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, Y1 + 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.
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 0.5 g of roots (fresh weight) 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.), Erlenmeyer flasks, each containing 35 ml of the nutrient medium. Individual adventitious roots, which developed at 25 ml Erlenmeyer flasks with 35 ml of a liquid medium. The growth index (G.I.) of the culture was 0.5 g of roots (fresh weight) in 250 ml Erlenmeyer flasks with 35 ml of the nutrient medium. The roots were maintained at 25˚C, on a gyratory shaker (100 r.p.m.), Erlenmeyer flasks, each containing 20 ml of the nutrient medium.

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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 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.

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 antibody 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 (Jug/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, Y1 and Y2) were of standard morphology and were the largest in the chromosome complement (Fig. 1). The X chromosome was slightly longer than the Y1 chromosome and significantly longer than the Y2 chromosome. Our C-banding studies failed to reveal constitutive heterochromatin within Y chromo-
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,Y) 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 chromocenters (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 chromocenters tended to separate into smaller fluorescent spots following 5-azaC treatment. The frequency of meristematic nuclei with 4-7 chromocenters increased in comparison with untreated material. The frequency of nuclei with 2 or 3 larger chromocenters 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).

**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 dimethylated at lysine 4 and methylated at lysine 9 (Fig. 3). Immunostaining 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

| Table 1. Number of nuclei with sex bodies in males of *Rumex acetosa*, n=200 |
|------------------|-----------|-----------|-----------|-----------|
|                  | I         | II        | III       | X         |
| Root meristem    |           |           |           |           |
| adult plants     |           |           |           |           |
| seedlings        | 26        | 22        | 28        | 25.3      |
| *in vitro*       | 174       | 168       | 171       | 171.0     |
| Root hairs       | 186       | 190       | 183       | 186.3     |

I-III - replications; X - mean value; LSD = 12.31 (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 - immediately 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         |
| Control          | 46.7      | 44.3      | 53.0      | 45.7      |
| 0-D              | 30.0      | 53.3      | 44.0      | 68.0      |
| 7-D              | 23.0      | 41.7      | 49.3      | 82.0      |

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

**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-H3K9Me signals; d and f - anti-H3K4Me signals. (mono-) - monomethylated; (di-) - dimethylated; (tri-) - trimethylated. Bar: 20 µm
able that dispersion of these bodies is directly caused by global DNA demethylation. It is more likely that de-
methylation indirectly causes the response by altering the ex-
pression 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 treat-
ment of the facultatively heterochromatinized human X chromosome [24, 42].

In *Rhoeo*, 5-azaC treatment did not alter interphase
condensation of constitutive heterochromatin, but dis-
rupted the large collective chromocentres seen in un-
treated 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 be-
came 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 subte-

dromeric 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 gener-
ations. However, in some plants/tissues demethylation
induced in this way seems to be reversible and is most
effective shortly after treatment [7]. Our indirect obser-
vations, based on the presence/absence of condensed
bodies within meristematic nuclei in *Rumex*, suggest a
resetting of methylation patterns shortly after the treat-
ment 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 hap-
pen 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 hete-

rochromatin [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 mo-
dification in interphase-condensed sex chromatin in
plants.

It has been suggested [21] that there are some differen-
tes in the mechanism of H3K9 methylation in facultative
and constitutive heterochromatin. Most probably, in con-
stitutive heterochromatin, both histone and DNA methyl-
a
lation 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 heterochro-
matin [32] and dimethylated to facultative heterochro-
matin (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 mono-
methylated and dimethylated H3K9.

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