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Cytochemical heterochromatin differentiation in *Sinapis alba* (*Cruciferae*) using a simple air-drying technique for producing chromosome spreads*

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Abstract: The fluorochrome and Giemsa chromosome banding patterns and the Ag-NOR histochemical staining of *Sinapis alba* are described. Two major types of heterochromatin can be distinguished, one of which contains GC-rich DNA. The observations are discussed as they relate to the known satellite DNAs of *S. alba.* – A simple air-drying technique for producing spreads of plant mitotic chromosomes is presented. Different materials and staining techniques were tested showing that the method has wide applications.

Sinapis alba has been for long a popular experimental plant in both physiological and cytological studies and, more recently, this species was also used in molecular investigations (e.g. CAPESIUS 1983, GRELLET & al. 1986, HALLDÉN & al. 1987). In a review by NAGL (1982) on "cell growth and nuclear DNA increase by endoreduplication and differential DNA replication", *S. alba* is one of the few examples of flowering plants with differential DNA replication. This is based on earlier cytophotometric DNA data by CAPESIUS & STÖHR (1974), who had measured the relative DNA content per cell-nucleus in the growing hypocotyl of *S. alba.* An increase of endopolyploidy during elongation growth was detected and it was suggested that the DNA in heterochromatin is overreplicated in the endopolyploid nuclei. This statement required further substantiation by biochemical methods. Therefore, a satellite DNA of *S. alba* has been isolated and its structure has been characterized in some detail by restriction enzyme mapping and sequencing (CAPESIUS 1979, 1983). However, the relationship of this highly repetitive nuclear component to heterochromatin and chromosome banding and to differential endomitotic heterochromatin replication has not yet been established.

The karyomorphology of *S. alba* was described by TSCHERMAK-WOESS & HASITSCHKA (1953) who have investigated meristematic cells and endopolyploid nuclei of differentiated tissues. They have shown that *S. alba* exhibits a nuclear structure

* Dedicated in gratitude to Prof. Dr ELISABETH TSCHERMAK-WOESS on the occasion of her 70th birthday.

classified as "Prochromosomenkern". In this type, the heterochromatin (usually centromere- and NOR-associated) forms dense chromocentres at interphase, whereas the euchromatic chromosome material is decondensed and hardly detectable. Endopolyploid nuclei have a cytological advantage over diploid nuclei: They exhibit, on an enlarged scale, a picture of the different chromatin fractions. Some of which may not be detectable in diploid nuclei because of their small total size (TSCHERMAK-WOESS 1963). In *S. alba* the occurrence of a minor heterochromatin fraction ("Körnchen") in 64-ploid nuclei, which was not discernable in diploid nuclei, was reported by TSCHERMAK-WOESS & HASITSCHKA (1953).

In order to link the cytological data with recent knowledge about the organization of highly repeated sequences in *S. alba,* we have decided to analyze the karyotype of *S. alba* by fluorochrome- and Giemsa-banding techniques. These observations should provide a basis for subsequent in situ hybridization experiments using satellite DNA clones and probes of ribosomal DNA, thus elucidating the reassessment of differential DNA replication in this species.

In the context of chromosome banding, we report on a novel air-drying method for producing spreads of plant mitotic chromosomes. Plant chromosome preparations are usually made by squashing previously softened or macerated tissue. Although this method has proved to be useful in a variety of applications for more than half a century, it is recognized, that the quality of chromosome squashes does not always meet the standard set by the air-drying technique which is widely used in mammalian and human cytogenetics. In this report we describe an air-drying technique which is simple, reliable, and particularly useful when banding techniques are to be applied. A similar method reported by AMBROS & al. (1986) has been demonstrated to be indispensable for molecular cytogenetics such as high resolution in situ hybridization with nonradioactive probe labelling and immunocytochemical detection methods.

Materials and methods

Seeds of *Sinapis alba* L. (*Brassicaceae, Cruciferae*) were kindly provided by Prof. INGRID CAPESIUS, University of Heidelberg. The seed sample was the same as that previously employed for DNA isolation (CAPESIUS 1979, 1983). Material of the other species used in preliminary test of the spreading method (see below) was obtained from the Botanical Garden of the University of Vienna, except for *Spirodela polyrrhiza* (L.) SCHLEID. clone 7110, which was kindly supplied by Prof. E. LANDOLT, ETH, Zürich.

Seedlings were grown under semi-sterile conditions on wet filter paper in Petri dishes. For chromosome preparations by the air-drying method, excised roots were pretreated with either cholchicine (0.05%, aqueous solution, applied at room temperature for 2 – 4 h) or 8-hydroxyquinoline (0.5%, aqueous solution, applied at 16 – 18 °C for 4 – 5 h). The roots were fixed in a freshly prepared mixture of absolute ethanol/glacial acetic acid (3 : 1) or methanol/glacial acetic acid (3 : 1). The meristems were fixed for a minimum of one hour and then stored in the fixative in the deep-freezer until required.

Buffer solution for chromosome spreading: 0.01 M citric acid-sodium citrate buffer pH 4.5 – 4.8 prepared from a ten times concentrated stock solution consisting of three parts of 0.1 M tri-sodium citrate and two parts of 0.1 M citric acid.

Enzyme solution. A mixture of pectinase, 20 – 40% (v/v), (Sigma, from *Aspergillus niger*, P-5146, obtained as glycerol-containing stock solution) plus cellulase, 2 – 4% (w/v), (Calbiochem 21947, or Onozuka R-10) is made up with the above 0.01 M citric acid-sodium

citrate buffer. The enzyme solution should be stored deep-frozen. It can be reused repeatedly, i.e. for up to ten and more digestions following purification by brief centrifugation.

Air drying method. In the following preparation procedure, the cells are spun down at about 4000 rpm in small conical centrifuge tubes (10 ml volume) at each step to change the solutions. The supernatant fluid is carefully removed with a Pasteur pipette and the pellet is resuspended in approximately 5 ml liquid.

(1) Transfer fixed plant material in buffer, dissect and collect meristematic tissues. – (2) Rinse twice in buffer. – (3) Soften the tissue in enzyme solution at 37 °C for 1 – 2 h (the optimal condition varies with different plants and should be tested empirically). Large particles can be carefully dispersed by sucking the suspension through a Pasteur pipette. – (4) Wash twice in buffer and finally suspend the pellet in a drop of buffer to prevent sticking together of protoplasts. – (5) Add an excess of freshly prepared fixative and change it twice. – (6) Finally suspend the pellet in a small amount of fixative and drop this suspension onto ice-cold tilted slides which are then air dried. – (7) After air-drying the slides should be aged overnight or longer before further processing.

Comments on the air-drying method. The above chromosome preparation technique is simple and reliable. Only a few stages are critical, which means that many steps of the procedure can be modified without introducing significant differences. The critical points are: the choice of the pectinase (some products may partially destroy the chromosomes) and the duration of the enzymatic digestion, which varies with different plants and tissues. A slightly increased treatment time is indicated with the prolonged storage of the fixed plant tissue. To keep the maceriation time to a minimum, the meristematic tissue can be dispersed mechanically with needles or by sucking the partially digested suspension repeatedly through a Pasteur pipette. Care should be taken when handling extensively digested material which consists mainly of a protoplast suspension, because losses due to shearing are likely.

Apart from *Sinapis alba*, other plants (e.g. *Vicia faba* L., *Pisum sativum* L., *Crepis capillaris* L., *Calla palustris* L., and *Spirodela polyrrhiza* (L.) SCHLEID, which exhibit great differences with respect to chromosome size and number, were used to test the utility of the air drying technique. In all cases excellent spreads of mitotic chromosomes were obtained (Fig. 1).

Chromosome banding methods. The following staining techniques have been applied with good results after preparation by air drying:

Giemsa C-banding as described by SCHWARZACHER & al. (1980), fluorochrome staining and counterstaining, including the tri-staining technique with chromomycin A_3/distamycin/DAPI, and staining with the DAPI/actinomycin D (SCHWEIZER 1981), fluorescent staining with the drug D 287/170 (SCHNEDL & al. 1981), Ag-NOR-staining according to the improved procedure of KODAMA & al. (1980).

Results and discussion

The air-drying method for plant chromosome spreads. The air-drying method used for making chromosome preparations of *S. alba* (and tested with other plant species, see Fig. 1) has several advantages over conventional squashes. Chromosomes are usually well spread and there is no exaggerated stretching or distortion. The cytoplasm is well dispersed which implies that the chromosomes are usually directly accessible to nuclear dyes. Also with certain applications it may be crucial that air-dried chromosomes have never been exposed to diluted acetic acid. With fluorochrome- and Giemsa-banding techniques the results using air-dried chromosome spreads were superior to those obtained by conventional squashes, most likely

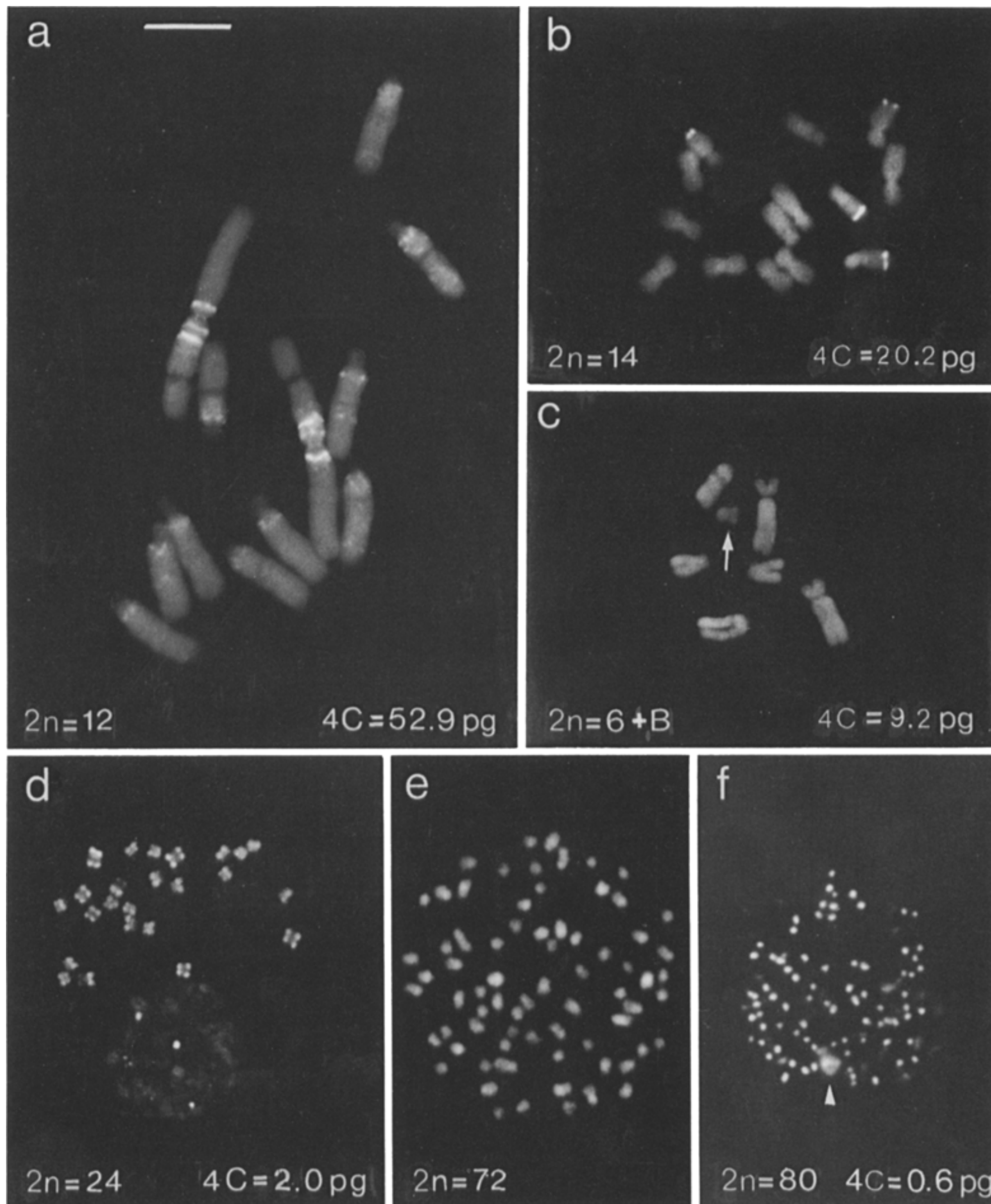

Fig. 1. Examples of air dried mitotic metaphase chromosome spreads stained with DAPI/actinomycin D (*a–d*) or with the dye D 287/170 (*e,f*). Bar = 10 μm. *a Vicia faba*, and *b Pisum sativum*; note in both species the occurrence of DAPI/actinomycin D-positive C-bands; *c Crepis capillaris* complement with one Iso-B-chromosome (arrow); *d Sinapis alba; e Calla palustris; f Spirodela polyrrhiza* (arrowhead points to a weakly stained chloroplast which was artificially transposed by the spreading process). Diploid chromosome numbers and nuclear DNA contents (where known) are indicated. Average 4 C-values were taken from BENNETT & SMITH (1976) and BENNETT & al. (1982) except for *S. polyrrhiza* (G. GEBER, unpubl.)

because it produces chromosomes almost free from overlying cell wall material and cytoplasmatic components. AMBROS & al. (1986) have demonstrated that the preparation of plant chromosomes by spreading and air-drying is particularly useful in conjunction with special immunocytochemical techniques such as in situ hybridization by biotin-streptavidin/peroxidase-DAB. This method will probably also be valuable in conventional in situ hybridization experiments using ^{3}H-labelled probes. Better resolution of the autoradiographic image is expected because the chromosomes in air-dried spreads are somewhat larger than in Feulgen-squashes, and because of a better contact between the photographic emulsion and the chromosomes and nuclei.

There have been repeated attempts to utilize the preparation technique of mammalian chromosomes for plant material. Mammalian chromosomes methods essentially include a short-term tissue or cell culture, a colcemid pretreatment, the hypotonic solution pretreatment followed by fixation and spreading of chromosomes by air-drying. MURATA (1983) reported a similar procedure for the preparation of plant chromosomes using protoplasts derived from plant cell or callus culture. The method has limitations because the production of viable protoplasts from meristematic tissues is technically difficult, and, moreover, single cell cultures are available only from a limited number of species. In our method and also in the related methods by HIZUME & al. (1980) and AMBROS & al. (1986), the meristematic tissue is enzymatically macerated subsequent to the fixation and then converted into a single cell suspension. This means that well spread plant metaphase chromosome preparations can be obtained in the absence of any hypotonic treatment, which is the "conditio sine qua non" for good mammalian metaphase spreads (HSU 1952). The contrast between animal and plant cells in their requirement for hypotonic treatment may possibly be due to inherent differences in cellular osmotic pressures.

Chromosome banding of *Sinapis alba*. The karyotype of *S. alba* with 2n = 24 is shown in Fig. 3. Conventional chromosome staining by the Feulgen-reaction or by aceto-carmin staining confers little differentiation along the chromosome arms, so that karyotyping has to depend on chromosome size and centromere position. The location of the centromere is in most chromosomes similar (submedian) and therefore it is very difficult to recognize the homologous chromosomes or to distinguish chromosomal groups. The 1 C nuclear DNA content of *S. alba* (2n = 24) is 0.5 pg (BENNETT & al. 1982), which means that the average chromosome size is comparable to that of *Arabidopsis thaliana* (1 C = 0.2 pg; 2n = 10) (SCHWEIZER & al. 1987).

Heterochromatin staining was done by the C-banding technique of SCHWARZACHER & al. (1980). All chromosomes of *S. alba* have centromeric/paracentromeric C-band material and at least two larger chromosome pairs are terminally banded in one arm (Fig. 2 *d*). Some of the large chromosomes in *S. alba* have very prominent blocks of C-heterochromatin showing that differences in the total amount of heterochromatin do occur between chromosomes. As it was difficult to identify homologous chromosomes after C-banding, it was not possible to decide if there occur C-band polymorphisms between homologous chromosomes. The relative proportion of C-banded metaphase chromosome length (total haploid karyotype length is set 100%) varied between 36 and 53% depending on the contraction of the metaphase chromosomes. The average amount of C-band material (44%) is prob-

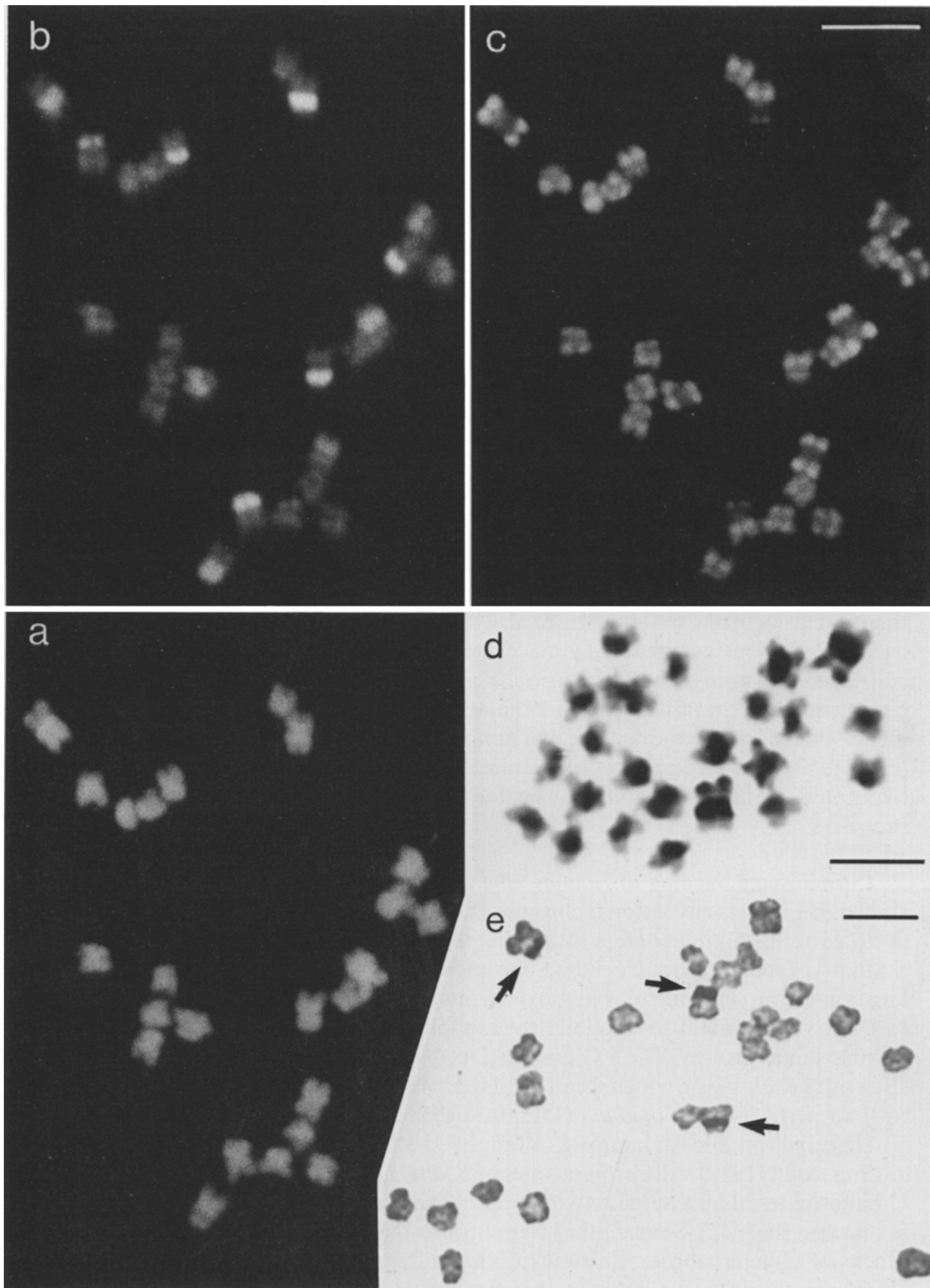

Fig. 2. Air-dried chromosome spreads of *Sinapis alba* (2 n = 24) after fluorochrome banding (*a*–*c*), *d* Giemsa C-banding and *e* Ag-NOR staining (active NORs marked by arrows). Bars = 5 µm. In *a*–*c* the same metaphase spread was first stained with chromomycin/distamycin/DAPI to show the DA-DAPI (*a*) and the chromomycin (*b*) fluorescence patterns. The preparation was then destained and restained with DAPI/actinomycin D (*c*)

Fig. 3. The same *S. alba* metaphase chromosomes as shown in Fig. 2 *a*–*c* arranged in order of decreasing length. Only the largest chromosome pair can be safely identified. *a* DA-DAPI fluorescence; *b* chromomycin fluorescent pattern; *c* DAPI/actinomycin fluorescence

able an overestimation because C-bands tend to swell by the Giemsa C-band staining procedure, whereas euchromatic chromosome regions tend to lose chromatin and therefore appear starved.

It has been demonstrated that certain types of heterochromatic C-bands can be distinguished by fluorochrome staining on the basis of overall base-composition differences (SCHWEIZER 1981). We have therefore applied the tristaining method with chromomycin A_3, distamycin A, and DAPI, to air-dried chromosome spreads of *S. alba*. After fluorescent microscopical examination and photography of the so-called DA-DAPI fluorescent banding pattern and of counterstain-enhanced chromomycin (CMA) fluorescence pattern, the preparations were destained and restained by DAPI/actinomycin D (AMD). Figures 2–4 show in *a, b,* and *c*, the three staining patterns observed. Counterstain enhanced chromomycin A_3 fluorescence (Figs. 2 *b*, 3 *b*, and 4 *b*) is considered to be indicative for GC-rich heterochromatin (see SCHWEIZER 1981). Bright DAPI fluorescence, which is not quenched by actinomycin D-counterstaining, is indicative for high AT-richness of satellite DNA and heterochromatin respectively (Figs. 1 *d*, 2 *a*, 3 *a*, and 4 *a*). DAPI/distamycin-positive bands (DA-DAPI bands) are considered to harbour moderately AT-rich satellite DNAs (see also MOYZIS & al. 1987).

A striking karyotype differentiation was obtained in *S. alba* by chromomycin A_3 counterstain-enhanced fluorescent staining (Figs. 2 *b* and 3 *b*). At least 12 chro-

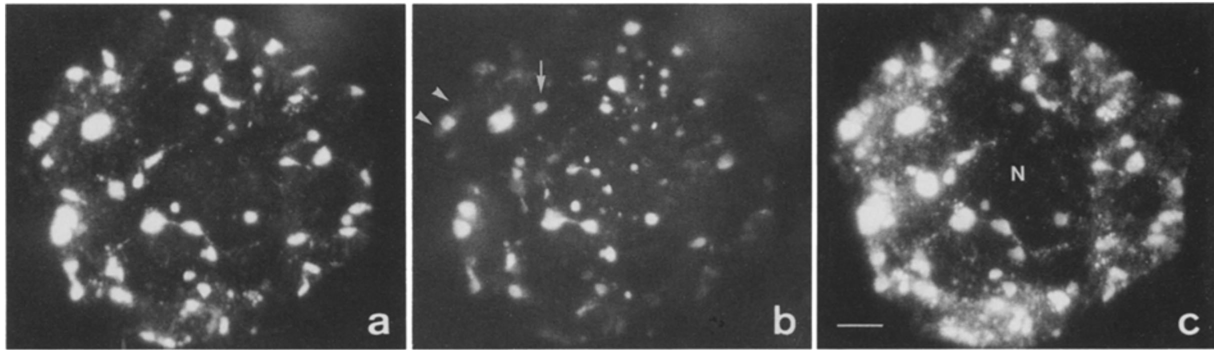

Fig. 4. *S. alba*, endopolyploid nucleus from root tip after fluorochrome staining. *a* DAPI fluorescence; *b* chromomycin fluorescence; *c* DAPI/actinomycin fluorescence. Sequential fluorescent staining allows the distinction of types of endochromocentres (some examples marked). Arrow points to an endochromocentre that is chromomycin$^+$/DAPI$^-$; arrowheads point to endochromocentres that are chromomycin$^-$/DAPI$^+$. There also occur chromocentres that are positively stained with both dyes. N Nucleolus. Bar = 5 μm

mosomes have a chromomycin-positive chromosome region; in four pairs this band type is particularly clearly defined. Thus, almost half of the C-band material (about 20% of total chromatin) has to be considered as relatively GC-rich. It is most likely, that some but not all of the chromomycin-positive regions harbour ribosomal DNA. Only one to three active nucleolus organizer regions were detected by the silver histochemical staining method (Ag-NOR-staining) (Fig. 2 *e*). The difference in number of active NORs probably reflects genetic differences between samples rather than methodological variations. The silver histochemical method for active NORs was highly reproducible when air-dried plant chromosome spreads were used (see also HIZUME & al. 1980, KODAMA & al. 1980).

The fluorescent heterochromatin staining patterns of *S. alba* chromosomes obtained by DAPI/actinomycin D is in general the reverse of the chromomycin pattern. However, certain chromosome regions may exhibit medium chromomycin and bright DAPI fluorescence. Probably the telomeric C-bands (Fig. 2 *d*) belong to this latter category. From the DAPI/actinomycin staining pattern of mitotic interphase nuclei and metaphase chromosomes it can be concluded that *S. alba* has also little amounts of AT-rich heterochromatin. Distamycin/DAPI staining produces a rather uniform fluorescence within and between *S. alba* metaphase chromosomes (Figs. 2 *a* and 3 *a*). A comparison of the fluorescent staining pattern and of the C-band pattern reveals that there are C-bands (probably on all chromosomes) which are not highlighted by fluorochromes. These regions do harbour satellite DNAs which are devoid of base-pair clusters of predominantly one type. On the basis of the mitotic metaphase fluorescent staining behaviour two major types of C-bands and a minor fraction (C) can be distinguished in *S. alba:* Type A: CMA$^+$, AMD/DAPI$^-$; B: CMA$^-$, AMD/DAPI$^-$; C: CMA$^-$, AMD/DAPI$^+$.

Endopolyploid nuclei (Fig. 4) exhibit a staining response by the three fluorochrome methods which only partly parallels the pattern recorded on mitotic chromosomes. In interphase nuclei the base-composition-dependent response of dye fluorescence is overrided to some extent by chromatin packaging differences. There are chromocentres which are chromomycin bright and DAPI pale; other chromo-

centres may be bright with both chromomycin and the two DAPI methods. A third group of chromocentres exhibits pale chromomycin fluorescence (despite high chromatin density) and bright DAPI fluorescence.

Fluorescent staining of endopolyploid nuclei (Fig. 4) confirms an earlier observation of TSCHERMAK-WOESS & HASITSCHKA (1953) that, apart from the very compact large endochromocentres, there also occur numerous smaller dot-like chromocentres which (because they apparently have no tendency to fuse) are interpreted as representing a different type of heterochromatin.

Heterochromatin and satellite DNA from *S. alba*. CAPESIUS (1979) analyzed the DNA from *S. alba* seedlings by neutral CsCl and also by Ag^+/Cs_2SO_4 density gradient centrifugation. In CsCl density gradient centrifugation a heavy shoulder is found at a density of 1.703 g/cm^3. This satellite DNA component is seen as a "light" band upon Ag^+/Cs_2SO_4 gradient centrifugation. Filter hybridization of the satellite with 18 and 25 S rDNA showed that this 1.703 g/cm^3 satellite fraction is enriched in ribosomal RNA cistrons. The proportion of this satellite DNA component of total nuclear DNA is about 10 – 15%, as estimated from the published ultracentrifugation profiles (CAPESIUS 1979). We conclude, on the basis of fluorochrome studies, that this 1.703 g/cm^3 satellite is localized in the chromomycin-bright regions (Figs. 2 *b* and 3 *b*) though the proportion of this component estimated by the banding method was somewhat higher (20%). It is not clear, which of the chromomycin-positive bands are "ribosomal bands". We have seen a maximum of 3 active NORs per diploid metaphase nucleus. The actual number of rDNA loci and their relation to chromomycin bands has yet to be determined by in situ hybridization in conjunction with banding methods.

In the *S. alba* genome another satellite DNA component occurs, which is not resolvable by neutral CsCl ultracentrifugation. This fraction, however, can be resolved upon Ag^+/Cs_2SO_4 gradient centrifugation as a "heavy" satellite amounting approx. 12% of the total nuclear DNA. The bouyant density of this satellite in neutral CsCl equals that of main band DNA, and therefore, it was named the "cryptic satellite". This cryptic satellite component of *S. alba* was analyzed in some detail by restriction enzyme mapping and sequencing (CAPESIUS 1983). It is a tandemly organized highly repeated fraction with a 172 bp basic repeat unit, which occurs in at least two structural variants. Recent detailed sequence comparisons have shown, that this satellite is related to the "alphoid-like" satellites as described for animals (GRELLET & al. 1986). It is likely, that it is localized in the centric C-bands which are not highlighted by chromomycin staining.

Using DNA clones of either satellite DNA type in combination with nonradioactive in situ hybridization methods it should be possible to further analyze the relationship between heterochromatic bands, NORs and highly repetitive DNAs.

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