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R. Hasterok · G. Jenkins · T. Langdon · R.N. Jones
J. Maluszynska

Ribosomal DNA is an effective marker of *Brassica* chromosomes

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Abstract Simultaneous fluorescence in situ hybridisation with 5S and 25S rDNA probes enables the discrimination of a substantial number of chromosomes of the complement of all diploid and tetraploid *Brassica* species of the "U-triangle", and provides new chromosomal landmarks for the identification of some chromosomes of this genus which were hitherto indistinguishable. Twelve out of 20 chromosomes can be easily identified in diploid *Brassica campestris* (AA genome), eight out of 16 in *Brassica nigra* (BB genome), and six out of 18 in *Brassica oleracea* (CC genome). Furthermore, just two rDNA markers permit 20 out of 36 chromosomes to be distinguished and assigned to either the A or B genomes of the allotetraploid *Brassica juncea*, and 18 out of 38 chromosomes identified and assigned to the A or C genomes of the allotetraploid *Brassica napus*. The number of chromosomes bearing rDNA sites in the tetraploids is not in all cases simply the sum of the numbers of sites in their diploid ancestors. This observation is discussed in terms of the phylogeny and variability within the genomes of the species of this group.

Keywords *Brassica* · Double-target FISH · 5S rDNA · 25S rDNA

Introduction

The genes for 45S rRNA and 5S rRNA are organised in tandem arrays at one or more loci in the eukaryotic genome. In some species, such as *Saccharomyces cerevisiae* and *Marchantia polymorpha*, the two classes of rDNA repeats are interspersed (Sone et al. 1999), whereas in the chromosomes of higher plants 45S and 5S rDNA are physically separate and map independently. However, in some species such as *Arabidopsis thaliana* (Murata et al. 1997), *Petunia hybrida* and *Crepis capillaris* (Montijn et al. 1999), *Phaseolus vulgaris* (Moscone et al. 1999) and *Oryza officinalis* (Shishido et al. 2000), these two classes of rRNA genes co-localise on the chromosomes bearing the nucleolar organising regions. The high copy number and tandem organisation of these genes together provide useful markers for chromosome identification and karyotyping in diverse plant genera such as *Trifolium* (Ansari et al. 1999) and *Aegilops* (Badaeva et al. 1996). Indeed, the simultaneous fluorescence in situ hybridisation (FISH) of 18S-5.8S-25S and 5S rDNA probes enabled the identification of all of the chromosomes of plant species such as *Hordeum vulgare* (Leitch and Heslop-Harrison 1993), *A. thaliana* (Murata et al. 1997; Fransz et al. 1998) and *Pinus elliottii* (Doudrick et al. 1995). This approach has also been put to good use in the Triticeae, where comparative mapping of the rRNA genes has revealed a complex evolutionary rearrangement of the chromosomes (Castilho and Heslop-Harrison 1995; Taketa et al. 1999).

The genus *Brassica* contains a number of species of outstanding agronomical importance and, as a consequence, has received considerable attention in the context of crop improvement by both conventional plant breeding and biotechnological programmes. These approaches often require reliable chromosome identification and karyotypical analysis in order, for example, to assay chromosome variation following in vitro culture and regeneration, to characterise substitution or addition lines, or simply to integrate genetic and physical maps. Unfortunately, the inordinately small chromosomes and the symmetry of the karyotype of *Brassica* species have in the past thwarted attempts at chromosome identification by conventional cytological means. Not surprisingly, therefore, these intractable species were subject to FISH analysis with robust probes able to tag particular chro-

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R. Hasterok (✉) · J. Maluszynska
Department of Plant Anatomy and Cytology, Silesian University, Jagiellonska 28, 40-032 Katowice, Poland
e-mail: hasterok@us.edu.pl
Tel: +48-(32)-2009-553, Fax: +48-(32)-255-58-73

R. Hasterok · G. Jenkins · T. Langdon · R.N. Jones
Institute of Biological Sciences, University of Wales, Aberystwyth, Ceredigion, SY23 3DD, UK

mosomes of their complements. In 1993, Maluszynska and Heslop-Harrison were the first to describe the number of 18S-5.8S-25S rDNA loci in *Brassica* diploids (*Brassica nigra, Brassica oleracea* and *Brassica campestris*) and allotetraploids (*Brassica carinata, Brassica juncea* and *Brassica napus*). They ascertained that all of the species had more than one pair of rDNA loci and that the allotetraploids had fewer rDNA loci than the sum of those of their diploid ancestors. Further investigations described more precisely the genomic distribution of rDNA sites on prometaphase and metaphase chromosomes (Snowdon et al. 1997; Fukui et al. 1998; Hasterok and Maluszynska 2000a,b), and later determined their transcriptional activity (Hasterok and Maluszynska 2000c). These investigations demonstrated the utility of 45S rDNA to identify some chromosomes of the complement and to facilitate construction of their karyotypes.

This paper extends the investigations described above in presenting a simple but robust method of identifying a substantial number of the chromosomes of all of the species of the classic "U-triangle" (U 1935) through the simultaneous use of 25S and 5S rDNA FISH probes. In addition, the comprehensive coverage of all the key species sheds new light upon the phylogenetic relationships and evolutionary pathways of this genus.

Material and methods

Plant material

Seeds of the following diploid and allotetraploid species were obtained from commercial sources: *B. nigra* var. occidentalis (genome BB; 2n=2*x*=16), *B. oleracea* var. capitata cv Kamienna Glowa (genome CC; 2n=2*x*=18), *B. campestris* var. rapifera cv Goldball (genome AA; 2n=2*x*=20), *B. carinata* cv s-67 (genome BBCC; 2n=4*x*=34), *B. juncea* cv Malopolska (genome AABB; 2n=4*x*=36), and *B. napus* cv Marita (genome AACC; 2n=4*x*=38).

Chromosome preparation

Seeds were germinated on filter paper moistened with tap water at 22.5°C in the dark for 2–3 days. Further treatment is according to Maluszynska and Heslop-Harrison (1993) with some modifications. Briefly, whole seedlings were treated with 2 mM 8-hydroxyquinoline for 1 h at room temperature, fixed in 3:1 methanol – glacial acetic acid and stored at –20°C until required. Fixed seedlings were washed in 0.01 M citric acid – sodium citrate buffer (pH 4.6–4.8) for 20 min and digested enzymatically in a mixture comprising 20% (v/v) pectinase (Sigma), 1% (w/v) cellulase (Calbiochem) and 1% (w/v) cellulase "Onozuka R-10" (Serva) for 100 min at 37°C. The root tips were squashed in a drop of 45% acetic acid, the coverslips were removed by freezing, the preparations were post-fixed in 3:1 ethanol:glacial acetic acid, followed by dehydration in absolute ethanol and air drying.

DNA probes

The 5S rDNA probe was amplified and labelled with rhodamine-4-dUTP (Amersham Pharmacia) from the wheat clone pTa794 (Gerlach and Dyer 1980), using PCR with universal M13 sequencing primers under the following conditions: 94°C×1 min, 35 cycles of 94°C×40 s, 55°C×40 s, 72°C×1 min, one cycle of 72°C× 5 min. The 25S rDNA probe was obtained by nick translation with digoxygenin-11-dUTP (Roche) of a 2.3 kb sub-clone of the 25S rDNA coding region of *A. thaliana* (Unfried and Gruendler 1990).

Double-target fluorescence in situ hybridisation

Methods of in situ hybridisation were adapted with some modifications from Schwarzacher and Heslop–Harrison (2000). In short, slides were pre-treated with DNase-free RNase (100 μg/ml) in 2×SSC for 1 h at 37°C, washed in 2×SSC for 5 min, post-fixed in 1% aqueous formaldehyde in PBS buffer for 10 min, washed in 2×SSC for 15 min, dehydrated in an ethanol dilution series, and air-dried. Probe DNA was mixed to a concentration of 100 ng per slide with 50% de-ionised formamide, 10% dextran sulphate, 2×SSC and 1% SDS. The probe DNA and substrates were denatured at 70–72°C for 5 min and allowed to hybridise overnight at 37°C in an Omnislide in situ hybridisation system (Hybaid). Slides were washed stringently for 10 min in 20% de-ionised formamide in 0.1×SSC at 42°C, followed by detection of digoxygenin by FITC-conjugated anti-digoxygenin antibodies. The chromosomes were mounted in Vectashield (Vector Laboratories) containing 2.5 μg/ml of 4´,6-diamidino-2-phenylindole (DAPI; Serva), and photographed onto Fuji Provia 400 ISO colour reversal film with an MC100 camera attached to a Zeiss Axioplan epifluorescence microscope. Images were scanned electronically and processed uniformly using Micrografx Picture Publisher software.

Results and discussion

Chromosome markers

Figure 1 shows the results of simultaneous FISH of 25S and 5S rDNA probes to the somatic metaphase chromosome complement of the three diploid and three allotetraploid species of *Brassica*. In each image, the rDNA signals are large and discrete, and typify those seen in a large number of replicate cells analysed in each species. Ribosomal DNA sites effectively mark eight different chromosomal types across the six species, of which six are unique to, and diagnostic for, the A, B or C genomes (Table 1). Chromosomal type I (Table 1) is the preserve of the A genome and bears the secondary constriction, and a large 25S locus closely linked to a smaller 5S site in its short arm. The nucleolar organising chromosome type VI of genomes B and C is similar, but does not possess a 5S site in the short arm, and its secondary constriction may be less distended as a result either of differences in rDNA activity or less susceptibility to squashing during chromosome preparation. It has been shown previously that only these two chromosomes bear transcriptionally active 45S rRNA genes (Hasterok and Maluszynska 2000c). Chromosome type II of the A genome is the only other chromosome to bear both 25S and 5S rDNA sites, which are in close apposition or co-localised in the long arm. Co-localisation of rRNA genes has been noted in the related species *Sinapis alba* and *Raphanus sativus* (Schrader et al. 2000), which raises the question as to whether the juxtaposition of these sites by chromosome rearrangement is simply fortuitous, or is highly conserved and has functional significance. In prokaryotes and some eukaryotes, such as *S. cerevisiae* and

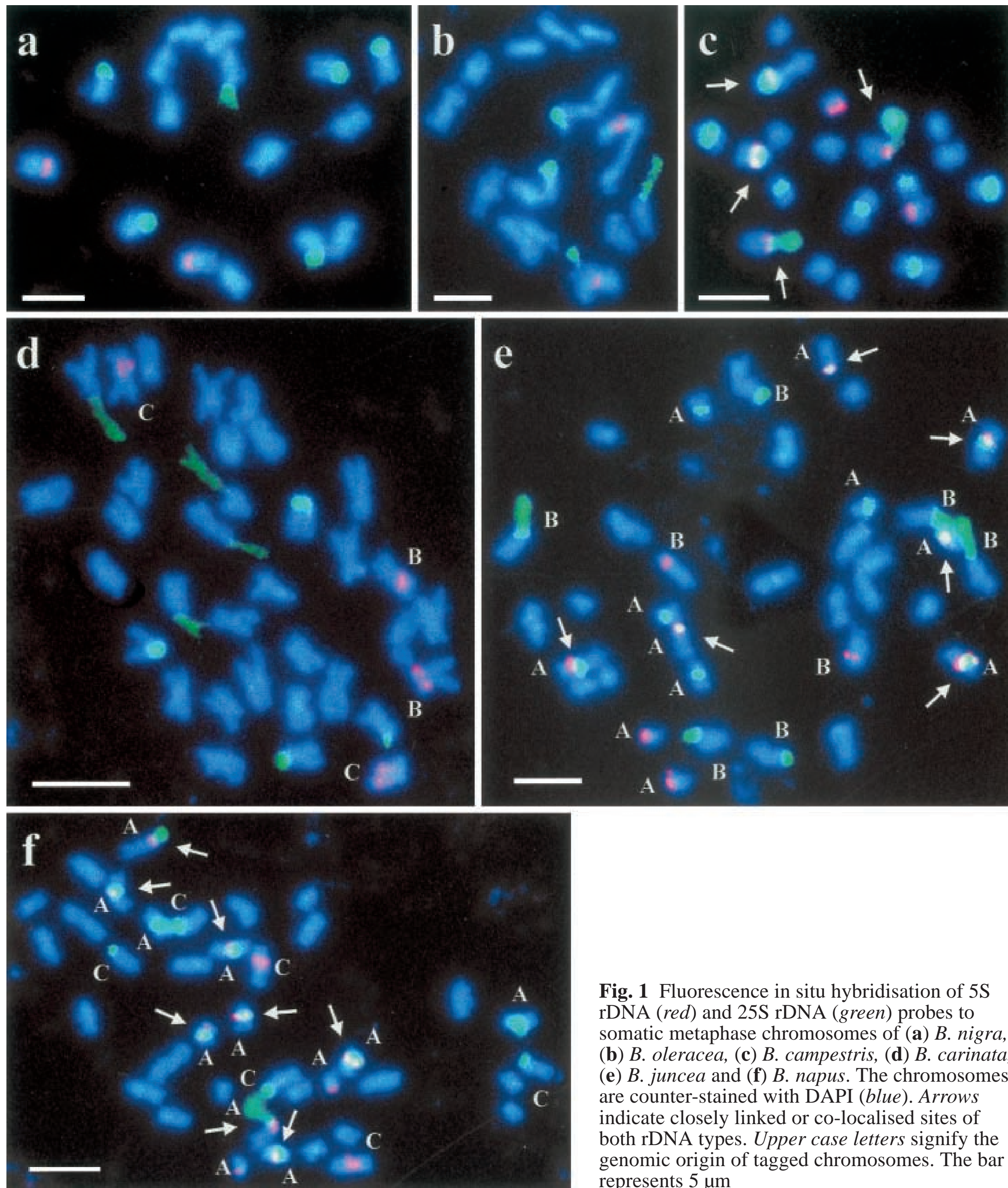

Fig. 1 Fluorescence in situ hybridisation of 5S rDNA (*red*) and 25S rDNA (*green*) probes to somatic metaphase chromosomes of (**a**) *B. nigra,* (**b**) *B. oleracea,* (**c**) *B. campestris,* (**d**) *B. carinata,* (**e**) *B. juncea* and (**f**) *B. napus*. The chromosomes are counter-stained with DAPI (*blue*). *Arrows* indicate closely linked or co-localised sites of both rDNA types. *Upper case letters* signify the genomic origin of tagged chromosomes. The bar represents 5 μm

M. polymorpha, 5S rDNA loci are in close proximity to the 16S-23S or 18S-5.8S-26S rDNA loci, respectively, which could facilitate the biogenesis of ribosomal subunits. The preferential localisation of 5S rDNA near the nucleolus in interphase nuclei of plants would tend to support this proposal (Montijn et al. 1999).

Chromosome types III and IV are found exclusively in the B and C genomes respectively, the former having one proximal 5S rDNA site in the long arm, the latter two adjacent sites which are particularly distinct on longer pro-metaphase chromosomes. Chromosome type V is confined to the A genome, and is a small submetacentric with a terminal 5S rDNA site in the long arm. Chromosome type VII has a prominent, distal 25S rDNA site in the B and C genomes, and chromosome type VIII of the A genome only bears a large proximal 25S rDNA site in its long arm.

Double-target FISH with 5S and 25S rDNA probes enables the discrimination of a substantial number of chromosomes of the complement of the *Brassica* species. *B. campestris* has the highest number of rDNA loci of the diploids, which marks 12 out of the 20 chromo-

Table 1 The numbers and types of chromosomes bearing 5S rDNA (*red*) and 25S rDNA (*green*) loci in the six species of *Brassica*

			5S+25S		5S			25S		
Chromosomal type			I	II	III	IV	V	VI	VII	VIII
Genome			A	A	B	C	A	B, C	B, C	A
Species	2n									
B. nigra	$2x$=16	BB	–	–	2	–	–	4	2	–
B. oleracea	$2x$=18	CC	–	–	–	2	–	2	2	–
B. campestris	$2x$=20	AA	2	2	–	–	2	–	–	6
B. carinata	$4x$=34	BBCC	–	–	2	2	–	4	4	–
B. juncea	$4x$=36	AABB	2	4	2	–	2	4	2	4
B. napus	$4x$=38	AACC	2	6	–	2	2	2	2	2

somes of its complement. Eight out of 16 chromosomes are marked in *B. nigra* and six out of 18 chromosomes are marked in *B. oleracea*. The ability to distinguish particular chromosomes of the complement has utility in the allotetraploids, where the majority of the marked chromosomes can be assigned to one or other of their constituent genomes.

Variation in chromosome patterns of rDNA loci

The expectation is that the allotetraploids would possess the sum of the chromosomal types of their diploid forebears. However, examination of the data in Table 1 shows clearly that this is not the case. *B. carinata* (BBCC) has two type-VI chromosomes less than expected. Both *B. juncea* (AABB) and *B. napus* (AACC) have more type-II chromosomes and fewer type-VIII chromosomes than would be expected from the karyotype of one of their ancestors, *B. campestris*. Type-II is morphologically similar to type-VIII, but has an additional, co-localised site in its long arm. It is possible that the ancestral donor of the AA genome had more type-II chromosomes and fewer type-VIII than extant genotypes of *B. campestris*. Alternatively, the genome of *B. campestris* may have itself undergone change since the evolution of the tetraploid species. Polymorphism in rRNA genes has been reported on the basis of cytogenetic and molecular analyses of varieties of *B. nigra*, *B. oleracea* and *B. napus* (Kianian and Quiros 1992; Maluszynska and Heslop-Harrison 1993; Snowdon et al. 1997; Fukui et al. 1998; Hasterok and Maluszynska 2000a, b, c). Variation in the numbers of 5S rDNA loci is also apparent in comparisons of varieties of *B. napus* (Kamisugi et al. 1998; Schrader et al. 2000), and is even between different populations of the same cultivar of *B. juncea* (Hasterok and Maluszynska 2000c). Variation in the chromosomal patterns of 5S and 45S rDNA loci is not uncommon and has been reported in many other plant species (Hanson et al. 1996; Moscone et al. 1999; Shishido et al. 2000). Different mechanisms have been invoked to explain this phenomenon, such as chromosome rearrangement, unequal crossing-over, gene conversion and transpositional events (Schubert and Wobus 1985; Leitch and Heslop-Harrison 1993; Hall and Parker 1995). There is recent evidence for extensive and rapid genome re-structuring in polyploids (Soltis and Soltis 1995), including synthetic polyploids of *Brassica* (Song et al. 1995), which could explain some polymorphism of the 5S and 25S rDNA loci in *B. juncea* and *B. napus*.

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