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Rumex acetosa Y chromosomes: constitutive or facultative heterochromatin?

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Abstract: Condensed Y chromosomes in *Rumex acetosa* L. root-tip nuclei were studied using 5-azaC treatment and immunohistochemical detection of methylated histones. Although Y chromosomes were decondensed within root meristem in vivo, they became condensed and heteropycnotic in roots cultured *in vitro*. 5-azacytidine (5-azaC) treatment of cultured roots caused transitional dispersion of their Y chromosome bodies, but 7 days after removal of the drug from the culture medium, Y heterochromatin recondensed and again became visible. The response of *Rumex* sex chromatin to 5-azaC was compared with that of condensed segments of pericentromeric heterochromatin in *Rhoeo spathacea* (Sw.) Stearn roots. It was shown that *Rhoeo* chromocentres, composed of AT-rich constitutive heterochromatin, did not undergo decondensation after 5-azaC treatment. The Y-bodies observed within male nuclei of *R. acetosa* were globally enriched with H3 histone, demethylated at lysine 4 and methylated at lysine 9. This is the first report of histone tail-modification in condensed sex chromatin in plants. Our results suggest that the interphase condensation of Y chromosomes in *Rumex* is facultative rather than constitutive. Furthermore, the observed response of Y-bodies to 5-azaC may result indirectly from demethylation and the subsequent altered expression of unknown genes controlling tissue-specific Y-inactivation as opposed to the global demethylation of Y-chromosome DNA.

Key words: Y chromosomes - Heterochromatin - Methylation - Histone H3 - *Rumex*

Introduction

The sorrel, *Rumex acetosa* L. is one of the few dioecious plant species which have sex chromosomes (XX + 12 autosomes in females and XY_1Y_2 + 12 autosomes in males). Sex-determination in sorrels depends on the X/autosome ratio [45, 46] and about ten *Rumex* species from the section *Acetosa* are characterized by the same dosage sex chromosome system [29]. The two sorrel Y chromosomes are considered late-replicating and are condensed within interphase nuclei [22, 41, 46]. The authors referred to these chromosomes as 'heterochromatic', yet it is unclear whether they represent facultative or constitutive heterochromatin. The heterochromatic nature of *Rumex* Y chromosomes is of special interest as it contrasts with the euchromatic nature of Y chromosomes in other plant species [1, 17]. In addition, facultative heterochromatin is extremely rare in Angiosperms [18, 19].

Using conventional staining techniques, sorrel Y chromosomes appear heteropycnotic within some interphase and prophase nuclei [30, 41]. Fluorescence studies [22, 34, 37] have revealed that sorrel Y chromosomes are almost entirely DAPI-positive, thus indicating that they are enriched with AT-rich repetitive sequences. In spite of this, C-banding studies [9, 34, and our unpublished results] have failed to detect constitutive heterochromatin within the Y chromosomes. It was subsequently concluded [9, 34] that heterochromatinization of *Rumex* Y chromosomes is not constitutive, but rather facultative. Further evidence supports the facultative character of sorrel Y heterochromatin. First, the Y chromosomes are not heterochromatinized in all cells

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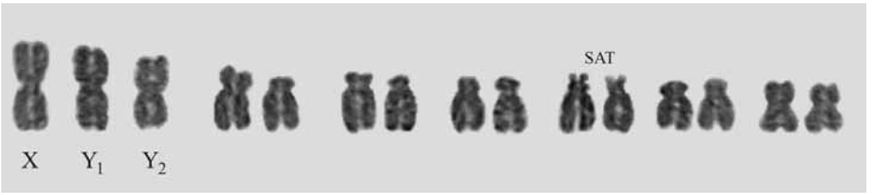

Fig. 1. Chromosome complement of male *Rumex acetosa* 2n = 12 + XY_1Y_2 with sex chromosomes indicated.

and tissues, and frequently form heteropycnotic bodies in somatic tissues, but only rarely in root-tip meristems [22, 30, 41]. Second, the results of DNAse sensitivity assays on root-tip cell Y chromosomes are similar to those of *Rumex* autosomes [9]. These studies suggest that Y chromosomes are probably transcriptionally active, at least in meristematic cells, where heterochromatinization is not generally observed.

The cytogenetic and molecular analysis of sorrel sex chromosomes has been hampered by a lack of convenient experimental model systems. *In vitro* root cultures proved to be very useful for the analysis of *Silene latifolia* sex chromosomes [8, 25, 38, 39, 43].

In this paper we have studied condensed Y chromosomes in sorrel root-tips cultured *in vitro* using 5-azaC treatment and immunohistochemical detection of methylated histones.

Materials and methods

Material. Plant material was obtained from seeds of commercial *R. acetosa* L. cultivar 'Lionski' (Polan, Cracow). *R. spathacea* (Sw.) Stearn plants were of our own stock and were previously cytologically analysed [14, 15].

Root culture initiation and maintenance. Petiole fragments (ca. 1 cm long), excised from aseptically grown seedlings of *R. acetosa*, were inoculated in a liquid MS medium [26] containing 1/2 strength macronutrients. Individual adventitious roots, which developed at sites of cuttings, were excised, and separately inoculated in 250 ml Erlenmeyer flasks, each containing 20 ml of the nutrient medium. The roots were maintained at 25˚C, on a gyratory shaker (100 r.p.m.), in the dark, and were subcultured every five weeks by inoculating 0.5 g of roots (fresh weight) in 250 ml Erlenmayer flasks with 35 ml of a liquid medium. The growth index (G.I.) of the culture was measured as the ratio of the final fresh weight of roots, five weeks after inoculation, to the fresh weight of the inoculum.

Adventitious roots (male line RAY1, and female line RAX1) developed from 10% of petiole explants, three weeks after their inoculation in the modified MS medium. The roots grew well in the hormone-free medium. Their G.I. value was ca. 14.

Cytology. Root-tip meristems and hairs of *R. acetosa* were taken from young (2-3 day) seedlings, adult plants, or from roots cultured *in vitro*. Roots of *R. spathacea* were taken from plant cuttings incubated in glass jars filled with tap water. Roots were fixed for 30 min. in a mixture of 96% ethanol and glacial acetic acid (3:1). After maceration in 1N HCl (60˚C for 5 or 10 min.) or in buffered pectinase (40%) + cellulase (4%), the root tips were rinsed in distilled water and squashed in 45% acetic acid. Cover slips were ripped off after freezing in dry ice. DAPI-stained preparations were mounted in Vectashield (Vector) and analysed by fluorescence microscopy. Karyotyping was undertaken on chromosomes conventionally stained using the Feulgen method.

5-azacytidine treatment. Roots of *R. acetosa* were cultured in daily renewed medium containing 5 μM 5-azaC for 3 days. After this treatment, roots were carefully washed in fresh medium and cultured without the drug. *R. spathacea* roots were treated in the same way with 5-azaC at the same concentration, diluted in tap water. Each experiment was repeated three times. The material not treated with 5-azaC served as a control. For each cell collection, the morphology of 200 nuclei was analysed and the frequency of nuclei with condensed chromatin bodies was calculated. For the obtained results, analyses of variance were undertaken. The mean values were compared using the Student t-test.

Histone immunostaining. Root tips of untreated cultured roots of *R. acetosa* were fixed for 30 min. with 3.7% formaldehyde in PBST (phosphate-buffered saline, pH 7.3 + Tween 20, final concentration 0.1%) at room temperature and washed 3 × 10 min. in PBST. The root-tips were then squashed without maceration in a drop of PBST and after freezing in liquid nitrogen, the coverslips were removed and slides were transferred immediately into cold PBS. After washing (3 × 5 min. in cold PBS), the preparations were blocked with 3% BSA (bovine serum albumin) in PBST for 15 min. at room temperature in a humid chamber. After blocking, the specimens were incubated with the primary antibodies (rabbit polyclonal antibodies against methylated histone H3 at lysine 9 and lysine 4) diluted in PBS with 0.01% sodium azide (1:250 H3K9 and 1:100 H3K4) overnight at 4˚C. Antibodies against methylated H3K9 were developed at the Research Institute of Molecular Pathology, Vienna Biocenter, and methylated H3K4 were from Abcam (Cambridge, UK). The next day, the slides were left for 30 min. at room temperature and then rinsed (3 × 10 min. in PBS) and incubated for 1h at room temperature in a humid chamber with secondary antibody (anti-rabbit conjugated with Cy-3 atibody from goat, Abcam, Cambridge, UK, ab6939) diluted 1:150 in PBS with 0.01% sodium azide. After washing (3 × 10 min. in PBS), the slides were counterstained and mounted in Vectashield (Vector Laboratories, Burlingame, USA) with DAPI (1μg/ml). The reliability of the used method was assayed by applying simultaneously a universal anti-histone H1 mouse monoclonal antibody (1:50, Abcam, Cambridge, UK, ab4269) which was detected with anti-mouse antibody conjugated with FITC from goat (1:200, Abcam, Cambridge, UK, ab5999). The antibodies were kindly provided by Prof. Dieter Schweizer (University of Vienna).

Results

Rumex chromosomes in vivo and in vitro

The karyotypes of all the male and female R. acetosa seedlings analysed were typical and corresponded to the previously published data [22, 29, 34]. The metacentric sex chromosomes (X, Y_1 and Y_2) were of standard morphology and were the largest in the chromosome complement (Fig. 1). The X chromosome was slightly longer than the Y_1 chromosome and significantly longer than the Y_2 chromosome. Our C-banding studies failed to reveal constitutive heterochromatin within Y chromo-

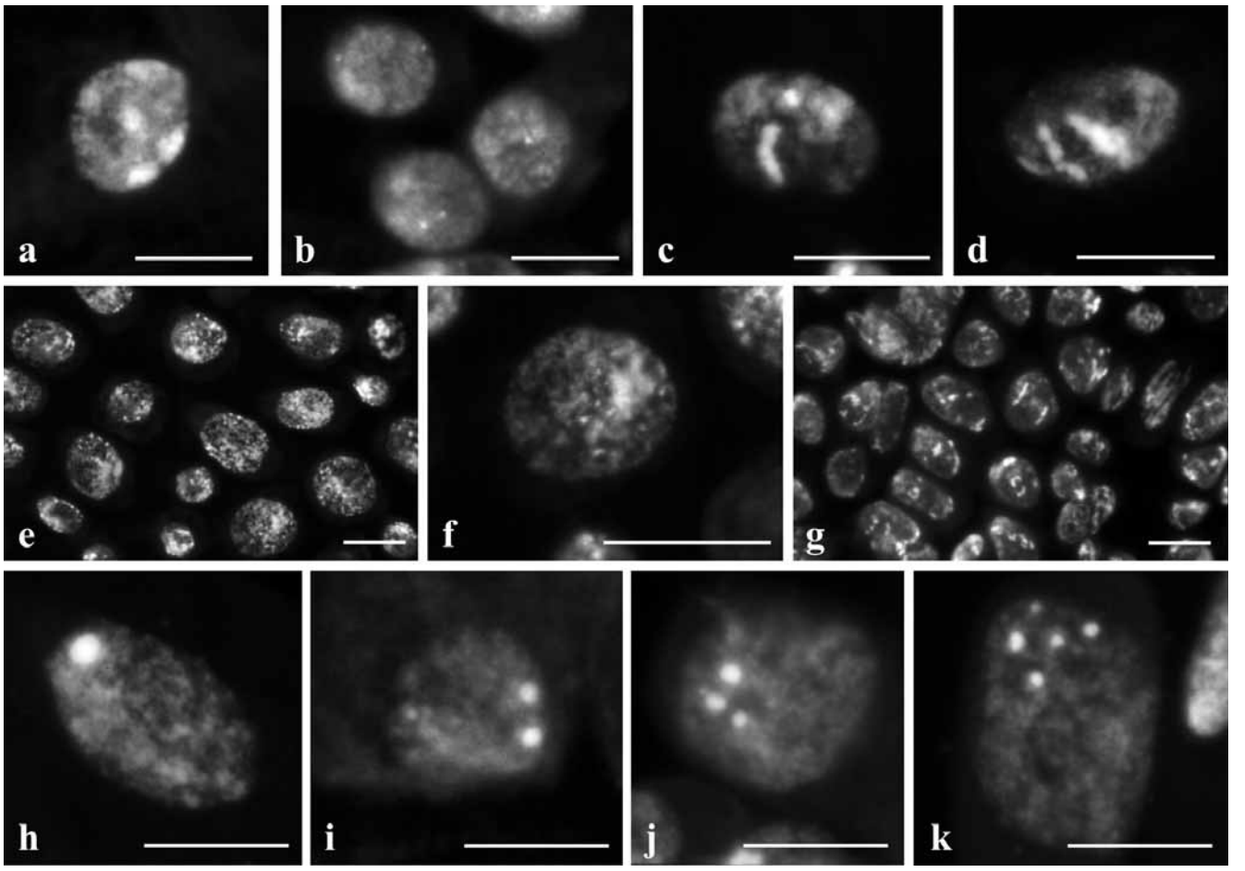

Fig. 2. DAPI-positive chromatin-bodies within nuclei of male *Rumex acetosa* (a-g) and within nuclei of *Rhoeo spathacea* (h-k). a - root hair (seedling); b - root meristem (seedling); c and d - root meristem (*in vitro*), without 5-azaC treatment; e and f - root meristem (*in vitro*), immediately after 5-azaC treatment; g - root meristem (*in vitro*), 7 days after 5-azaC treatment; h - k - root meristem, nuclei with 1, 2, 3 and 5 heterochromatic bodies. Bars: 20 μm

somes (data not shown). Although it has been previously suggested that *R. acetosa* Y chromosomes are hypervariable for centromere localization [44], both Y chromosomes were morphologically stable in all the male seedlings.

The long-term male root culture (RAY1 line) analysed here was karyologically stable. 15 chromosomes (12 autosomes + XY_1Y_2) of standard morphology were invariably observed in metaphase cells derived from these roots. No signs of mitotic disturbances - such as anaphase bridges, chromosome fragments, irregularly shaped nuclei or micronuclei were observed.

Differences in Y chromosome condensation in vivo and in vitro

The nuclear structure of *Rumex* male and female plants was distinct. Male nuclei of non-meristematic tissues usually possessed 1-4 large, oval-shaped or elongated DAPI-positive bodies. Such bodies were not observed in female nuclei (data not shown). In root-hair cells, chromatin bodies were abundant and large, while in meristem cells of male roots, the DAPI-positive segments were usually small and not numerous (Table 1, Fig. 2a,b). As FISH signals of Y-specific *RAYS* sequences (*R. acetosa* Y chromosome-specific sequence) colocalize with male-specific chromatin bodies [22, 37], it is likely that the male-specific chromatin bodies we observed correspond to Y chromosomes (or their substantial parts).

In male roots cultured *in vitro* (RAY1 line), we were surprised to detect DAPI-positive nuclear bodies not only within root-hairs, but also within meristematic cells (Tab. 1, Fig. 2c,d). Nuclear bodies of this kind were not observed in either meristematic or non-meristematic tissues of female roots cultured *in vitro* (RAX1 line).

The effect of 5-azaC on chromatin condensation

In *Rumex* cultured male roots (RAY1 line), male-specific segments disappeared in 90% of root-tip nuclei immediately after 5-azaC treatment (Fig. 2e,f). Seven days after removing 5-azaC from the culture medium, the percentage of nuclei with DAPI-positive bodies in-

creased from 10% to 71.8% (Fig. 2g), a percentage comparable to that in nuclei from the untreated root cultures (Tab. 2). Thus, it seems that male-specific chromatin in meristematic tissue of cultured roots recondenses after transferring roots to fresh medium without 5-azaC.

In *R. spathacea*, a species with distinct chromocentres (Figs. 2h-k) formed by AT-rich constitutive heterochromatin [14 , 15, 16], treatment of roots with 5-azaC did not affect the percentage of DAPI-positive segments compared to untreated roots (97.2% and 94.7% for treated and untreated roots, respectively). However, the large, single, collective chromocentres tended to separate into smaller fluorescent spots following 5-azaC treatment. The frequency of meristematic nuclei with 4-7 chromocentres increased in comparison with untreated material. The frequency of nuclei with 2 or 3 larger chromocentres remained almost unaltered in 5-azaC-treated material. Importantly, the observed effect of 5-azaC on interphase chromatin organization in *Rhoeo* seems to be irreversible (Tab. 3).

Table 1. Number of nuclei with sex bodies in males of *Rumex acetosa*, n=200

	I	II	III	X
Root meristem				
adult plants	26	22	28	25.3
seedlings	8	10	12	10.0
in vitro	174	168	171	171.0
Root hairs	186	190	183	186.3

I-III - replications; X - mean value; LSD = 5.54 (α=0.05)

Table 2. Cultured roots of male *Rumex acetosa*; number of nuclei with sex bodies in meristem cells after 5-azaC treatment, n=200

	I	II	III	X
Control	174	168	171	171.0
0-D	13	22	25	20.0
7-D	138	154	139	143.0

0-D - immediatelly after treatment; 7-D - 7 days after treatment; I-III - replications; X - mean value; LSD = 12.31 (α=0.05)

Table 3. Root meristem cells of *Rhoeo spathacea*; mean number of nuclei with and without chromocentres in meristem cells after 5-azaC treatment, n=200

	a	b	c	d	e
Control	46.7	44.3	53.0	45.7	10.3
0-D	30.0	53.3	44.0	68.0	4.7
7-D	23.0	41.7	49.3	82.0	4.0

a - one collective chromocentre; b - two chromocentres; c - three chromocentres; d - more chromocentres; e - without chromocentres; LSD = 10.8 (α=0.05)

Histone H3 methylation in sex bodies

The assembly of higher order chromatin structures in eukaryotes is linked to the covalent modifications of histone tails [5, 11, 28]. Heterochromatinization of *Rumex* Y chromosomes in *in vitro* cultured roots offers a unique opportunity to study the link between H3 methylation and epigenetic regulation of sex chromatin condensation in plants. Because lysines can accept three methyl groups, we tested the presence of monomethylated, dimethylated and trimethylated lysine 4 and 9 residues in H3 histone within interphase nuclei of *Rumex* using appropriate antibodies.

Our findings suggest that DAPI-positive sex bodies observed within male nuclei were globally enriched with H3 histone, heavily demethylated at lysine 4 and methylated at lysine 9 (Fig. 3). Immunostaing with antibodies against mono- di- and trimethylated H3K4 showed no signals within sex bodies and speckles evenly distributed throughout the euchromatic parts of nuclei. In case of H3K9, positive immunolabelling was obtained only for monomethylated and dimethylated forms of this residue. In all analysed preparations, the staining was predominantly localized to DAPI-positive sex bodies (Figs. 3a,b). No positive signals, either within condensed, or within uncondensed chromatin were obtained with antibodies against trimethylated H3K9 (data not shown).

Discussion

Rumex Y chromosomes: constitutive or facultative heterochromatin?

C-banding is the best method for cytological discrimination between constitutive and facultative heterochromatin. We were, however, unable to obtain unambiguous C-banding patterns for *R. acetosa* chromosomes. A failure to detect heterochromatin within *Rumex* Y chromosomes using C-banding was previously reported [9, 34]. However, reports to the contrary [37, and references cited therein] suggest that sorrel Ys are composed of constitutive, C-banded material.

From the cytological point of view, non-functional, highly repetitive sequences of constitutive heterochromatin are generally species-specific rather than tissue-specific, and by definition are permanently condensed at interphase [6]. In contrast, facultative heterochromatin is only structurally silenced (condensed) in certain cell types or developmental stages [6, 27]. *In vivo*, *R. acetosa* Y chromatin was usually condensed in interphase nuclei of non-cycling, differentiated cells and tissues, but not in meristematic cells, where larger DAPI-positive chromatin bodies were observed only occasionally [22, 30, 41]. In roots cultured *in vitro*, however, Y chromosomes were also condensed in the majority of meristematic cells. Taken together, these

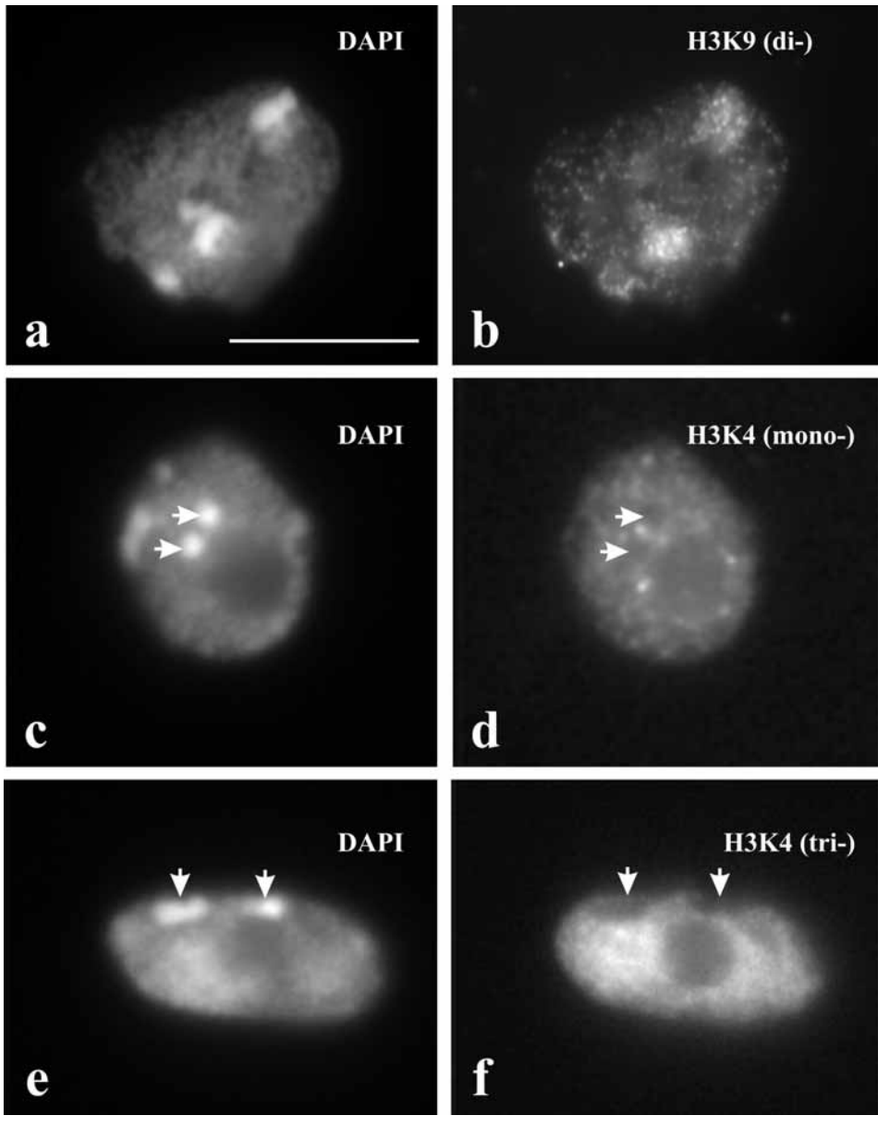

Fig. 3. DAPI-staining (a, c, e) and immunolabelling (b, d, f) of meristematic cell nuclei of male *Rumex acetosa* roots (*in vitro*); b - anti-H3K9^{Me} signals; d and f - anti-H3K4^{Me} signals. (mono-) - monomethylated; (di-) - dimethylated; (tri-) - trimethylated. Bar: 20 μm

observations suggest that Y-chromosome condensation at interphase is subject to epigenetic modification during development *in vivo* and culture conditions *in vitro*. These observations strongly support the existing view on the facultative character of Y heterochromatin in this species [9, 30, 34].

Constitutive heterochromatin is generally more highly methylated than bulk DNA [10, 20, 40]. In plants and mammals, C-banding-positive, highly repetitive sequences are 5-methylcytosine-rich [12, 33]. It has been suggested that DNA sequence repetition might trigger DNA methylation in constitutive heterochromatin [23, 36]. On the other hand, hypermethylation is distinctly absent from both the inactive human X chromosome [3] and facultative heterochromatin in the endosperm nuclei of *Gagea lutea* [18]. Thus, facultative heterochromatinization does not seem to be obligatorily correlated with global DNA hypermethylation. A lack of sex-specific differences in the level of DNA methylation in male and female nuclei of fully expanded *R. acetosa* leaves has also been reported [22]. This finding supports the facultative character of interphase Y-heterochromatin within the nuclei of *R. acetosa* differentiated cells.

The effect of 5-azaC on chromatin condensation in Rumex and Rhoeo

We compared the response of AT-rich *Rumex* Y chromatin to 5-azaC to that of constitutive heterochromatin with similar base composition from *R. spathacea*. In *Rumex*, 5-azaC treatment caused transitional dispersion of condensed segments within meristematic nuclei of cultured male roots. As heterochromatic sex bodies in males lack hypermethylated DNA [22], it is less prob-

able that dispersion of these bodies is directly caused by global DNA demethylation. It is more likely that demethylation indirectly causes the response by altering the expression of unknown genes, which control the tissue-specific condensation/decondensation of sex chromatin. In a similar manner, the reactivation of genes silenced by DNA methylation ensued after 5-azaC treatment of the facultatively heterochromatinized human X chromosome [24, 42].

In *Rhoeo*, 5-azaC treatment did not alter interphase condensation of constitutive heterochromatin, but disrupted the large collective chromocentres seen in untreated cells. Condensed chromosome segments tended to separate after drug administration, at least in some nuclei, but the frequency of cells with and without condensed chromatin remained unaltered. The spatial reorganization of interphase chromatin after 5-azaC treatment, in which normally colocalizing domains became separated, has also been observed in other plant species, for instance in *Triticum* [13, 35] and *Triticale* [7]. The observed spatial disturbances at interphase in these plants concern silenced transgenes [35] and subtelomeric constitutive heterochromatin [13], that is parts of the genome which are usually hypermethylated.

It has been suggested [2], that DNA demethylation induced by 5-azaC could persist for many cell generations. However, in some plants/tissues demethylation induced in this way seems to be reversible and is most effective shortly after treatment [7]. Our indirect observations, based on the presence/absence of condensed bodies within meristematic nuclei in *Rumex*, suggest a resetting of methylation patterns shortly after the treatment with 5-azaC is interrupted.

Our observations in *Rumex* and *Rhoeo* are consistent with suggestions [11] that the heterochromatic state of constitutive heterochromatin in plants is not controlled by DNA methylation, whereas the heterochromatic state of facultative heterochromatin is under the epigenetic control of DNA methylation. Most probably, the *de novo* methylation of many developmentally regulated DNA sites may not occur once in development, but may happen repeatedly as embryonic cells divide [4].

H3K4 and H3K9 methylation patterns in sex bodies

Strong methylation of lysine 4 (K4) in histone H3 is typical for transcriptionally active euchromatin, whereas demethylation of lysine 4 and methylation of lysine 9 (K9) are linked to gene-silencing and assembly of heterochromatin [21, 40]. In higher eukaryotes, histone methylation in facultative heterochromatin has only been studied within inactive human X chromosomes [5, 31]. Facultatively inactivated human X chromosomes are globally enriched with methylated H3K9, but largely lack methylated H3K4. Our findings with antibodies against H3K9 and H3K4 suggest that modifications of histone H3 in male sex bodies within *Rumex* nuclei are generally the same as those in the inactive human X chromosome. This is the first report of histone tail modification in interphase-condensed sex chromatin in plants.

It has been suggested [21] that there are some differences in the mechanism of H3K9 methylation in facultative and constitutive heterochromatin. Most probably, in constitutive heterochromatin, both histone and DNA methylation irreversibly stabilize transcriptionally silent chromatin domains, whereas in facultative heterochromatin these chromatin modifications are reversible.

In mammals, trimethylated H3K9 is preferentially localized to constitutive pericentromeric heterochromatin [32] and dimethylated to facultative heterochromatin (inactivated X chromosome) [31]. On the other hand, it was shown that in *Arabidopsis* constitutive heterochromatin is deprived of trimethylated H3K9, and silent chromatin in this species is enriched with mono- and dimethylated form of this histone. It was shown that male sex bodies in *Rumex* are enriched with monomethylated and dimethylated H3K9.

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