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Stable chromosome fission associated with rDNA mobility

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A spontaneous chromosome fission in the plant *Hypochoeris radicata* has been characterized by Feulgen staining, *in situ* hybridization of the rDNA probe pTA71 and silver staining for active nucleolus organizing regions. The parental acrocentric chromosome has no detectable ribosomal genes at the centromere, but both fission derivatives possess active NORs at their centric ends. In fission heterozygotes, pachytene configurations studied by synaptonemal complex spreading show that the ribosomal cistrons form short arms on each telocentric which pair together to form a triradial. The paired short arms are associated with the single nucleolus at pachytene. It is proposed that the origin and stabilization of the fission rearrangement involved transposition of rDNA from the nucleolus organizing region of chromosome 3 into the centromeric region of chromosome 1.

Key words: chromosome fission, NOR, NOR movement, rDNA

Introduction

The occurrence of Robertsonian (Rb) rearrangements, *i.e.* centric fusion or fission, is well documented in a wide range of organisms. However, possible mechanisms that give rise to such rearrangements have been the subject of much debate. According to classical cytogenetic theories, a stable chromosome must have one centromere and two telomeres. Thus Rb rearrangements must result from a two-break reciprocal translocation, fusion involving the loss of a small centric fragment and fission requiring a donor chromosome to provide a centromere and telomeres. Holmquist & Dancis (1979) suggest, however, that reversible fusion and fission is possible without the loss or gain of material if telocentric chromosomes have centromeres which overlap telomere sequences. Fusion of telocentrics thus brings the two centromeres together to act as one and produces latent telomere sequences within the region. Fission then can only take place if the ancestral chromosome has such latent telomere sequences within the centromere region.

Reports of Rb rearrangements, especially fusion, are common in animals, in which they are thought to have played an important part in karyotypic evolution (White 1978). The involvement of Rb rearrangement in plant karyotypic evolution is less usual but has been discussed by Jones (1978). Isolated cases of centric fission in plants have been reported in *Nigella doerfleri* (Strid 1968), *Crocus minimus* (Brighton 1978), *Tradescantia andreuxii* (Jones 1978) and *Vicia faba* (Schubert & Rieger 1990).

This paper reports the characterization of a centric fission in the plant *Hypochoeris radicata* (Compositae). This chromosome rearrangement arose spontaneously during the somatic development of one individual of an experimental population (Parker *et al.* 1982) and both products of the fission are mitotically and meiotically stable.

In the present study the involvement of a nucleolus organizing region (NOR) is implicated in the spontaneous centric fission rearrangement. NORs, generally observed as secondary constrictions along the length of Feulgen-stained chromosomes, are the location of genes coding for ribosomal RNAs (rRNAs) other than the 5S component (Gall & Pardue 1969). The genes are present as tandem repeats and the copy number can vary enormously between species (Long & Dawid 1980) and between individuals within species. The chromosomal location of ribosomal genes (rDNA) can be determined by *in situ* hybridization and their activity can be established by silver staining. Both these techniques have been applied to the fission chromosomes of *H. radicata*, demonstrating that the fission has been accompanied by transfer of active ribosomal cistrons to the split centromere.

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Materials and methods

Chromosome preparations

Young root tips were pretreated with 0.05% colchicine for 2 h, fixed in 3:1 ethanol–acetic acid at room temperature and stored at −20°C.

For standard staining root tips were hydrolysed in 1 M HCl at 60°C, stained with Feulgen and squashed in 2% lactopropionic orcein.

For *in situ* hybridization and silver staining root tips were rehydrated in 0.01 M citrate buffer, digested in 1% cellulase (Sigma), 2% pectinase (Sigma) for 30 min at 37°C and squashed in 45% acetic acid.

In situ hybridization

In situ hybridization of the wheat ribosomal probe pTA71 (Gerlack & Bedbrook 1979) was carried out according to the method of Clark *et al.* (1989). Briefly, the probe was labelled with biotin using a nick translation kit (BRL). Slides were pretreated with RNase (200 μg/ml in 2 × SSC, 1% bovine serum albumin) and chromosomal DNA was denatured by immersing the slides in 70% deionized formamide in 2 × SSC at 60°C for 90 s. After ethanol dehydration, slides were hybridized with the denatured probe cocktail [25 μl of labelled probe (40 μg/ml), 50 μl of deionized formamide, 5 μl of carrier (salmon sperm DNA 10 mg/ml), 20 μl of 50% dextran sulphate] for 4 h at 37°C. Following washing to remove unbound probe, sites of hybridization were detected using the Vectastain *Elite* ABC kit (Vector Lab). Slides were counterstained with Leishman's stain and mounted in DPX mounting medium (R.A. Lamb).

Silver nitrate staining

Silver nitrate staining for active nucleolus organizing regions (NORs) was achieved using a modified method of Lacadena *et al.* (1984). Briefly, slides were immersed in 2 × SSC at 60°C for 30 s then rinsed in sodium citrate solution (0.04 g/l trisodium citrate adjusted to pH 3 with formic acid). Staining was carried out in a moisture chamber at 60°C using a freshly prepared solution at 50% silver nitrate in sodium citrate solution. Each slide was covered with nylon mesh and stained for 8–15 min. After rinsing thoroughly with distilled water and air drying, slides were mounted in DPX.

Synaptonemal complex preparations

Synaptonemal complexes were prepared by a modified method of Albini & Jones (1984) as follows: pollen mother cells (PMCs) were released from five unfixed, prophase I florets into 45 μl of ice-cold digestion medium (0.4% cytohelicase; IBF Biotechnics). After 6 min digestion and removal of large fragments of anther wall, the cell suspension was transferred onto a 60-μl drop of 2% Lipsol/0.01% sodium dodecyl sulphate (SDS) on a plastic-coated slide (1% cellulose triacetate). After 6 min 120 μl of fixative (4% paraformaldehyde, 1.5% sucrose, pH 8.5) was added and the suspension was spread over the slide using a mounted needle. The preparations were air dried for at least 12 h. Before staining, ridges of deposited sucrose were removed from the slides by rinsing gently in distilled water. Slides were stained with 50% silver nitrate solution under nylon mesh for 30 min in a moisture chamber at 60°C. Suitably spread nuclei were located by light microscopy, transferred to EM grids and examined with a JEM 1200EXII electron microscope. Measurements were made from photographic prints using a Summagraphics digitizing tablet linked to a BBC microcomputer.

Results

Wild-type *Hypochoeris radicata* (2*n* = 8)

H. radicata has three pairs of acrocentric chromosomes (1–3) with arm ratios of about 1:2 and one smaller submetacentric pair (4). Pairs 1 and 2 are morphologically indistinguishable. There is only one secondary constriction which is located subterminally on the short arm of pair 3 (Figure 1a).

In situ hybridization with the ribosomal gene probe pTA71 (Gerlach & Bedbrook 1979), revealed two sites of hybridization per interphase nucleus. In metaphase preparations the two signals were located subterminally on the short arms of an acrocentric pair, presumably the NO chromosomes, pair 3 (Figure 1b).

Silver staining detected two active NORs in the complement, again located at the standard NO position, subterminally on an acrocentric pair (Figure 1c). In interphase cells either a single large nucleolus or two similar-sized, smaller nucleoli were observed.

Fission heterozygotes (2*n* = 9)

In the chromosome complement of fission heterozygotes one of the acrocentric chromosomes, designated chromosome 1, is replaced by its fission products, chromosome 1^S and chromosome 1^L (Parker *et al.* 1982). Secondary constrictions are visible on the standard NO chromosome, pair 3. In these plants the procentric ends of the fission chromosomes 1^S and 1^L also carry satellite structures which are morphologically identical to the NORs of the standard pair 3 (Figure 2a)

After *in situ* hybridization with the rDNA probe pTA71, four signals were seen in each interphase nucleus. Two of the signals were noticeably smaller than the other two (Figure 2b). In metaphase preparations two signals were located, as in the wild-type, on the short arm of an acrocentric pair. The remaining two signals, usually smaller in size, were located at the centric ends of chromosomes 1^S and 1^L (Figure 2c). Although the *in situ* detection technique is not quantitative, differences in signal size can be interpreted as differences in ribosomal gene copy number (Maluszynska & Schweizer 1989). This would indicate that the centric regions of the fission chromosomes 1^S and 1^L

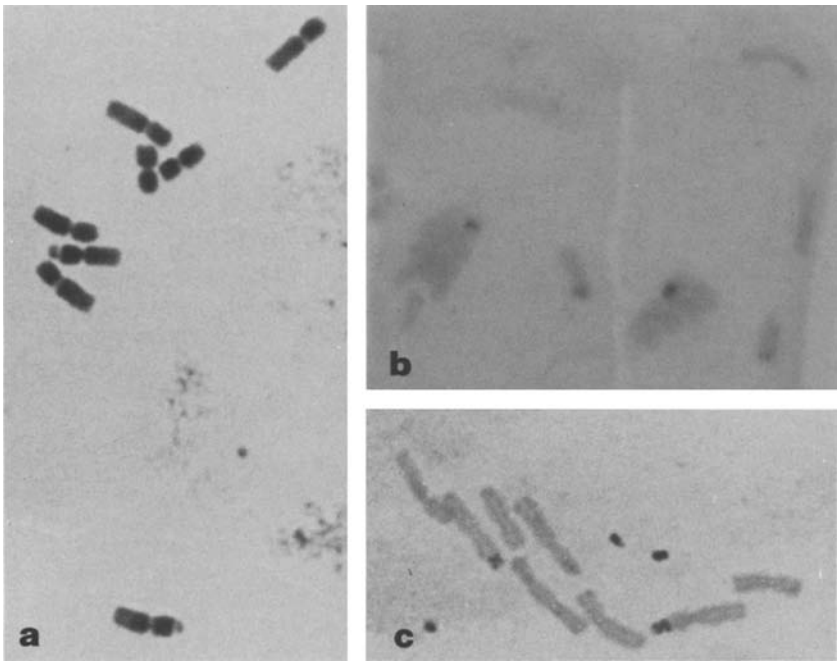

Figure 1. Metaphase chromosomes of wild-type *Hypochoeris radicata* ($2n = 8$). **a** Feulgen-stained chromosome complement (×2500). **b** *In situ* hybridization of the ribosomal gene probe, pTA71, showing two signals per cell (×2000). **c** Silver-stained complement showing two active NORs per cell (× 2500).

each bear considerably fewer rDNA copies than the standard chromosome 3.

Silver staining of metaphase chromosomes showed that all four rDNA sites in the complement are active in nucleolus formation (Figure 2d). Most interphase cells (91%) possessed either a single, large nucleolus or two similar-sized, smaller nucleoli as in the wild-type individuals. The remaining cells, however, contained one or two small micronucleoli in addition to the standard one or two major nucleoli (Figure 2e). If NOR activity is a direct reflection of ribosomal gene number, then the two micronucleoli probably correspond to the smaller sites of rDNA hybridization located procentrically on chromosomes 1^S and 1^L.

At pachytene in surface-spread synaptonemal complex preparations the fission chromosomes 1^S and 1^L can be seen to pair with their homologous regions of the standard chromosome 1, so forming a trivalent. This was present in most cells as a triradial configuration. Both chromosome 1^S and 1^L have small short arms, and these usually pair with each other (Figure 2f). The relative size of these short arms an be expressed in terms of centromere indices (0.050 for 1^S; 0.024 for 1^L). These values were calculated using the position of visible centromere structures present in some nuclei. The rDNA activity of chromosomes 1^S and 1^L were also reflected in these meiotic preparations by the association of the short arm of the triradial with the nucleolus (Figure 2g).

Fission homozygotes ($2n = 10$)

In the chromosome complement of fission homozygotes both members of the chromosome 1 pair are replaced by their fission products. In these plants secondary constrictions are visible on the standard NO chromosome pair and also procentrically on all four fission chromosomes (Figure 3a).

In interphase nuclei a total of six rDNA hybridization sites were detected. Observation of metaphase cells showed the location of these signals to be on the short arm of an acrocentric pair and at the centric ends of all four fission chromosomes (Figure 3b). The signals located on the fission chromosomes were again noticeably smaller than those on the acrocentric pair, suggesting a lower copy number for the ribosomal cistrons on chromosome 1^S and 1^L.

Silver staining showed that all six rDNA sites in the fission homozygote complement are active in nucleolus formation (Figure 3c). Again most interphase cells (75%) contained a single, large nucleolus or two similar-sized nucleoli. However, the remaining 25% of interphase cells carried four micronucleoli in addition to major nucleoli.

Discussion

By the use of Feulgen staining, *in situ* hybridization and silver staining it has been shown that, in all karyomorphis of *Hypochoeris radicata*, (i) the number and location of rDNA sites corresponds exactly to the number and location of secondary constrictions and (ii) that all of these sites are active in nucleolus formation. Thus active NORs exist at the subtelomeric region of the short arm of chromosome 3 and near the centromeres of chromosomes 1^S and 1^L when they are present in the complement. The centric fission of chromosome 1 was originally detected in some of the flowering shoots of a single plant bearing wild-type

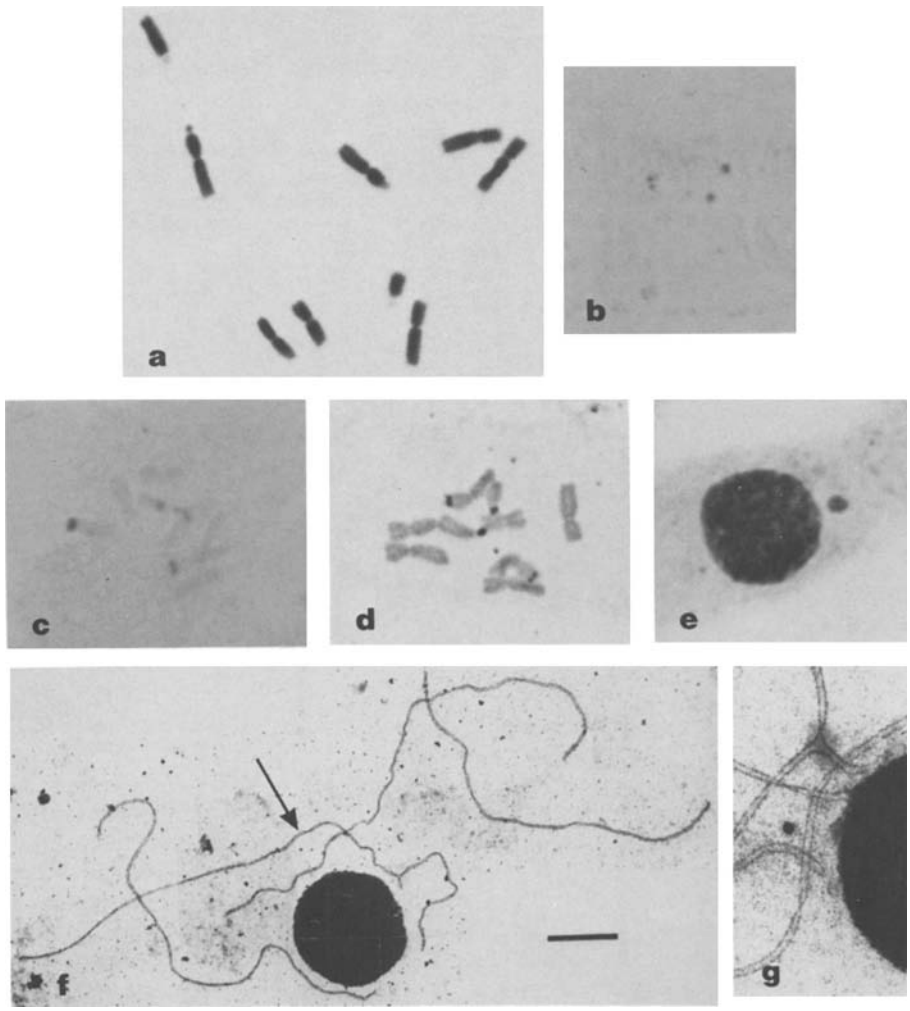

Figure 2. The fission heterozygote ($2n$ = 9). **a** Feulgen-stained chromosome complement (×2500). **b** Interphase nucleus after *in situ* hybridization with the ribosomal probe, pTA71. Note that two of the four signals are smaller (×2000). **c** Metaphase chromosomes after *in situ* hybridization with the ribosomal probe, pTA71. Signals are present on an acrocentric pair and also procentrically on both fission chromosomes (×2000). **d** Silver staining for active NORs showing four sites per cell (×2500). **e** Silver-stained interphase cell showing a micronucleolus in addition to the major nucleolus (×2500). **f** Electron micrograph of a pachytene nucleus. The trivalent is arrowed. Bar = 5 μm. **g** Electron micrograph showing the small arms of the fission chromosomes in association with the nucleolus (×8500).

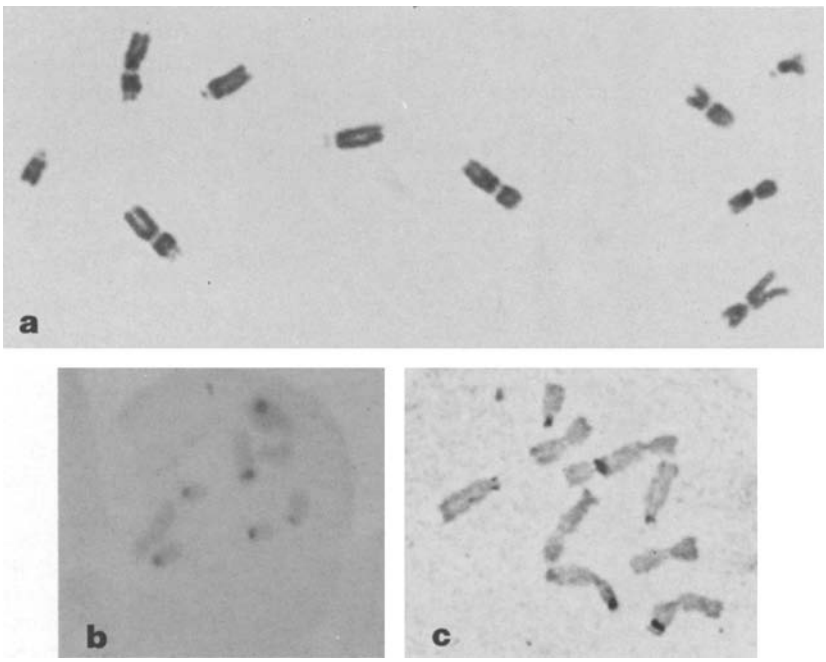

Figure 3. Metaphase chromosomes of the fission homozygote ($2n$ = 10). **a** Feulgen-stained chromosome complement (×2500). **b** *In situ* hybridization of the ribosomal probe, pTA71 showing six signals per cell. These are located on an acrocentric pair and on all four fission chromosomes (×2000). **c** Silver-stained complement showing NOR activity at all six rDNA sites (×2500).

roots (Parker *et al.* 1982). The present study has shown that rDNA is present immediately distal to the centromere of both fission chromosomes 1^S and 1^L, yet no detectable *in situ* hybridization signal exists at the centromere region of the standard chromosome 1. The question then arises as to how the rearrangement occurred. There are a number of possibilities:

(1) rDNA was transposed from the NOR of chromosome 3, into the centromeric region of chromosome 1. This insertional translocation perhaps caused instability of the centric region and was succeeded by a fission event.
(2) Centric fission of chromosome 1 was followed by translocation of rDNA to both 1^S and 1^L from the NOR of chromosome 3.
(3) A latent rDNA site within the centromeric region of chromosome 1 underwent amplification and this generated instability.

In a number of reported cases NOR location varies between individuals without any other gross karyotypic changes. In *Allium cepa*, for example, Sato (1981) reported variation in NOR number and position, and a similar pattern of variation was also found in *A. cepa*, *A. fistulosum* and their hybrids (Schubert *et al.* 1983, Schubert 1984, Schubert & Wobus 1985). Supernumerary NORs, all subterminal in position, have been found in *A. flavum* (Loidl & Greilhuber 1983) and in some natural populations of *A. schoenoprasum* (Bougourd & Parker 1976). NOR variability is also common in amphibians. In a detailed study of the treefrogs, *Hyla chrysoscelis* and *H. versicolor*, Wiley *et al.* (1989) found variable rDNA sites. They concluded that the genes appeared to have been precisely excised or inserted into the chromosomes without disturbance of banding patterns, thus behaving like mobile genetic elements. In *Triturus vulgaris meridionalis* there exist rDNA sites which are additional to the standard NOR sites. These supernumerary locations vary between individuals and are inherited in a Mendelian fashion (Batsioni *et al.* 1978). Ribosomal DNA site polymorphism has also been reported in *Bufo terrestris* (Foote *et al.* 1991). NOR polymorphism could be due to rDNA translocation or transposition but, in amphibians, the possibility exists that additional rDNA sites may result from reinsertion errors during rDNA amplification in oocytes.

Subrepeating sequences from the ribosomal intergenic spacer region have been found at chromosomal locations other than rRNA gene sites in *Vicia faba* (Maggini *et al.* 1991). The abnormal sites are located at chromosome ends and in all heterochromatic regions. In relation to these reports, it is interesting to note the distribution of heterochromatin in wild-type *Hypochoeris radicata*, *i.e.* at the NOR of chromosome 3 and at the centromere of another acrocentric pair (1 or 2) (Parker *et al.* 1982).

In maize, at least 20 interchanges are known with a breakpoint in the NOR (Philips *et al.* 1972), while in humans the most common structural rearrangements are Robertsonian (Rb) translocations involving the NO chromosomes 13, 14, 15, 21 and 22. It has been proposed that close association of rDNA regions within the nucleoli could facilitate genetic exchange between non-homologues. Such exchanges could lead to homogenization of rDNA sequences (Arnheim *et al.* 1980, Krystal *et al.* 1981) or Rb translocation if the exchange occurred within an inverted segment (Stahl *et al.* 1983, Guichaoua *et al.* 1986). Characterization of Rb translocation breakpoints in humans (Cheung *et al.* 1990) has shown that they may occur within the rDNA region or proximal to the rDNA, within alphoid sequences, and may result in monocentrics or dicentrics. Choo *et al.* (1988) suggested the importance of alpha satellite sequences in Rb translocation since the proportion of such sequences carried by each NO chromosome correlates with their relative involvement in these rearrangements.

The structure and function of telomeres is well known (Zakian 1989). Their importance in chromosome stability lies in their ability to protect ends from degradation and facilitate end replication. Thus, for the products of a centric fission to be stable, the process must necessarily involve (i) the presence of telomeric sequences at the centromere region of the ancestral chromosome, (ii) donor material to provide telomere repeats or (iii) the centric ends to be 'healed' by the addition of telomere repeats *de novo* (Werner *et al.* 1992). The pericentric location of telomeric sequences has been demonstrated in a number of animal species (Meyne *et al.* 1990) and also in some individuals of *Vicia fiba*, where they may provide for centric fission (Schubert 1992).

With respect to the fission rearrangement in *Hypochoeris radicata* and the supply of donor material to provide telomeric sequences, it is interesting to note the findings of Maggini *et al.* (1991). These authors found that repeat sequences from the ribosomal intergenic spacer in *Vicia faba* also hybridized to chromosome ends. In view of this homology and the existence of telomere-like sequences in subtelomeric regions of other species, it is tempting to speculate upon the presence of telomere-like sequences in the ribosomal intergenic spacer. Interestingly, the presence of telomeric sequences scattered through the entire nucleolus organizing region has recently been recorded in the eels *Anguilla anguilla* and *A. rostrata* (Salvadori *et al.* 1995). If this were a general case, it could explain the capacity of rDNA to apparently 'cap' broken ends of human chromosomes (Zankl & Huwer 1978) and perhaps also to stabilize the fission chromosomes in *Hypochoeris radicata*.

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