Chromosome banding and DNA methylation patterns, chromatin organisation and nuclear DNA content in *Zingeria biebersteiniana*

R. CREMONINI*[1], M. RUFFINI CASTIGLIONE*, V.G. GRIF**, V.V. KOTSERUBA**, E.O. PUNINA**, A.V. RODIONOV**, O.V. MURAVENKO***, K.V. POPOV***, T.E. SAMATADZE*** and A.V. ZELENIN***

*Department of Botanical Sciences, University of Pisa, Via Luca Ghini 5, I-56126 Pisa, Italy**
*Laboratory of Biosystematics and Cytology, Komarov Botanical Institute, RAS, St. Petersburg, Russia***
*Laboratory of Functional Morphology of Chromosomes, Enghelgardt Institute of Molecular Biology, RAS, Moscow, Russia****

Abstract

Chromosome structure and chromatin organisation of a two-chromosome model cereal *Zingeria biebersteiniana* (Claus) P. Smirnov were studied: nuclear DNA content was determined by microdensitometric analysis after Feulgen staining; Feulgen absorption at different thresholds of absorbance in interphase nuclei also provided evidence on the organisation of chromatin, allowing quantitative estimation of condensed chromatin within interphasic nucleus. The DNA methylation pattern of *Z. biebersteiniana* metaphase chromosomes was examined with a specific monoclonal antibody. 5-methyl-cytosine residues are present in several chromosome sites and differences may be present between corresponding regions of homologues. Chromosome banding pattern reveals large bands in the centromeric regions of each chromosome, showing constitutive heterochromatin; by fluorochromes staining pericentromeric blocks are evidenced. After the cold and 9-aminoacridine pre-treatments and after aceto-carmine and aceto-orceine staining, respectively, the metaphase chromosomes were analysed by image analysis system revealing a segmentation of the chromosome body that resembles Giemsa/Reverse banding in animal chromosomes.

Additional key words: chromosomes, fluorochromes, image analysis, *Poaceae*.

Introduction

Plants with very low chromosome numbers are of interest as single model systems for the examination of the structural organisation of their chromosomes. Many studies have been carried out on the karyotype evolution in the angiosperms with only two chromosomes in their haploid complement (Jackson 1973, Watanabe *et al.* 1975, Yonezawa 1981, Ikeda 1987, Watanabe and Smith-White 1987, Stedje 1988, Leitch *et al.* 1997) and such studies provides useful information on the origins and organisation of chromosomes.

Previous reports have described the karyotype of *Z. biebersteiniana* by light and electron microscopy, by fluorochrome banding and the cytological localization of ribosomal RNA genes by fluorescent *in situ* hybridization FISH (Bennett *et al.* 1986, 1995, Kotseruba 2001). Moreover Saunders and Houben (2001) have analysed the organisation of the pericentromeric heterochromatin that is composed of members of the *Zbcen-1* tandem repeat family, intermingled with accumulated putative *copia*-like retrotrasposon sequences.

In the last six years some reports (Ruffini Castiglione *et al.* 1995, Frediani *et al.* 1996, Široký *et al.* 1998, Castilho *et al.* 1999) have analysed, with different purposes, the DNA methylation pattern of metaphase

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Abbreviations: a.u. - arbitrary units; FISH - fluorescent *in situ* hybridization; G/R - Giemsa/Reverse
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[1] Corresponding author; fax: (+39) 050 551345, e-mail: cremonio@dsb.unipi.it

chromosomes of different plant species by using a monoclonal antibody against 5-methylcytosine (anti-5-mC). In view of the interest of this problem and in order to obtain more information about 5-mC in plant chromosomes, we studied the DNA methylation pattern of metaphase chromosomes of *Zingeria biebersteiniana*, the species with the lowest number of chromosomes in the family of *Poaceae* (n=2); Tsvelev and Zhukova (1974) suggested that this main number is the result of strong reduction of the probable primary main number in *Poaceae* n=7.

To study the specific organisation of *Z. biebersteniana* chromosomes we compare patterns of methylation and the chromosome banding patterns (C-, fluorochromes, cold and 9-aminoacridine pre-treatments) and, since two distinct reports on *Z. biebersteiniana* nuclear DNA content are present in literature with different 4C-values (Bennett *et al.* 1986, Sorokin and Punina 1992) we reinvestigate the DNA amount .in *Z. biebersteiniana* genome by Feulgen reaction.

Material and methods

Germination of seeds: Seeds of *Z. biebersteiniana* (Claus) P. Smirnov, collected in Volvograd region, Russia, and kindly supported by Dr. L.F. Savelyeva, were germinated on the moist filter paper in Petri dishes at 26 °C in darkness up to root length 10 - 12 mm.

Nuclear DNA content: 1 cm long roots were fixed in ethanol-acetic acid (3:1, v/v), squashes were made under a cover slip in a drop of 45 % acetic acid after a treatment with a 5 % aqueous solution of pectinase for 15 min at 37 °C with the addition of 0.001 M EDTA in order to inhibit the activity of DNase, if present (Berlyn *et al.* 1979). The coverslips were removed by the dry-ice method and the squashes were hydrolysed in 5 M HCl at room temperature for 30 min and stained by Schiff reagent. After staining the slides underwent three washes, each of 10 min interval, in SO_2 water prior to dehydration and DPX mounting.

Squashes of the root tips of *Vicia bithynica* were concurrently stained for each group of slides and used as internal standard. Absorptions measured in *V. bithynica* preparations were used to convert relative Feulgen arbitrary units into picograms of DNA. Feulgen DNA absorptions in individual cell nuclei, in the post synthetic condition (G2), were measured at the wavelength of 550 nm using a *Leitz MPV3* (Wetzlar, Germany) integrating microdensitometer equipped with a computer.

Different thresholds of absorbance: With the same instrument and at the same wavelength, the Feulgen DNA absorptions of chromatin fractions with different condensation levels were determined through measurements of one and the same nucleus after selecting different thresholds of absorbance according to the method of Cremonini *et al.* (1993, 1994). The percentage of Feulgen absorptions at different thresholds of absorbance were mathematically processed in order to obtain the exact position of the inflexion point in the curve, where the residual Feulgen absorption represents the cytophotometrically determined condensed chromatin.

Immunological localisation of 5-mC-rich chromosome regions: 1 cm long roots were treated with a 0.3 % aqueous solution of colchicine for 4 h at room temperature. Squashes were prepared as reported in the section Nuclear DNA content. The preparation, the analysis of the cross-reaction profile and the binding properties of the anti 5-mC used have been described in a previous paper (Podestà *et al.* 1993).

Cytological preparations were treated with NaOH 0.07 M for 2.0 m and dehydrated in 70 % and 95 % ethanol. After treatment with a blocking solution (1 % BSA in PBS 0.1 M pH 7.4, added with 0.1 % *Tween 20* and 1.5 % normal goat serum) for 15 min at room temperature, the slides were incubated with anti 5-mC diluted 1:100 in the solution A (1 % BSA in PBS 0.1 M pH 7.4, added with 0.1 % *Tween 20*) for 2 h at room temperature. After 3 × 10 min washes in the solution A, the slides were incubated for 1 h in gold conjugated goat anti-mouse (*British Biocell International*, Llanishen, Cardiff, UK) diluted 1:100 in the solution A.

The unbound gold conjugated was removed by four washes, the first in solution A/distilled water (1:1, v/v), the following washes in distilled water only. For light microscopy, the gold signal was enhanced with silver (Silver enhancing kit for L.M., *British Biocell International*): three drops of initiator and three drops of enhancer were mixed together and placed on the slides in the dark. After 5 min, the reaction was stopped in tap water and the slides were then stained with Giemsa, air dried and mounted in DPX. Ten metaphase plates for ten root tips were analysed.

The binding of anti 5-mC revealed high specificity for 5-mC residues in the DNA, as shown by the results (data not reported) of a number of negative control studies: *1*) a treatment with a non-immune goat serum at the same dilution as the primary antibody; *2*) further dilution of the primary antibody; *3*) the omission of the secondary gold conjugate antibody; *4*) the omission of the DNA denaturation step.

C-banding: The seedlings were treated for 3 h with 0.05 % colchicine solution containing 1 mg cm^{-3} of 9-aminoacridine. The seedlings were fixed in 45 % acetic acid. Squashed preparations were made from a cell suspension in 45 % acetic acid. Coverslips were removed by the dry-ice method and the slides were air dried; the staining procedure was carried out according to Badaev *et al.* (1985).

Fluorochrome staining using *Hoechst 33258* and olivomycin: Seedlings were pre-treated in 0.05 % (m/v) colchicine for 2 h at the room temperature and subsequently heated in acetocarmine. Squash preparations were made from the root-tip and apex coleoptile meristem of the seedlings. The cytological slides have been prepared by the standard method removing the cover-slip by freezing. Air-dried slides were stained with *Hoechst 33258* and olivomycin according to Holmquist (1992) and Schwarzacher and Schweizer (1982), respectively. The mounting solution was a mixture of McIlvane's buffer (pH 4.2) and glycerine.

To exhibit complementary banding pattern on the same metaphase plate, preparations were stained by olivomycin then restained by *Hoechst 33258.* Fluorescence was observed by excitation with ultraviolet light (365 nm) using an *Opton* (*Zeiss*, Jena, Germany) fluorescence microscope with different filter sets for *Hoechst 33258* and olivomycin.

G/R-like banding after cold and 9-aminoacridine pre-treatments: The technique developed by Raskina and Rodionov (1992) was followed in order to obtain G/R-like bands in *Z. biebersteiniana* metaphase chromosomes after cold pre-treatments. Seedlings were pre-treated in iced water at 2 - 3 °C for 18 h and subsequently fixed in ethanol-acetic acid (1:1, v/v). Fixed seedling roots were stained with heating in 4 % aceto-carmine up to boiling and squash preparations were made from root-tip meristems.

To obtain G/R like banding after 9-aminoacridine treatment, the seedlings were treated with 1 mg cm^{-3} 9-aminoacridine solution at 20 °C overnight, then fixed in ethanol-acetic acid (3:1) and stained by acetoorcein solution for 24-28 h at room temperature (1 % orcein in 45 % acetic acid). The root tips were cut off and squashed in 45 % acetic acid to obtain temporary chromosome preparations. The slides were observed, photographed and subsequently the microphotographs were examined by image analysis system. Computer program *Videotest-Karyo 1.5* (*Ista-Videotest*, St. Petersburg, Russia) enabled the production of ideograms from selected metaphases both from G/R like and from C-banded slides.

This software uses algorithms based on drawing the chromosome axis, determination of margins of bands. All operations were run in the interactive regime and using thus created idiograms the karyological parameters were determined.

Results

Chromosome morphometric data, nuclear DNA content and thresholds of absorbance: Chromosome morphometric data by image analysis system are summarised in Table 1. For nuclear DNA determination we tested two hydrolysis methods (cold hydrolysis: 30 min, 5 M HCl at room temperature and hot hydrolysis: 7 min 1 M HCl 60 °C, data not reported) and observed that the different methods gave similar measurements of nuclear DNA content. Using the cold hydrolysis procedure, the DNA content for *Z. biebersteiniana* is 6.06 pg of DNA in 4C pre-prophase nuclei. The mean Feulgen absorption, the surface area and the nuclear DNA content are summarised in Table 2 where the values of *Vicia bithynica* meristematic nuclei are also reported. We have chosen *Vicia bithyinica* as internal standard, among our available standards, because the value of *V. bithynica* is the lowest and the closest to *Z. biebersteiniana* value.

The results of the analysis, on 4C pre-prophase nuclei, at different thresholds of absorbance are reported in Fig. 1. In order to compare the values of the curves of absorbance we chose pre-prophase nuclei in each sample, having Feulgen absorption values corresponding to 4C content and the same surface area. Statistical analysis

Table 1. Chromosome morphometric data of *Zingeria biebersteiniana.* Means of fifty determinations carried out in five root meristems ± SE.

Chromo-some	Length of arms [μm] short	long	Total length [μm]	Centromeric index (s/s + l) [%]
I	3.6 ± 0.3	3.7 ± 0.3	7.3 ± 0.6	49.3 ± 0.1
II	3.3 ± 0.3	3.5 ± 0.3	6.8 ± 0.6	47.5 ± 0.2

Table 2. Mean Feulgen absorption, surface area of pre-prophase nuclei (4C) and nuclear DNA content in *Zingeria biebersteiniana.* Means of fifty determinations carried out in five root meristems ± SE (* - values from Frediani *et al.* 1992).

Plant species	Absorbance [a.u.]	Surface area [μm^2]	DNA amount [pg]
Z. biebersteiniana	406.53 ± 10.64	3.61 ± 0.09	6.06 ± 0.03
*V. bithynica**	1187.93 ± 19.58	10.96 ± 0.36	18.30 ± 0.12

(data not reported) does not show significant differences either within the 4C DNA amounts, in the pre-prophase nuclei of the same slide or between roots collected from different seeds.

At absorbance thresholds 26 the Feulgen absorption of *Z. biebersteiniana* is reduced to zero and the Feulgen absorption of *V. bithynica* is reduced to zero at threshold of absorbance 23.. For *Z. biebersteiniana* the inflexion point is at absorbance threshold 13 and the residual absorption is 46.40 %.

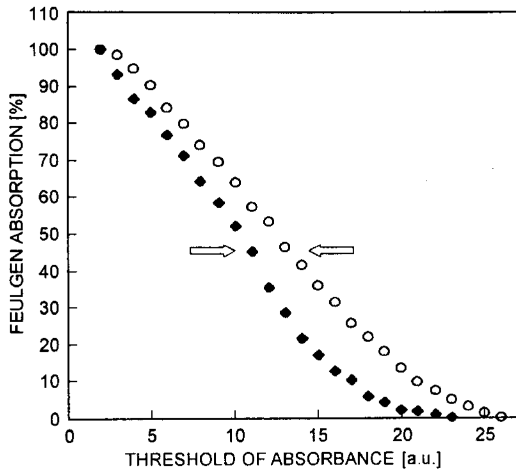

Fig. 1. Percentage of Feulgen absorption at different thresholds of absorbance of 4C interphase nuclei in *Vicia bithynica* (*closed rhombs*) and *Zingeria biebersteniana* (*open circles*). *Arrows* indicate the position of the inflection point.

Immunological localisation of 5-mC residues: In the chromosome pairs of *Z. biebersteiniana* 5-mC residues are present in several chromosome sites, even if they are particular abundant in the telomeric and/or subtelomeric regions and in certain intercalary bands. For pair I anti 5-mC binding is appreciable, as a rule, in the satellite and in the regions surrounding the secondary and primary constriction. For pair II, anti 5-mC binding is appreciable in telomeric regions, in the proximal region of the short arm and in certain intercalary bands (Figs. 2*A*, 3*A*)

If the behaviour of homologous chromosome regions in the different cells is taken into account, the following can be noted: the frequency of anti-5-mC binding changes among chromosome regions of different pairs; moreover corresponding region may or may not display comparable methylation levels in the two chromosomes of the same pair. In connection with this, three types of labelling are observed: both unlabelled, both labelled and one labelled and the other unlabelled (Table 3).

C-banding: C-banding reveals large bands in the centromeric regions of each chromosome (Fig. 2*B*). By chromosome image analysis system of C-banded chromosomes, other interstitial bands can be recognised both on the short and on the long arms of each chromosome (Fig. 3*B*). C-bands near secondary constriction are polymorphic in mean size.

Fluorochrome staining: Karyotype of *Z. biebersteiniana* is symmetrical: the two chromosomes pairs are similar in size (Table 1) and they show fluorescent pericentromeric blocks of heterochromatin (Fig. 2*C*,*D*); however, they are identified by a presence of secondary constriction that is localised in the region of fluorescence fading and smaller heterochromatic blocks in the first pair (Fig. 3*C*). The same fluorescent pattern is also present in prometaphase chromosomes (figure not shown).

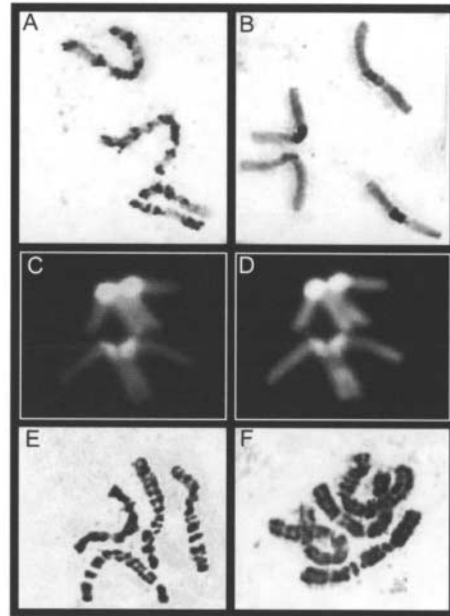

Fig. 2. Metaphase chromosome bandings (magnification 4000×): *A* - 5-mC-rich regions in metaphase chromosomes as revealed by immunogold technique; *B* - C-banding; *C* - olivomycine staining; *D* - *Hoechst 33258* staining; *E* - cold pretreatment; *F* - 9-amino acridine pre-treatment.

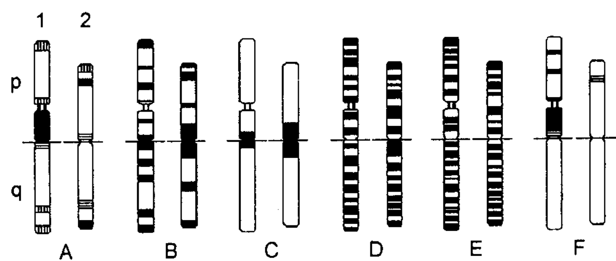

Fig. 3. Ideogram of haploid set (*1* - *2*) of chromosomes with different banding patterns (*A* - *E*) and *in situ* hybridisation (*F*). *A* - 5-mC banding (*vertically stripped parts of columns* - labelling percentage > 80 %, *full parts* 60 - 80 %, *horizontally stripped parts* 40 - 60 %); *B* - C-banding; *C* - fluorochrome banding; *D* - G/R-like banding after cold pre-treatment; *E* - G/R-like banding after 9-aminoacridine pre-treatment; *F* - ribosomal DNA localisation (*full parts of columns* - 18-26 S rDNA, *horizontally stripped parts* - 5S rDNA; redrawn from Bennett *et al.* 1995).

Table 3. Percentage of chromosome pair of *Zingeria biebersteiniana* in which bands are both labelled, both unlabelled or one labelled and the other unlabelled. Twenty chromosome pairs were analysed for each of six samples.

Chromosome bands	Pair I both labelled	only one labelled	both unlabelled	Pair II both labelled	only one labelled	both unlabelled
a	82	18	-	74	22	4
b	68	32	-	61	22	17
c	70	-	30	43	18	39
d	36	18	46	39	39	22
e	72	28	-	48	26	26
f	72	28	-	-	-	-

G/R-like banding: The staining of chromosomes after the cold and 9-aminoacridine pre-treatments results in similar segmentation of chromosome body that resembles G/R-banding in animal chromosomes and the clear banding pattern is revealed in metaphase (Fig. 2*E,F*, 3*D,E*).

Discussion

Nuclear DNA content and cytophotometrically determined heterochromatin: The differences obtained in the Feulgen absorption values in *V. bithynica* and in *Z. biebersteiniana* reflect real differences in the nuclear DNA content, as we have analysed pre-prophase nuclei (4C) from tissues in the same developmental stage and all squashes were stained concurrently. Our determination of nuclear DNA content differs from the data reported by Bennett and Smith (1991) and Sorokin and Punina (1992) (7.4 and 8.0 pg, respectively). This divergence may be due to the different seed accession, staining and determination methods used.

The residual Feulgen absorption at the inflexion point may represent the cytophotometrically determined heterochromatin component in interphase nuclei (Cremonini *et al.* 1993, 1994) since instrument at lower thresholds of absorbance reads all the chromatin fractions, both less condensed and more condensed, and at higher thresholds only the more condensed chromatin fraction. The position of the inflexion point may correspond to a particular thresholds of absorbance designated as the "cut-off point" by Havelange and Jeanny (1984) which separate the condensed from the dispersed chromatin.

Our determination of condensed fraction of chromatin (46.4 %, Fig. 1) may be more accurate if compared with the method of Havelange and Jeanny (1984) where the determination of the "cut-off point" and consequently of the more condensed chromatin component was exclusively due to the interpretation of the investigator.

5-mC-rich regions: 5-mC is present in several specific chromosomal location and this fact remains partially in line with reports of a preferential distribution of 5-mC in constitutive heterochromatin. In fact in *Z. biebersteiniana* highly methylated regions are not always located in the chromosomal site positive to C-banding and Q-banding (proximal region of the long arm of chromosome pair II). These findings reflect a structural heterogeneity of the heterochromatic portion on plant genome and could suggest an evolutionistic significance to some epigenetic changes involving specific DNA sequences at particular genomic locations (Heslop-Harrison 2000, and references therein). Moreover extensive cytosine methylation changes in centromeric and telomeric regions may result non-random, and probably involved in the phenotypic variation and genome organisation as in *Zizania latifolia* introgression lines (Liu *et al.* 2001).

In many species, NORs are 5-mC-enriched (Houchins *et al.* 1997, Schubeler *et al.* 2000). In *Z. biebersteiniana* only the main site of 18S+26S-rRNA genes exhibits 5-mC-positive signal after immunological reaction. Our findings (Fig. 3*F*) according to the distribution of ribosomal genes by Bennett *et al.* (1986)) shows that the minor sites of 18S+26S-rRNA genes of 1p-arm were anti-5-mC-negative. The 5S-rRNA site of the chromosome 1 is located within 5-mC-enriched region, the 5S rRNA site of chromosome 2 is close to or within 5-mC-rich region.

Our results (Table 4) also indicate that differences may exist between corresponding regions of homologues and might support a different methylation pattern. Bearing this in mind, it is worth noting that some corresponding regions always show an identical behaviour in homologues, while other regions, as a rule, bind anti-5-mC in only one chromosome of the pair. This situation may also involve bands belonging to the same chromosome pair. Further indications supporting the view that anti-5-mC binding effectively reflects a differential distribution of methylated bases between corresponding regions of homologues, include the close association between the presence of 5-mC in specific

regions and its absence in others of the same chromosome.

These data confirm previous results obtained with the same antibody in *Allium cepa* (Ruffini Castiglione *et al.* 1995) and *Vicia faba* (Frediani *et al.* 1996). To date, a certain amount of data is available in literature describing the behaviour of homologous chromosomes. In our material the differences between homologous chromosomes could be reasonably explained by a different organisation of chromatin in corresponding regions of the homologues, which could reflect different transcriptional activities. In this connection we can hypothesize that both specific and variable presence of methylated clusters distributed in karyotypes are likely 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 (Attwood *et al.* 2002, Li *et al.* 2002).

C-banding: C-banding is considered as the main approach for constitutive heterochromatin identification. The C-banding pattern of *Z. biebersteiniana* metaphase chromosomes correspond in general to the C-banding previously described by Semenov and Semenova (1975) and Bennett *et al.* (1986, 1995). The comparison of C-band pattern with the 5-mC-rich regions distribution showed there are two kind of C-heterochromatin in *Z. biebersteiniana*, either 5-mC positive or negative. 5-mC-enriched C-bands are located in pericentromeric and telomeric regions of chromosome 1, in centromeric region of 2p-arm, and on both telomeres of chromosome 2. Both distal interstitial C-bands of chromosome 2 and 1q-arm are 5-mC-enriched also. C-bands that are 5-mC-negative after immunological reaction are centromeric C-band of 2q, proximal interstitial C-band of 1q, and both interstitial bands of 1p.

Fluorochrome staining: For fluorochrome staining we used *Hoechst 33258* and olivomycin. These complement one another: *Hoechst 33258* binds with four or more AT bp (Murray and Martin 1988) and olivomycin binds with the sequences containing two or more GC bp (Van Dyke and Dervan 1983). Since the chromosomes stained with olivomycin do not reveal other heterochromatic block and CG fluorescence is observed topologically in the same region where it is observed when *Hoechst 33258* is used, it is possible to affirm that 5-mC-positive pericentromeric heterocromatin blocks in *Z. biebersteiniana* can not be considered as pure AT-enriched site as previously stated by Bennett *et al.* (1995). Most probably their composition is mixed and therefore AT-clusters are intermittent with GC-rich sequences as happens in W chromosomes of chicken (Rodionov *et al.* 1984). We never observed fluorescence in the telomeric and in the interstitial C-bands in either case of *Hoechst 33258* or olivomycin staining. This might mean that these bands carry DNA with nucleotide composition that is close to the AT/GC composition of *Z. biebersteiniana* euchromatin.

G/R-like banding: Our methods demonstrate the phenomenon of G/R like banding of *Z. biebersteniana* chromosomes but the number of blocks varies according to the method, condensation degree and different mitotic phase. The close-situated prometaphase blocks usually form aggregated blocks in metaphase.

Clear interstitial G-like bands were observed in plant chromosomes by acetocarmine staining (Murata and Orton 1984), different proteolitic treatments (Yang and Zhang 1988) and actinomycin D pre-treatment (Kakeda *et al.* 1990), cold pre-treatment (Raskina and Rodionov 1992). A system of alternating dark and light bands throughout the length of the plant chromosome arms is usually referred to as G-banding. The dark interstitial bands of plant chromosomes would correspond to the pachytene chromomeres of meiotic chromosomes and would be a structure homologous or analogous to the animal euchromatic G-positive bands.

Therefore, it might be more correct to refer to dark stained interstitial bands of *Z. biebersteiniana* chromosomes induced by cold-pre-treatment, as an equivalent to R-bands of mammalian chromosomes because in some characteristics features, such as a higher degree of chromatin condensation, later replication and lower gene density, the animal G-positive bands are more similar to these on heterochromatin than R-bands (Comings 1978, Rodionov 1985, Holmquist 1992 and Craig and Bickmore 1994).

5-mC-rich DNA in mammalian chromosomes is located in and around the secondary constrictions, in GC-enriched R-bands and in some GC-enriched regions (Vasilikaki-Baker and Nishiola 1983, Barbin *et al.* 1994, Montpellier *et al.* 1994, Miniou *et al.* 1994). Comparable situation occurs in our material where the regions around the secondary constriction and the telomeric regions are 5-mC-enriched. It is very speculative, but probably the 5-mC-enrichment of telomeric regions of *Z. biebersteiniana* chromosomes resembles the 5-mC-enrichment GC-enriched R-bands (T-bands) of mammalian chromosomes in spite of it was shown that subtelomeric regions of cereal chromosomes are gene-enriched (Gill *et al.* 1993).

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