversible addition of an acetyl group. There are many data showing a positive correlation between the addition of acetyl groups to core histones, particularly H4 and H3, and transcriptional activity. For example, in chromatin identified as active by greater susceptibility to digestion by

DNA endonucleases, core histones are generally more highly acetylated than those of inactive chromatin. Immunocytological data on various eukaryotic models show that the core histone underacetylation occurs in facultatively heterochromatinised nuclei, chromosomes or chromosome regions, too.

Plant chromosomes were first characterised for the distribution of H4 acetylated isoforms in *Vicia faba*. It was found that acetylation at positions of lysine 5, 8 and 12 is correlated with the intensity of transcription (Houben et al. 1996). DNA methylation and histone acetylation were also studied in facultative heterochromatin regions of pentaploid endosperm nuclei of *Gagea lutea*. This facultative heterochromatin was found to be partly hypermethylated (Buzek et al. 1998a), but it displayed a conspicuous depletion of H4Ac5, 8 and 12, but not 16. Western analyses of H4 histones from *G. lutea* leaves showed that the most abundant form is diacetylation and that H4Ac16 seems to be first acetylated and H4Ac5 as the last (Buzek et al. 1998b). These data indicate that the order of H4 acetylation in plants is similar as in mammals and that H4 may be diacetylated prior to nucleosome assembly as described in humans. The final status of histone acetylation reflects a nuclear chromatin structure and function and it is achieved by multiple histone acetyltransferases and deacetylases. Histone acetylation studies on maize embryos have shown that histone deacetylase serves a specific function in the dry embryo and could be a prerequisite for DNA repair processes, while total histone acetyltransferase activity increases during germination which is obviously connected with the activation of metabolic and mitotic processes.

Using specific polyclonal antisera raised against acetylated isoforms of histone H4 we analysed their distribution along chromosomes in the dioecious *Silene latifolia*. All the distal-subtelomeric chromosome regions, on both the sex chromosomes and autosomes, displayed strong signals of H4 acetylation at N-terminal lysines 5, 8, and 12. These acetylated domains correspond to the very early replicating distal chromosome regions as revealed by 5-bromodeoxyuridine pulses followed by the indirect immunofluorescence microscopy (Vyskot et al. 1999). The distribution of H4 acetylated at lysine 16 was uniform along the chromosomes. The unique distal-subtelomeric H4 acetylation was also observed in other *Silene* species, but not in two non-related plants tested (tobacco and onion). These results as well as our FISH data with a complex cDNA probe reveal that *Silene* species possess the specific distal-subtelomeric location of euchromatin, gene-rich regions on chromosomes.

Binding of chromatin remodelling proteins

A group of specific binding proteins was first described in *Drosophila* (Polycomb-group, PcG) playing a key role in regulation of homeotic gene expression. These regulatory proteins are characterised by a common motif (chromodomain) and have been recently found in many eukaryotic organisms, including mammals and plants. They occur especially in both constitutive and facultative heterochromatin, so probably bound to non-transcribed DNA sequences. *CURLY LEAF* (*CLF*) is a gene necessary for a specific expression of floral homeotic genes in *Arabidopsis* and its product possesses a high homology to the Polycomb-group (Goodrich et al. 1997). CLF protein seems to be a negative regulator of *AGAMOUS* gene required for a proper formation of carpels and stamens. The wild-type allele of *CLF* is necessary to suppress transcription of *AGAMOUS* gene in leaves and other vegetative tissues. A recessive (loss-of-function) *clf* mutation leads to an ectopic expression of floral homeotic genes and has a pleiotropic effect on plant development (e.g. curly leaves, early flowering, homeotic floral transformations). Recently, another Polycomb-like regulatory protein - medea - with a strong maternal effect has been identified in *Arabidopsis*. A recessive mutation of the gene is expressed by a giant embryo formation which results in seed abortion (Grossniklaus et al. 1998). It has a property of a classical maternal gene, since its allele of paternal origin is imprinted and cannot complement a defect of the maternal allele. A gene encoding a chromodomain of *Drosophila* Polycomb-group protein was also transferred into tobacco plants to study its phenotypic effects. Transgenic plants displayed various abnormalities in their leaves and flowers. In axillary shoot buds an enhanced expression of a homeodomain gene *Nt-HD2*, which is down-regulated in wild-type leaves, was found (Ingram et al. 1999). Taken together, both animals and plants have obviously evolved Polycomb-like protein repressive system which plays an important epigenetic role in their development.

Another group of specific chromatin modulators, recently also described in plants, are SWI2/SNF2-like proteins (Jeddeloh et al. 1999; Amedeo et al. 2000). These proteins are obviously tightly connected with DNA methylation since their mutations lead to a loss of genomic methylation and/or a release of transcriptional silencing.

Epigenetic phenomena

Several endogenous genes have been shown to be subject to parental imprinting in the mouse and human: either only paternal or only maternal alleles are active in the progeny. In these cases, the methylation state of alleles is often determined by their parent-of-origin. If the parent specific scheme of the monoallelic gene expression fails, serious human genetic disorders can occur (e.g. Praeder-Willi and Angelman diseases). In angiosperm plants, genomic imprinting seems to be restricted to endosperm tissue (e.g. endosperm size factors, R-locus, and zein and tubulin encoding genes in maize), but the above-mentioned gene *MEDEA* is probably the first example of a parentally imprinted gene in embryo. In some experiments on transgenic mice it was found that the maternally inherited transgene was methylated and silenced, while the paternally derived constructs were not modified. Other recent data indicate that one of most important roles of DNA methylation is a defense mechanism against transposons: if their promoters are methylated, the transposons are not mobile and, due to frequent 5-mC to thymine transition mutations, they could even be destroyed. Another example of the role of DNA methylation in mammals is the inactive (lyonised) X chromosome which contains hypermethylated CpG islands and which displays some other features of chromatin inertness: histone H4 and H3 hypoacetylation and late replication (for review, see Jamieson et al. 1996).

Plant cells cultured *in vitro* are subjected to growth stress conditions which often result in a great phenotype heterogeneity in population of regenerants. This phenomenon called somaclonal variation can reflect both changes in nucleotide DNA sequences and methylation patterns. We studied DNA methylation of two repetitive sequences in tobacco nuclear genome in the course of *in vitro* dedifferentiation and differentiation. Using 5-mC sensitive restriction enzymes and DNA/DNA hybridization with 25S-rDNA probe it has been shown that during the early phase of callus induction prominent changes in the methylation pattern occur which are stably maintained during subsequent callus growth. The following protoplast recovery and plant regeneration have again displayed some more modifications of the methylation status. Comparing the patterns of Ro plants with the original plant material and the calli it can be assumed that both share in the resulting methylation status. The experiments analyzing the a family of non-transcribed highly repetitive sequences have displayed a quite monotonous methylation status thus indicating no random methylation perturbations in silent DNA sequences (Vyskot et al. 1993).

Cytosine methylation represents one of the mechanisms which protects eukaryotic organisms against intrusive DNA sequences. This fact could be a serious problem preventing expression of transgenes in crop plants. In our experiments we introduced the *Drosophila* gypsy-like element *Dm111* and the selectable dominant *nptII* gene via a Ti-vector into tobacco plants in order to check the structural and functional stability of transgenes in plants and their progeny. Southern blot analyses clearly showed that transgenes were integrated intact and did not suffer from any gross DNA rearrangements. Contrary to this structural stability, not all the transgenic plants and their offspring displayed the original and stable expression of the *nptII* gene. The levels of the nptII enzyme strongly varied in individual plants and did not depend on the copy number of the *nptII* gene. Both the unstable *nptII* expression during the individual plant development in one original transgenic tobacco and some irregularities in segregation ratios after self-pollination indicated that epigenetic effects due to methylation of DNA modulated the expression of foreign genes in transgenic plants. This conclusion was supported by a spontaneous and 5-azacytidine-stimulated demethylation (Vyskot et al. 1989).

Polyploidy is quite common in plants: many species are thought to be of polyploid origin, either autopolyploid or allopolyploid. Polyploids are often characterised by their gigas effect and changed shape and texture of organs. Some data indicate that the RNA and protein contents per haploid genome of the polyploid plants are lower than those of the diploids. It is not clear yet, if all duplicate genes are equally expressed. Polyploids could

display various types of gene silencing. For example, the expression of ectopic transgenes, which are most often used to study gene silencing, is reduced in triploid compared with diploid hybrids. It has been demonstrated on many additive plant hybrids that preferential nucleolar silencing is caused by epigenetic interactions of parental genomes connected with DNA methylation and histone H4 and H3 hypoacetylation (Chen and Pikaard 1997). A majority of data on gene expression in polyploids presented so far have been obtained by analysis of isozymes: polyploids display a fitness advantage over diploids due to their increased biochemical diversity. Expression analyses (based on RT-PCR technique) indicate that in hybrid plants transcripts originated from both parents are expressed. In our work we have performed a number of karyological analyses on an autotetraploid cell line of the model dioecious plant *Silene latifolia* in order to show whether the double dosage of chromosomes in the autotetraploid cell line leads to chromatin modifications of the two supernumerary chromosome sets. Previous studies on the structure of terminal regions of *S. latifolia* chromosomes show that they consist of very short telomeres (Riha et al. 1998), followed by a block of subtelomeric repeats (Buzek et al. 1997) and a large region which is early replicating and rich in H4 acetylated histones (Vyskot et al. 1999). Immunofluorescence analyses did not indicate any global differences in the DNA methylation, histone H4 acetylation, and chromosome replication patterns which could arise as a consequence of the whole chromosome set duplication of the original diploid genome (Figure 6). Similarly, a number of silver-positive nucleoli roughly correlated to the ploidy level. The early replication and H4 hyperacetylation have been detected at all subterminal chromosome regions which indicates, together with cDNA *in situ* hybridisation patterns, the localisation of gene-rich regions (Siroky et al. 1999). More recent data comparing haploid and tetraploid *Saccharomyces cerevisiae* cell strains have shown that polyploidy has an ambiguous effect on gene expression: some genes are induced or repressed, while the others produce similar levels of mRNA in the haploid and tetraploid cells (Galitsky et al. 1999).

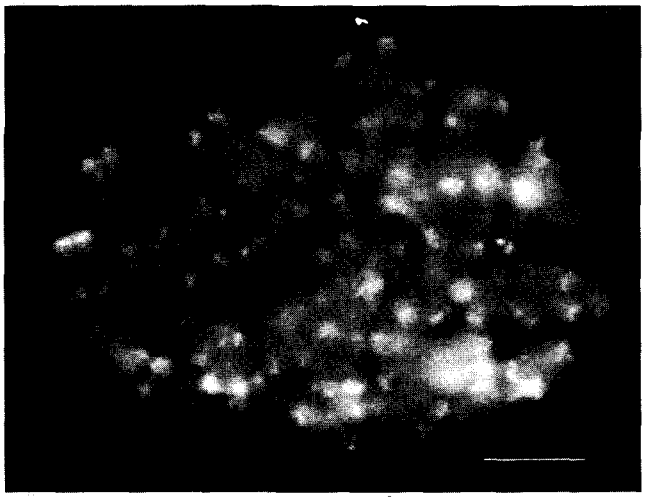

Figure 6. Indirect immunolabelling of root tip metaphase chromosomes of tetraploid *Silene latifolia* with a rabbit polyclonal antiserum raised against histone H4 acetylated at terminal lysine 5 and a fluorescein-labeled antirabbit secondary antibody (light signals). Strong H4 acetylation levels are evident at subterminal (gene-rich) regions of all chromosomes. Bar represents 10 μm.

Conclusion

Epigenetic processes represent a higher level of control of gene expression and obviously play an important role in individual development of animals and plants. They are also involved in inheritance of both mitotic and meiotic gene expression patterns (cell memory). Even though mechanisms of epigenetic inheritance have not been fully elucidated yet, they represent one of key processes in regulation of gene expression, cell memory and development. Recent data clearly indicate that two most important epigenetic mechanisms DNA methylation and histone deacetylation - are coupled: both methyl-CpG-binding protein and - DNA methyltransferase are associated with histone deacetylase activity.

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