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B Chromosomes in *Nierembergia aristata* (Solanaceae): Nucleolar Activity and Competition with the A Chromosomes

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Key Words
B chromosomes · *Nierembergia* · Nucleolar activity · Nucleolar dominance · Solanaceae

Abstract
B chromosomes are additional dispensable chromosomes that may be present in some individuals, populations, or species, which have probably arisen from the A chromosomes but follow their own evolutionary pathway. Supposedly, B chromosomes do not contain major genes except for ribosomal DNA (rDNA) sequences that have been mapped on the supernumerary chromosomes of many plants and animals. This paper is a new report of B chromosome occurrence in plants. B chromosomes with nucleolar organizing regions (NORs) were found in a diploid sample of *Nierembergia aristata* D. Don (sub nom. *N. stricta* Miers) (2n = 2x = 16). This is an extreme case in which B chromosomes possess not only strong nucleolar activity, as revealed by conventional staining methods, AgNOR and fluorescence banding, and fluorescent in situ hybridization (FISH), but also show nucleolar competition with the A chromosomes. The observed phenomenon could be analogous to the nucleolar dominance or 'differential amphiplasty' phenomenon that occurs in interspecific hybrids.

B chromosomes are additional dispensable chromosomes that may be present in some individuals, populations, or species, which have probably arisen from the A chromosomes but follow their own evolutionary pathway [Camacho et al., 2000]. B chromosomes do not pair or recombine with any of the A chromosomes at meiosis, and they have an irregular and non-Mendelian mode of inheritance [Jones and Rees, 1982]. They are morphologically different from the A chromosomes, and the size of B chromosomes does not generally exceed the size of the largest A chromosome in plant species [Jones, 1995]. B chromosomes are known to occur in approximately 15% of extant species [Beukeboom, 1994]. Among angiosperm species, 8% of monocots have B chromosomes versus only 3% of eudicots [Levin et al., 2005]. Among members of the Solanaceae, B chromosomes are known to appear in *Cestrum* L. (1–10 Bs), *Combera* Sandw. (1–5), *Datura* L. (1–6), *Fabiana* Ruiz et Pav. (1–7), *Leptoglossis* Benth. (1), *Nicandra* Adans. (1), *Nicotiana* L. (1–2), *Nierembergia* Ruiz et Pav. (1–5), *Pantacantha* Speg. (1–6), *Petunia* Juss. (1–2), and *Solanum* L. (1–3) [reviewed in Acosta, 2006].

Most B chromosomes are heterochromatic, which supports the idea that these elements are genetically inert. Nevertheless, some B chromosomes show transcriptional activity. In particular, many B chromosomes have been

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found to carry ribosomal genes [Maluszynska and Schweizer, 1989; Green, 1990; Jones, 1995; Dhar et al., 2002; Leach et al., 2005; Murata et al., 2006], and in a few instances, their activity has been inferred from positive silver nitrate staining [Beukeboom, 1994; Camacho et al., 2000] or based on molecular evidence [Leach et al., 2005]. Some B chromosomes are also composed of euchromatin. Empirical data have revealed that these chromosomes are not necessarily inert and that chromatin structure, repression by genes on A chromosomes, or DNA methylation processes may cause their inactivity [Silva and Yoyenaga-Yassuda, 2004].

This paper is a new report of B chromosome occurrence in plants. B chromosomes with nucleolar organizing regions (NORs) were found in a diploid sample of *Nierembergia aristata* D. Don (sub nom. *N. stricta* Miers) (2n = 2x = 16), a tuber-bearing solanaceus herb that inhabits flooded regions from Brazil, Paraguay, Uruguay, and northeastern and central Argentina [Cocucci and Hunziker, 1995]. This species is cytogenetically interesting because some populations exhibit polyploidy (2n = 6x = 48) [Di Fulvio, 1976, 1984; Acosta et al., 2006]. The present study reports an extreme case in which B chromosomes possess not only strong nucleolar activity, as revealed by conventional staining, AgNOR and fluorescence banding, and fluorescent in situ hybridization (FISH), but also show nucleolar competition with the A chromosomes.

Materials and Methods

Plant Material

The material analysed consists of 2 samples of *Nierembergia aristata* collected in the same place in different years:

Collection site: Argentina, Province Córdoba, Department San Justo, between Villa Concepción del Tío and Frontera Sur.

EDF 847: collected October 1989.

EDF 1015: collected January 1999.

The respective voucher specimens were deposited in the herbarium of Museo Botánico de Córdoba, Argentina (CORD).

Cytogenetic Procedures

Somatic chromosomes were observed in squashed root meristems obtained from seed germination. The root apices were fixed in a 1:3 acetic acid:ethanol mixture for 12 h after a pretreatment in 2 mM 8-hydroxyquinoline for 8 h at 8°C.

For conventional staining, root tips were stained according to Feulgen's technique with Schiff's reagent for 1.5 h in the dark after hydrolysis in 5 N hydrochloric acid for 50 min at room temperature [Jong, 1997]. Meristem cells were isolated, macerated, and squashed in a drop of 2% aceto-carmine. Slides were made permanent by removing the coverslip by freezing with liquid CO_2 [Bowen, 1956] and mounted in Euparal (Chroma, Germany).

For chromosome banding and fluorescent in situ hybridization (FISH), root tips were squashed and pectinase-cellulase macerated according to the method of Schwarzacher et al. [1980] at 37°C for 60 min. After removal of the coverslip with CO_2, slides were air dried, aged for 1 to 2 days at room temperature, and then held at –20°C until use.

Triple staining with chromomycin A_3, dystamicin A, and 4′-6-diamidino-2-phenylindole (CMA/DA/DAPI) [i.e. CDD staining] was carried out as described by Schweizer [1980]. Enhanced or reduced fluorescence of a chromosome segment is indicated in the text by attaching + or –, respectively, to the fluorochrome or fluorochrome combination. AgNOR staining was performed according to the Ag-I procedure of Bloom and Goodpasture [1976] with the modification of Kodama et al. [1980] using nylon cloth (mesh size 0.242 mm) instead of coverslips.

Fluorescent in situ hybridization (FISH) was performed according to the protocol of Moscone et al. [1996]. The 5S rDNA and 18S-25S (45S) rDNA repeated sequences were localised using the following DNA probes: pXV1, a 349-base pair (bp) fragment of the 5S rRNA gene repeated unit from *Beta vulgaris*, including the adjacent spacer [Schmidt et al., 1994]; and R2, a 6.5-kilobase (kb) fragment of the 18S-5.8S-25S rDNA repeat unit from *Arabidopsis thaliana*, including the internal transcribed spacers ITS1 and ITS2 and a short 5′ segment of the intergenic region (IGR) [Wanzenböck et al., 1997]. The first probe was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany), and the second probe was labeled with biotin-11-dUTP (Sigma), both by nick translation.

Somatic chromosomes and interphase nuclei were observed and photographed on a Leica DMLB epifluorescence microscope (Leica, Heerbrugg, Switzerland) equipped with a computer-assisted Leica DC 250 digital camera system. For epifluorescence microscopy, images were captured in black and white using appropriate filter sets in each case. Digital images were pseudocoloured (and combined in the case of FISH images) using the IM 1000 Leica software, then imported into Photoshop, version 7.0 (Adobe, San Jose, Calif., USA) for final processing.

Results

Nierembergia aristata EDF 847 have 2n = 16 chromosomes in their normal complement and display 5 metacentric and 3 metasubmetacentric chromosomes pairs. This taxon shows a symmetric karyotype, with small chromosomes of rather homogenous size (ranging from 2.83 to 1.88 μm) and 2 chromosome pairs carrying nucleolar organizing regions (pairs 2 and 7). The cytological studies that we carried out here revealed that 11 individuals (50 % of the total examined) of EDF 847 (fig. 1E) were aneuploids with 2n = 17 in all cells analysed (69). The extra chromosome was not recognisable due to the similar shape and size of most of the chromosomes of this species.

In the karyotype of *Nierembergia aristata* EDF 1015, no major structural differences were observed when it

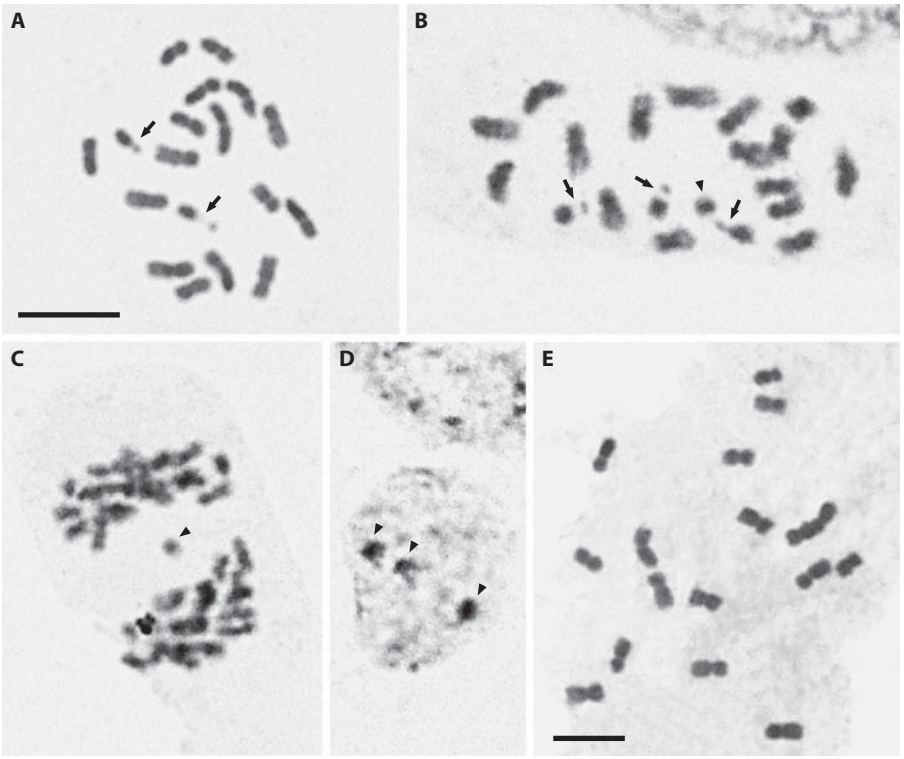

Fig. 1. Somatic metaphases, anaphase and interphase nucleus of *Nierembergia aristata* after conventional staining. **A–D** EDF 1015 sample. **E** EDF 847 sample. **A** Metaphase with 2n = 16 + 2 B. **B** Metaphase with 2n = 16 + 4 B. **C** Anaphase showing one B chromosome lagging. **D** Interphase nucleus with 3 condensed B chromosomes. **E** Metaphase with 2n = 17. Arrows indicate nucleolar organizing regions on B chromosomes. Arrowheads mark B chromosomes. Bars represent 5 μm.

was compared to the normal karyotype of EDF 847. However, this sample contained many individuals carrying B chromosomes (92%). The population contained a complete series of up to 5 B chromosomes per individual as follows: 0B (2 plants), 1B (4 plants), 2B (9 plants; fig. 1A), 3B (8 plants), 3 to 5B (2 plants; fig. 1B). Only one morphological type was observed. The B chromosomes were all similar, being subtelocentric and very small (0.90 to 1.31 μm) and having a length of 56% of the length of the smallest members of the A set. The B chromosomes were mitotically very stable, except when individuals had 3 or more B chromosomes; in these cases, some B chromosomes could be seen to be lagging at anaphase (fig. 1C). B chromosomes are visible in interphase nuclei as chromocentres, indicating that they are partly or wholly heterochromatic (fig. 1D). In addition, and more interestingly, satellites could be observed at the telomeres of the short arm of all B chromosomes. Finally, the low frequency of mitotic metaphases found in individuals without B chromosomes compared to seedlings with B chromosomes is of note.

To determine the structure of the B chromosomes and the associated satellites, fluorescent banding was performed using chromomycin A_3, dystamicin A and DAPI in a triple staining method (fig. 2A, B). In the sample analysed here (EDF 1015), the fluorescent chromosome banding pattern obtained by CMA/DA is generally the reverse of that seen using DA/DAPI. This species exhibits CMA/DA+ DA/DAPI– constitutive heterochromatin (hereafter designated as CMA+ DAPI–, i.e. chromomycin bright and DAPI dull), suggesting that *Nierembergia aristata* has GC-rich heterochromatin. The satellites are the only heterochromatic fluorescent segments. NORs are always located on the short arm of one metacentric (no. 2) and one metasubmetacentric (no. 7) chromosome pair and on the short arm of B chromosomes. Satellited chromosome pairs in *N. aristata* EDF 1015 that were revealed with this method agree very well with a karyotype constructed from chromosomes stained using Feulgen's technique, which was obtained from EDF 847. In interphase nuclei, it was possible to detect other signals corresponding to heterochromatin associated with B chromosomes, in addition to the bright bands belonging to chromosome pairs 2 and 7 (fig. 2C). The allocycly of the B's is noteworthy in that they remain condensed in the interphase nuclei.

Fluorescence in situ hybridization (FISH) revealed that *N. aristata* EDF 1015 has one pair of 5S and 2 pairs of 45S rDNA sites in its standard chromosome complement (fig. 2D). The 5S loci are intercalary on the short

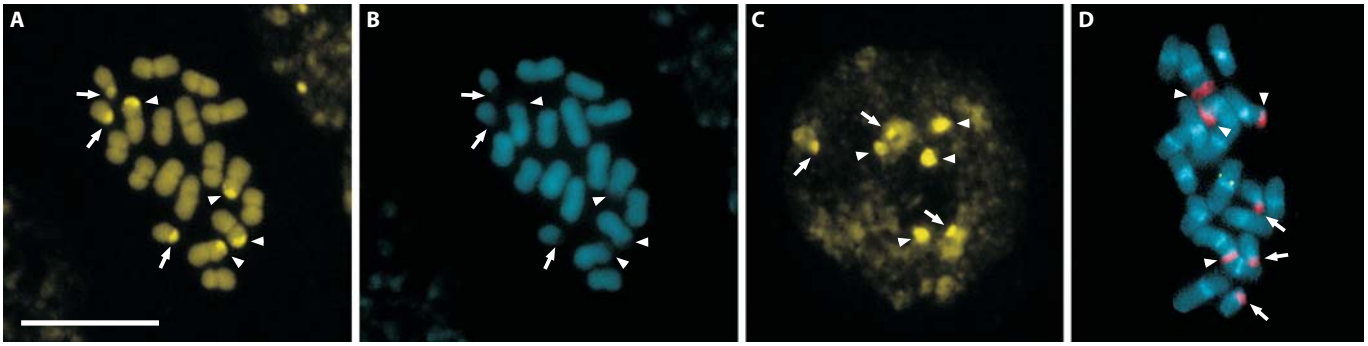

Fig. 2. Somatic metaphases and interphase nucleus of *Nierembergia aristata* EDF 1015. **A–C** Triple staining with CMA/DA/DAPI. **D** Double fluorescent in situ hybridization using probes for 45S and 5S. **A**, **B**, **D** Somatic metaphase chromosomes with 2n = 16 + 3 B. **C** Interphase nucleus with 3 B chromosomes condensed. **A**, **C** CMA/DA fluorescence. **B** DA/DAPI fluorescence. **D** Red signals indicate hybridization with the biotin-labeled 45S probe, which was detected with TRITC-conjugated antibodies, and green signals show hybridization to the digoxigenin-labeled 5S probe, which was detected with FITC-conjugated antibodies. Arrows indicate CMA+ DAPI– NOR-associated heterochromatin or 45S rDNA clusters on B chromosomes in **A–C** and **D**, respectively. Arrowheads mark CMA+ DAPI– NOR-associated heterochromatin or 45S rDNA clusters on normal chromosomes, in **A–C** or **D**, respectively. Bar represents 10 μm.

arms near the centromere of chromosome pair 1. All 45S clusters are terminal and correspond to the GC-rich heterochromatin bands. In addition, the B chromosomes were painted with the 45S probe, confirming that the fluorescent bands observed are rDNA sites.

The AgNOR banding that we carried out in EDF 1015 showed 2 nucleolar organizer region (NOR) loci (one major and one minor) on 2 chromosome pairs of the standard chromosome complement (fig. 3A). The major NOR site is located at the terminal region of the short arm of one metacentric chromosome pair (no. 2). The minor NOR site mapped close to the telomere of the short arm of one metasubmetacentric pair (no. 7). Additional AgNORs are present at the end of the short arms of the B chromosomes, indicating that the NORs of the B chromosome are transcriptionally active in organizing nucleoli (fig. 3C, E). Nevertheless, when B chromosomes with active organizing regions are present, independent of their number, only 2 NORs belonging to A chromosomes are active (corresponding to metacentric chromosome pair 2; see table 1). Thus, chromosome pair 2 bears active 45S sites, and pair 7 bears inactive ones. In individuals without B chromosomes, there are 4 NORs active on A chromosomes, which agree in number and size with the nucleoli observed in interphase nuclei (fig. 3B). However, all individuals with B chromosomes show only one nucleolus with NORs associated (from A and B chromosomes; fig. 3D, F).

The histochemical silver staining method is indicative of the transcriptional activity of rDNA [Maluszynska and Schweizer, 1989]. The strong silver-dots that were observed in B chromosomes compared to the NORs from A chromosomes may be a reflection of higher rRNA gene activity in the B chromosomes and/or of significantly higher amounts of rRNA genes in the B chromosomes.

Table 1. Silver-stained nucleolar organizer regions (AgNORs) visualised in somatic metaphase in *Nierembergia aristata* EDF 1015. The numbers in parentheses after absolute values indicate percentage of the total examined

2n	No. of individuals analysed	No. of metaphase cells examined	No. of AgNORs	
			A chromosomes	B chromosomes
16	2 (15%)	2 (11%)	4	–
16 + 1 B	3 (23%)	3 (16%)	2	1
16 + 2 B	4 (31%)	5 (26%)	2	2
16 + 3 B	4 (31%)	9 (47%)	2	3

Discussion

Chromosome counts and karyotypes from 2 populations of *Nierembergia aristata* are presented, including 2 new chromosome numbers for this species (2n = 17, 16 + 1–5 B). The normal chromosome number and karyotype obtained here agree with the previously determined gam-

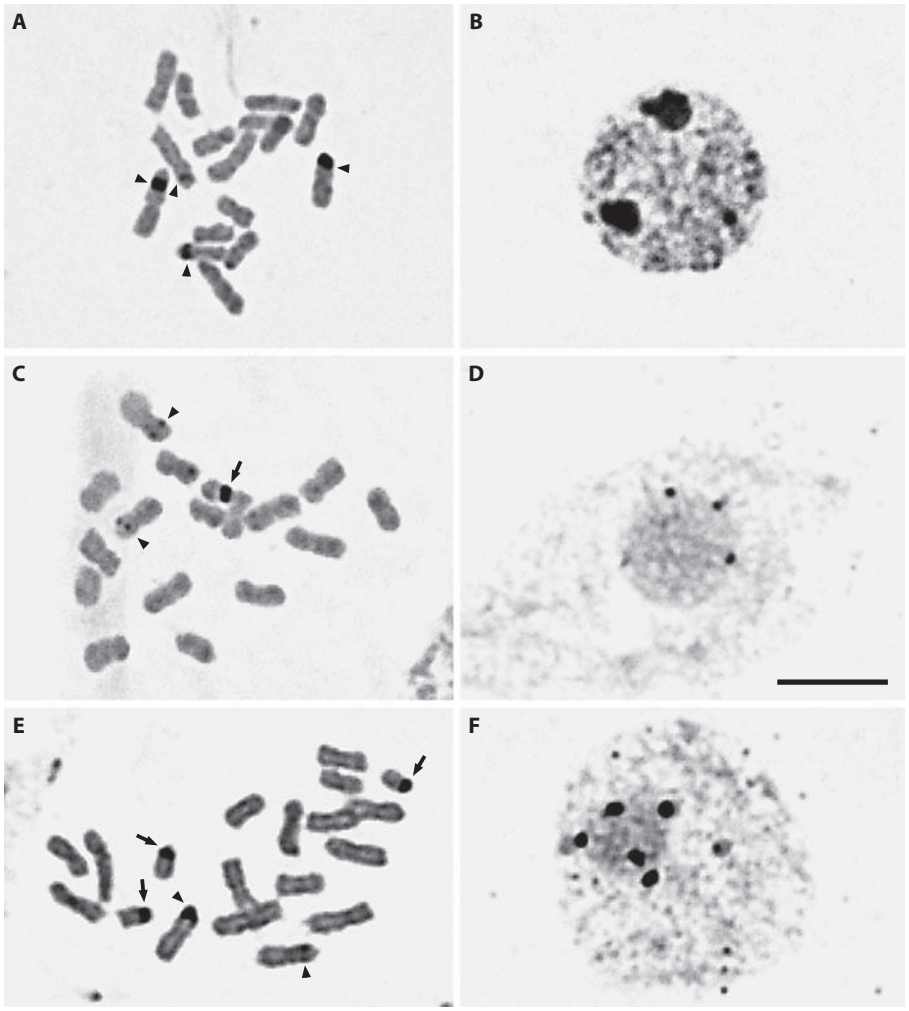

Fig. 3. Somatic metaphases and interphase nuclei of *Nierembergia aristata* EDF 1015 after AgNOR banding. **A**, **B** Individual with 2n = 16. **A** Metaphase with 4 AgNORs. **B** Interphase nucleus with 4 Ag-positive nucleoli. **C**, **D** Individual with 2n = 16 + 1 B. **C** Metaphase with 3 AgNORs. **D** Interphase nucleus with only one nucleolus showing 3 AgNORs. **E**, **F** Individual with 2n = 16 + 3 B. **E** Metaphase with 5 AgNORs. **F** Interphase nucleus with only one nucleolus showing 5 AgNORs. Arrows indicate AgNORs on B chromosomes. Arrowheads mark AgNORs on normal chromosomes. Bar represents 10 μm.

ete number published in 4 distinct populations by Di Fulvio [1976, 1984] and the karyotype formula reported in Acosta et al. [2006] and Tate et al. [2009].

The accessory chromosomes observed here have many characteristics that confirm their designation as B chromosomes, i.e. they are found in only some individuals of the population studied, and they show numerical variation between individuals (and within individuals in some cases). The highest B chromosome number (5) that we found in some individuals of *N. aristata* is remarkable. In the Solanaceae, *Cestrum parqui* L'Hér and *C. aurantiacum* Lindl. hybrids present up to 10 small B chromosomes, which is the highest B chromosome number registered in the family [Sýkorová et al., 2003]. In addition, the elevated percentage of individuals carrying B chromosome (92%) that were observed here is also interesting. This phenomenon was registered in *Allium schoenaprosum* L. and *Solanum ottonis* Hylander, with 65% and 100% of plants having B chromosomes, respectively [Zutshi and Kaul, 1974; Jones and Rees, 1982]. The low frequency of mitotic metaphase that we found in this study in individuals without B chromosomes compared to seedlings with B chromosomes is also noteworthy. There is clear evidence that B chromosomes can confer a selective advantage during the early stages of the life cycle. Some studies have shown that B-containing individuals survive better than individuals without B chromosomes between the seed and seedling stages and that B chromosomes have increased the rate of germination in these plants [Plowman and Bougourd, 1994].

In *N. aristata*, only one morphological type of B was observed, which is smaller than A chromosomes, as is common in B chromosomes of plants [Jones, 1995]. In

addition, they are subtelocentric, so they are readily identifiable in mitotic cells. B chromosomes are typically composed of repeated DNA sequences that vary in type and copy number [Camacho et al., 2000]. This heterochromatic nature has been described in many B's of plants [Battaglia, 1964; Jones and Rees, 1982]. In fact, the chromocenters observed in interphase nuclei could indicate that B chromosomes are partly or wholly heterochromatic. However, the metaphase chromosomes that were stained by triple staining (CMA/DA/DAPI) did not show heterochromatin blocks in B chromosomes, except at nucleolar organizing regions. Thus, if these chromosomes are heterochromatic, their repetitive sequences are not particularly rich in GC or AT base pairs.

The frequency of trisomic individuals found in the EDF 847 sample, which was collected in San Justo in 1989, could be related to the origin of B chromosomes in the same population collected 10 years later (EDF 1015). The traditional view, which is still widely accepted, is that B chromosomes are derived from the A chromosomes. The first step in this process is the occurrence of a mutation causing aneuploidy, such that a new chromosome originates due to non-disjunctions or other kinds of rearrangement, followed by gradual modification of this chromosome by successive mutations and structural modification until there is a complete loss of homology and the capacity to pair with the original A chromosome [Sen, 1974; Jones, 1975; Dnyansagar amd Pingle, 1979; Carr and Carr, 1982; Camacho et al., 2000; Page et al., 2001; Jones and Houben, 2003]. The origin of B chromosomes with NORs seems to be associated with chromosome fragmentation of an aneuploid A NOR-bearing chromosome, or it could be a consequence of the reported mobile nature of rDNA, with B chromosomes as the preferred 'landing sites' due to their neutral character [Jones and Houben, 2003].

A rapid evolution of extra chromosomes, which is probably what was observed here, has also been reported by Moens [1965], Tsuchiya [1969] and Dhar et al. [2002]. Green [1990] has suggested that B chromosomes undergo degeneration by Muller's ratchet mechanism, similar to what is seen for sex chromosomes. The new supernumerary chromosomes derived from the autosomal complement that create a trisomic condition are genetically covered by their autosomal progenitor sequences, as the Y is by the X. Thus, the supernumerary chromosomes are free to accumulate deletions and other kinds of mutations and to follow a unique evolutionary history within their population. Furthermore, the B chromosomes do not experience selective pressure because of the absence of pairing and recombination, so they will evolve quickly from one generation to another.

The present study reports an extreme case in which B chromosomes possess not only strong nucleolar activity but also exhibit nucleolar competition with A chromosomes. This phenomenon could be analogous to the nucleolar dominance or 'differential amphiplasty' phenomenon that occurs in interspecific hybrids. In many interspecies hybrids, ribosomal RNA (rRNA) genes inherited from one parent are transcribed, but the rRNA genes derived from the other progenitor are silent. This process could occur at the level of gene transcription, and cytosine methylation and histone deacetylation could be implicated in the rRNA gene silencing of one pair of chromosomes [Pikaard, 2000a, b]. The mechanisms by which dominant and under-dominant rRNA genes are discriminated in newly formed hybrids leading to the initial establishment of nucleolar dominance are poorly understood, though 2 hypotheses have been postulated: the species-specific transcription factor hypothesis and the enhancer-imbalance hypothesis. In the former, an rRNA gene promoter from one species is often not recognised in an unrelated species because of the incompatibility of their Pol I transcription factors, conceivably resulting in the complete silencing of one set of rRNA genes in the hybrid. The enhancer-imbalance hypothesis is appealing because it suggests a simple biochemical basis for discriminating among rRNA genes based on transcription factor binding affinities. Thus, an NOR with relatively few genes could still be dominant over an NOR with more genes if the former had a higher binding affinity for transcription factors that were present in limiting amounts. Our results are inconsistent with the predictions of the species-specific transcription factor hypothesis because the NOR silenced belong to the species. Therefore, our findings fit better with the second hypothesis indicating that the NORs on chromosome 2 and the B chromosomes are more effective in attracting factors that are essential for rRNA gene transcription. However, there is evidence that the transcription factors are not in limiting concentrations because the inactivation of the NOR on chromosome pair 7 is not related to the number of B chromosomes present. Furthermore, the NORs on B chromosomes are never observed to be inactive, independent of their numbers. Therefore, we conclude that the sequence types of the organizing regions could be important. Pontes et al. [2003] suggested that variation in nucleolar dominance is likely to result from relatively subtle genetic or epigenetic variation rather than dramatic genome-restructuring events, and it is not a fundamental trait of

Arabidopsis suecica due to species-specific differences inherent in its progenitors. The principal question, then, is whether the organizing nucleolar regions that we observed on the B chromosomes are similar to NORs on chromosome pairs 2 or 7.

Changes in NOR activity patterns associated with the presence of B chromosomes have also been reported in the grasshopper *Eyprepocnemis plorans*, in rye and in wheat. In the former, B chromosomes positively influence the activity of the X chromosome NOR; though in rye and wheat with rye B chromosomes, the presence of B chromosomes induces NOR and satellite condensation, indicating a reduction in rRNA gene expression from A chromosomes, as was observed here [Morais-Cecílio et al., 2000; Bakkali et al., 2001; Delgado et al., 2004].

Finally, having a B chromosome with active rDNA cistrons may be advantageous for an organism by increasing the number of ribosomes, which may indirectly promote the permanence of B chromosomes in the population [Beukeboom, 1994]. Indeed, the B chromosomes of *Nierembergia aristata* EDF 1015 are a suitable model system for studying the regulation of chromosome activity. Furthermore, the fact that A and B chromosomes contain rDNA enables us to study their origin and phylogeny as was carried out in *Crepis capillaries* and *Brachycome dichromosomatica* [Leach et al., 2005; Field et al., 2006]. Thus, to explain the possible origin of the B chromosomes in *Nierembergia aristata*, microdissection followed by microcloning of the B chromosomes appears to be the most promising technique to be utilised in future studies. Using this technique, comparisons of the internal transcribed spacer sequences within the ribosomal genes on the B and A (pairs 2 and 7) chromosomes could be carried out.

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