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Ribosomal RNA genes in B chromosomes of *Crepis capillaris* detected by non-radioactive *in situ* hybridization

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A non-radioactive *in situ* hybridization method using biotin-labelled rDNA has made it possible to localize rRNA genes not only at the secondary constriction in both homologous chromosomes No. 3 of *Crepis capillaris* but also in the B chromosomes occurring in the plants employed. Very clear dot-like rDNA signals at the telomeres of both arms were observed in all B chromosomes. Histochemical silver staining, which is indicative of transcriptional activity of rRNA gene clusters, resulted in both darkly-staining nucleolar constrictions of chromosomes No. 3 and silver deposits at the telomeres of Bs. We conclude that the B chromosomes of *C. capillaris* are isochromosomes with active rRNA genes located near both telomeres.

INTRODUCTION

Accessory or B chromosomes are known to occur erratically in a variety of plant and animal species and to exhibit variation both in morphology and number as well as behaviour and effects. Bs are usually smaller than the ordinary "A" chromosomes and they never share linkage homology with standard chromosomes as concluded from their failure to pair with A chromosomes at meiotic prophase (Jones and Rees, 1982). B chromosomes, by virtue of their DNA mass, exert a nucleotypic effect on their "host". Beyond that, they are an additional source of genomic DNA polymorphism. Genetically, B chromosomes are assumed to be inactive and empty. However, recent data suggest that they may exert a *trans*-regulatory effect on A chromosomes or even carry housekeeping genes themselves. An effect of B chromosomes on the expression of A chromosomal genes has been described by Oliver *et al.* (1982) for the liliaceous plant *Scilla autumnalis* Loidl (1982*a*, *b*), Guillen and Ruiz Rejon (1984) in several species of *Allium*, and Cabrero *et al.* (1987) in a grasshopper, have, on the basis of cytological observations, presented data which indicate the occurrence and activity of nucleolus organizer regions, *i.e.*, active rRNA cistrons, in accessory chromosomes.

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A novel *in situ* hybridization method by non-radioactive labelling and immunocytochemical detection of DNA hybrids has given us the means for rapid and precise detection of rRNA gene clusters. An accession line of *Crepis capillaris* known to contain B chromosomes (see Rutishauser and Röthlisberger, 1966) was used as a model system. The results obtained suggest that the Bs of *C. capillaris* are isochromosomes carrying minute amounts of rDNA at both telomeres.

MATERIALS AND METHODS

Our accession line of *Crepis capillaris* (L.) Wallr. containing B chromosomes originated from the collection of Professor A. Rutishauser, Zürich (see Rutishauser and Röthlisberger, 1966). It has been propagated since the early 1960s in various experimental Gardens; in the Botanical Garden of Vienna since 1974. The same provenance of *C. capillaris* was used by Brown and Jones (1976) in their studies, and seed material from their population was also given to us and later co-cultivated with our plants.

Seeds of *C. capillaris*, originating from open pollinated plants with different numbers of B chromosomes (0 to 3), were soaked for six hours in tap water at room temperature. Seeds were sown on wet filter paper in Petri dishes. After three to

four days, seedlings were placed in 0·02 M 8-hydroxyquinoline for two hours at room temperature and then for two hours at 4°C. The seedlings were fixed in methanol-glacial acetic acid (3:1) overnight and stored in 96 per cent ethanol in the deep freezer.

Root tip meristems were digested in 1 per cent pectinase and 2 per cent cellulase in sodium citrate buffer (pH 4·7) for one hour at 37°C. Squashing was performed in a drop of 45 per cent acetic acid. The preparation and the number of B chromosomes were checked under a phase contrast microscope. The coverslip was removed by freezing the slide and the preparations were then air-dried and stored in a desiccator over silica gel at 4°C.

In situ hybridization of rDNA probes was carried out using a biotin-avidin-FITC detection technique (Pinkel *et al.*, 1986) with some minor modifications (Hagemann, 1988). Ribosomal DNA probes (18S and 25S rDNA clones from *Cucurbita pepo*) were kindly provided by Professor V. Hemleben, Tübingen. The 2·5 kb insert of clone pRZ 197 contains the full 18S rRNA coding region and a small part of adjacent regions (ETS and ITS). The 7·8 kb insert of pRZ 268 contains a small part of the ITS, the 25S rRNA coding region, and a considerable part of the NTS. The spacer elements of *Cucurbita pepo* rDNA are expected not to cross-hybridize with genomic sequences of *Crepis capillaris*. Clones were labelled by nick-translation with Biotin-11-dUTP and a 1+1 mixture of both probes was used for *in situ* hybridization. Immunocytochemical biotin detection was achieved by sequential application of FITC-labelled avidin, biotinylated anti-avidin, followed again by FITC-avidin. Counterstaining was done with 2 μg/ml DAPI (4,6-diamidino-2-phenylindole) for one hour.

Chromosomal fluorescence was observed with a Leitz Ortholux fluorescence microscope equipped with a Ploem-Opak II and Leitz filter blocks A, E3 and I2/H2. Photographs were taken on Kodak Tri-X-Pan film.

A crude estimate for the relative amount of rDNA at the telomeres of B chromosomes was calculated from the size of spots observed at major NORs (No. 3) and Bs after *in situ* hybridization. The area of spots was measured in 15 chromosomes of each type on photographic pictures of metaphase plates using the digitizing tablet (4Teo Micro-DGT-PAD) connected with a DEC PDP 11/23 computer.

Silver staining was done according to the modified method of Bloom and Goodpasture (1976). Slides were incubated for 10 minutes in 0·01 M borate buffer (pH 9·2). Then a few drops of freshly prepared 50 per cent silver nitrate solution in distilled water were applied to each preparation. Slides were covered with a nylon mesh (Hizume *et al.*, 1980) and incubated in a moist chamber at 60°C for two hours and then washed in distilled water before air-drying.

RESULTS

Crepis capillaris ($2n = 2x = 6$) is especially suitable material for cytogenetic investigations because it has only three pairs of medium-sized chromosomes, which can be easily identified on the basis of their length and arm ratio. We have adopted in Fig. 1b the chromosome designations of Ambros *et al.* (1986). The longest chromosome is No. 1, the shortest No. 2 and the NOR-bearing chromosome is No. 3. This corresponds to chromosomes A, C and D respectively of the original Navashin classification. Following C-banding or fluorochrome banding, each chromosome type exhibits additional morphological traits, which allow chromosome identification in cases of mutational structural rearrangements as are encountered upon *in vitro* tissue culture (Ashmore *et al.*, 1981; Maluszynska *et al.*, in preparation). The B chromosomes of *C. capillaris* are smaller than the shortest A chromosomes (No. 2), *i.e.*, about the size of the short arm of chromosome 1 (around 3 μm). In the material used in this study, from 0 to 3 B chromosomes were present in root tip meristematic cells in different plants. Only one morphological type of B was observed. All Bs were metacentric and DAPI-fluorescent staining following *in situ* hybridization revealed that they harbour relatively large amounts of constitutive heterochromatin. The heterochromatin, covering about one third of total length, confers on these chromosomes a striking overall brightness when stained with DAPI subsequent to the *in situ* hybridization protocol (figs. 1(b), 2(b)).

In situ hybridization with biotin-labelled rDNA has made it possible to localize with very good resolution not only the major rRNA gene loci in the standard chromosome complement of *C. capillaris*, but also to look for additional minor rDNA sites on A and B chromosomes. In mitotic metaphases of root tip meristematic cells, a FITC-brilliant block was always seen at the secondary constriction (nucleolus organizing region; NOR) in both homologues of the acrocentric chromosome No. 3. This confirms earlier conclusions based on banding techniques, that *C. capillaris* has

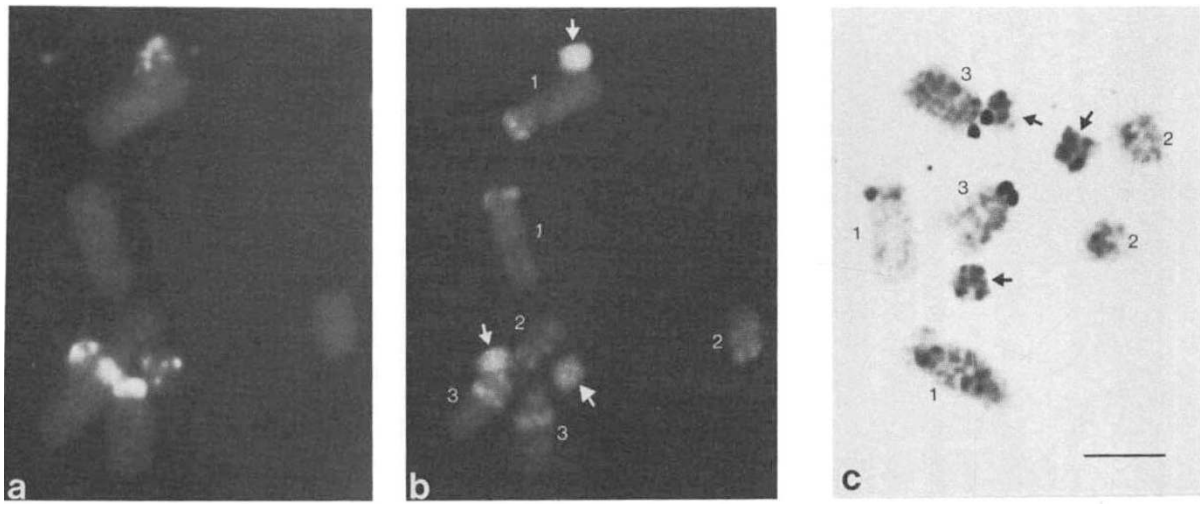

Figure 1 Full metaphase complement of *C. capillaris* with three Bs. (a) *in situ* hybridization with rDNA, FITC-fluorescence and (b) DAPI staining; (c) silver staining. Arrows indicate B chromosomes. Bar = 5 μm.

one pair of NOR-bearing chromosomes, and that rRNA cistrons are clustered at the paracentromeric constriction in the short arm of chromosome 3 (originally designed as chromosome D, see Röthlisberger, 1970) (figs. 1(a), 2(a) and 3).

On the remaining A chromosomes (Nos. 1 and 2) no rRNA gene sites could be detected. However, to our surprise, in all B chromosomes very clear rRNA gene signals were visible usually expressed as four dots at the telomeres of the chromatids of both arms. This result strongly supports the contention that the Bs of *C. capillaris* are iso-chromosomes. In interphase nuclei it was possible to detect, in addition to the bright No. 3 fluorescence associated with the nucleoli, smaller and weaker signals corresponding to rRNA cistrons of B chromosomes (fig. 4(a)). These observations were further substantiated by results obtained by silver impregnation ($AgNO_3$–NOR-staining). The histochemical silver staining method is indicative of the transcriptional activity of rDNA (Miller *et al.*, 1976). Ag-positive segments were observed at the secondary constriction of both nucleolar chromosomes No. 3. The Bs had pairs of much smaller and weaker but nevertheless discernable silver-dots at their telomeres (fig. 1(c), 3(b)). The weak and erratic silver staining of Bs as compared to the major NORs may be a reflection of lower rRNA gene activity in the Bs and/or of significantly lower amounts of rRNA genes in the accessory chromosomes. Both these reasons could explain why rDNA has not been detected previously in the B chromosomes of *C. capillaris*. The DNA–DNA *in situ* hybridization method used here is sensitive enough to detect minor amounts of rDNA (for further discussion see Ambros and Schweizer, 1987) irrespective of whether the rRNA genes are transcribed or not.

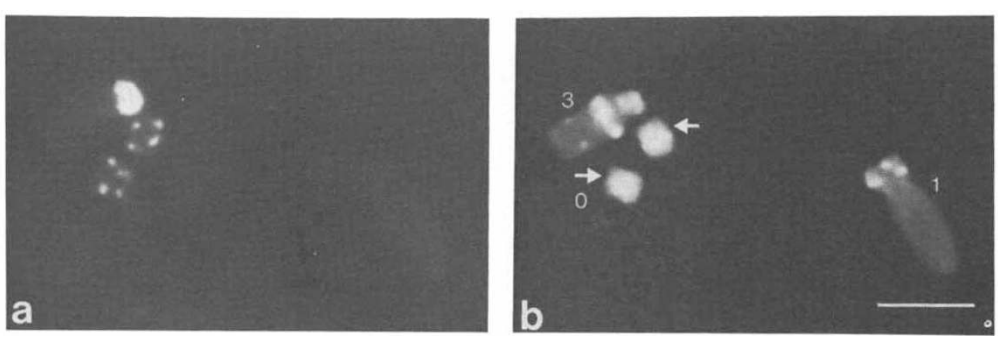

Figure 2 Part of a metaphase of *C. capillaris* with two Bs, chromosome No. 1, and No. 3 with bright NOR. (a) *in situ* hybridization with rDNA, (b) DAPI-staining, Arrows indicate B chromosomes. Bar = 5 μm.

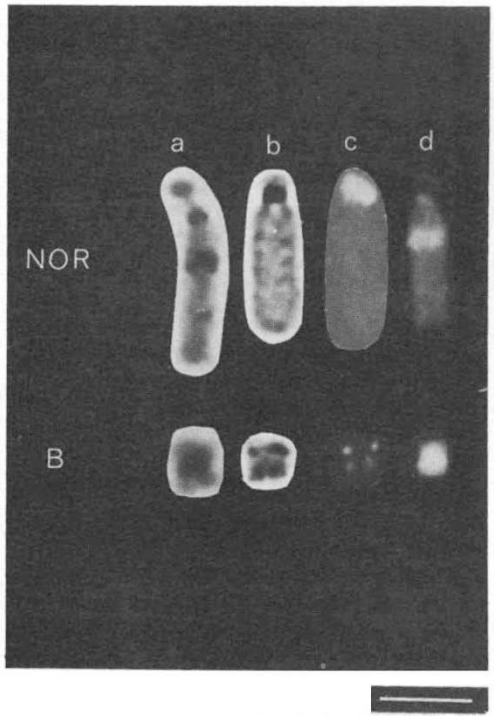

Figure 3 Comparison of differential staining by cytochemical methods of the NO- and B chromosome of *C. capillaris*. (a) C-bands, (b) silver staining, (c) *in situ* hybridization with biotinylated rDNA and an avidin-FITC/antiavidin detection method, (d) DAPI. Bar = 5 μm.

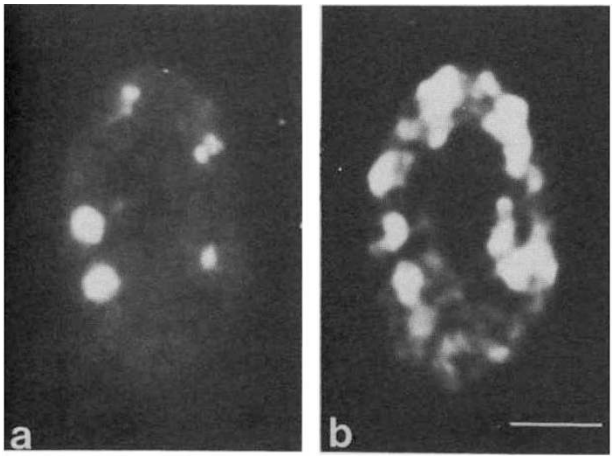

Figure 4 Interphase nucleus of *C. capillaris* ($2n = 6+3$ Bs) (a) *in situ* hybridization with biotinylated rDNA as detected by avidin-FITC. (b) DAPI staining. Bar = 5 μm.

As mentioned above, DAPI fluorescence staining performed after *in situ* hybridisation allowed the observation of a C-banding-like staining differentiation. The explanation for this phenomenon is that chromosome preparations in the *in situ* hybridization protocol, are subject to a treatment resembling C-banding and that DAPI can replace in this case the Giemsa dye. The obtained pattern of DAPI bands is thus consistent with Giemsa C-bands (fig. 1(b), 3(a, d)). The longest chromosome (No. 1) has bands at the distal region of the short arm; the shortest chromosome (No. 2) has submedian bands in the long arm and is also banded at the distal region of its short arm. The nucleolar chromosome (No. 3) has large DAPI-positive submedian bands in the long arm and two dots in a more distal region; also the centromeric part and NOR are C-band positive. The B chromosomes, as stated, are heavily banded, their centromeric/paracentric regions showing the brightest DAPI fluorescence in the karyotype (figs. 1(b), 2(b) and 3(a, d)).

DISCUSSION

B chromosomes have been found in a number of different species of the genus *Crepis* (reviewed by Jones, 1975; Jones and Rees, 1982). Rutishauser (1960*a*) was the first to describe Bs of *Crepis capillaris* from a small natural population near Schaffhausen (Switzerland). He described two morphological types of Bs which he called monocentric and dicentric. The chromomere pattern of these Bs suggested that their paracentromeric arm-regions are heterochromatic. This is in accord with our observations obtained by banding techniques, although we have seen in our plants only one morphological type of B chromosome, namely a small mono-metacentric.

In the majority of cases, Bs have been shown to be extensively or even entirely heterochromatic. This is true of the B chromosome of *C. capillaris* which has centromeric and pericentromeric heterochromatin amounting to 35 per cent of total chromosome length. The heterochromatic nature of the Bs of *C. capillaris* becomes evident by their conspicuous DAPI fluorescence after *in situ* hybridization and by their dark staining following C-banding (fig. 3). Autoradiographic studies of DNA replication in *C. capillaris* revealed heavy late labelling of the Bs showing that DNA synthesis of Bs is scheduled towards the end of the S phase as is characteristic for heterochromatic DNA (Abraham *et al.*, 1968).

rRNA cistrons in the B chromosomes of C. capillaris

To our knowledge this is the first report on the detection of rDNA in plant B chromosomes by the

use of an *in situ* hybridization technique. The methods employed here, *i.e.*, the non-radioactive immunocytochemical biotin/FITC-avidin/anti-avidin technique (Pinkel *et al.*, 1986), in conjunction with silver histochemical staining, allow us to conclude that the B chromosomes of *C. capillaris* bear rRNA genes at both telomeres, and that in somatic tissue some or all of these rDNA sites are usually transcribed. Because a rRNA gene-probe from a distant genus (*Cucurbita*) was used, the possibility of cross hybridization of spacer sequences can be excluded. We, therefore, conclude that the signals on B-telomeres reflect true rRNA gene clusters rather than rDNA-related satellite DNA sequences.

In other plant species nucleolar activity of B chromosomes has been observed in a limited number of cases (table 1, for animals see Jones and Rees, 1982). In the only known plant molecular study in this area, Flavell and Rimpau (1975) investigated wheat plants with a varying number of rye B chromosomes using rRNA/DNA filter hybridization. A positive correlation was found between the number of rRNA genes and the number of Bs in these addition lines. In two rye provenances with accessory chromosomes this correlation was not observed, and, on the basis of this result, it was concluded that not all Bs in rye are the same, and that those transferred into the wheat genetic background each carry about 650 ribosomal RNA genes.

The amount of nuclear DNA and the number of rRNA genes in *Crepis capillaris* was determined by Doerschug *et al.* (1976). The 1C DNA content is approximately 2 pg of which 0·4 per cent is represented by ribosomal DNA. The number of rRNA cistrons per haploid genome is about 2500. We have estimated from the relative sizes of the *in situ* hybridization spots of rRNA genes at the major NOR of chromosome 3 and at the telomeres of the Bs that one B chromosome carries less than 30 per cent of the total rDNA located at a major NOR. Therefore, Bs are likely to carry about 400 copies of rRNA cistrons at each telomere.

Association of B chromosomes with nucleolar activity has been observed by cytological methods in one population of *Allium flavum* (Loidl, 1982) suggesting that the B chromosome in these plants carries a nucleolar organizer region. The association of Bs with nucleoli was also found in another liliaceous species, *Allium sphaerocephalon* and *A. pallens* (Guillen and Ruiz Rejon, 1984; Loidl, 1982*b*). Similarly, in a biosystematic study of the genus *Calycadenia*, several types of B chromosomes were found, the meiotic behaviour of which suggested that they were carrying a nucleolar organizing region (Carr and Carr, 1982). In the grasshoppers *Chorthippus binotatus* and *Eyprepocnemis plorans* regulatory effects of supernumerary chromosome segements on the activity of NORs, and the activation of a latent nucleolar organizer region on a B chromosome fused to an autosome have been discovered (Cabrero *et al.*, 1986, 1987). A nucleolar B chromosome showing segregation-distortion has been reported by Bidau (1986) in the grasshopper *Dichroplus pratensis.*

Evolution of nucleolar B chromosomes

Our present knowledge of the molecular structure, cytology and behaviour of B chromosomes is still too rudimentary to allow the suggestion of a model

Table 1 Nucleolar B chromosomes in plants

Species	Method and observations	References
Allium flavum	Cytological detection of NOR and the association of Bs with nucleolus	Loidl (1982*a*)
Allium pallens	Cytological detection of NOR and the association of Bs with nucleolus	Loidl (1982*b*)
Allium sphaerocephalon	Cytological detection of NOR and the association of Bs with nucleolus	Guillen and Ruiz Rejon (1984)
Alopecurus pratensis	Cytological detection of association of Bs with major nucleolus; Formation of own nucleolus by Bs	Bosemark (1957)
Calycadenia sp.	Cytological detection of nucleolus association with Bs	Carr and Carr (1982)
Crepis capillaris	rDNA *in situ*-hybridization; rRNA cistrons at telomeres of Bs	Present study
Gibasis linearis	Cytological detection of NOR	Brandham and Bhattarai (1977)
Pennisetum typhoides	Cytological detection of NOR	Powell and Burton (1966)
Secale cereale	RNA/DNA hybridization; correlation of rRNA genes with number of Bs	Flavell and Rimpau (1975)
Tradescantia edwardsiana	Cytological detection of NOR	Stack (1974)

for their origin and evolution (for discussion see Battaglia, 1964; Jones and Rees, 1982; Amos and Dover, 1981). In *C. capillaris* earlier cytoevolutionary studies (Babcock, 1942; Togby, 1943) suggested that Bs occurring in species with reduced chromosome numbers may originate from fragments produced by unequal interchanges (see Jones and Rees, 1982; fig. 6,1). The symmetrical distribution of rDNA sites observed by us near the telomeres in both arms of the B chromosomes of *C. capillaris* appears to support the contention that they are isochromosomes. This indicates, that they could have arisen either from chromosome number 3 by centromere misdivision, leading to a telocentric fragment and subsequently to an isochromosome, as proposed by Kayano in 1956 for *Lilium callosum* (see Rutishauser, 1960*b*), or by an anomalous juxta-centromeric crossover (U-type exchange) resulting in an inverted duplication of a centric fragment, *i.e.*, a dicentric isochromosome such as described by Rutishauser (1960*a*). In the case of our *C. capillaris* Bs, amplification of centric heterochromatin and another chromosome break, in the secondary constriction, have to be invoked (see also Markarian and Schulz-Schaeffer, 1958) to explain the heterochromatic arm regions and the very terminal location of the rDNA spots.

Alternatively, one should also take into account the possibility of interchromosomal transposition of rRNA genes from standard NO-chromosomes to supernumerary chromosomes, which then could have originated from a different A chromosome (*e.g.*, Nos. 1 or 2). Based on the variability in size, number and chromosomal position of NORs in *Allium* species hybrids, Schubert and Wobus (1985) have presented a hypothesis on the interchromosomal mobility of NORs in plant genomes. Multichromosomal distribution of rRNA genes and their genetic exchange among nonhomologous chromosomes are known to occur, *e.g.*, in man and higher apes (Arnheim *et al.*, 1980).

Molecular and immunocytochemical techniques, such as those used in the present investigation, should help to solve some of the questions regarding the cytoevolution of "accessory" chromosomes.

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