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The large spacer of a nuclear ribosomal RNA gene from radish: organization and use as a probe in rapeseed breeding

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Summary. An Eco RI fragment spanning the spacer region separating two adjacent radish rDNA units was isolated and partially characterized. Although previous studies did not reveal obvious length heterogeneity in radish rDNA units, we observed the presence of several short repeats within this spacer, thus demonstrating that these repeats are not typical of species with variable length rDNA spacer. A short fragment containing two and one-half repeats was sequenced and used as a probe to demonstrate that this short sequence is highly specific for the genus *Raphanus*. We used these rDNA spacer sequences in preliminary assays for variability among 14 rapeseed cultivars and for introgression of radish rDNA in rapeseed to illustrate the usefulness of these probes.

Key words: rDNA – Large spacer – Repeated sequences – Variability – Radish – Rapeseed

Introduction

Few plant ribosomal genes have been characterized in detail. From a number of studies it is clear that their general organization is very similar to that of animal rRNA genes. As for animal rDNA, heterogeneity has been reported for a number of species (Vedel and Delseny 1987; Rogers and Bendich 1987). In some species, such as flax or soybean, the rDNA units are fairly homogeneous. In wheat, barley, rye, pea and broad bean they present length heterogeneity, and in onion and radish they have the same length but present sequence heterogeneity. Other species present both length and sequence heterogeneity. Heterogeneity is also evident at the species level, and it is very often possible to distinguish between different cultivars or different wild type populations by their restriction pattern (Appels and Dvorak 1982; Saghai-Maroof et al. 1984; Ellis et al. 1984; Doyle and Beachy 1985; Olmedilla et al. 1985; Appels et al. 1986; Jorgensen et al. 1987). Therefore, this heterogeneity might be very useful for plant breeding and evolutionary studies.

The molecular basis for this length heterogeneity has been recently elucidated. Variations in the copy number of a small repeated element within the large intergenic spacer have been described for wheat, broad bean, maize and rye (Appels and Dvorak 1982; Yakura et al. 1984; Flavell et al. 1986; Rogers et al. 1986; Appels et al. 1986; McMullen et al. 1986; Toloczycki and Feix 1986), and account for length heterogeneity. So far, complete sequence information on the large spacer region is limited to cereals, and partial sequencing data on dicot spacers are restricted to broad bean.

During the last few years we have been interested in analysing radish ribosomal genes (Delseny et al. 1983, 1984) and we observed that, in contrast with the preceding species, this plant did not show obvious length variation of the rDNA large spacer. Therefore, we found it interesting to investigate this region for the presence or absence of short repeats.

In this paper we determine the general organization of a cloned rDNA spacer of radish, and report sequencing information on a subclone containing two of the seven repeats which are present in the middle of the spacer. Hybridization of this subclone to other cruciferae species demonstrates that this short sequence is species specific. We present preliminary results showing

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that the spacer sequence can be useful for genetic analysis of rapeseed.

Materials and methods

Plant material

The origin of the seeds is as follows; radish seeds were purchased from Vilmorin, (*Raphanus sativus* cv Rond rose à bout blanc National), *Raphanus raphanistrum* seeds were obtained from Crucifer germplasm collection (Instituto Nacional de Investigaciones Agrarias, MADRID, Spain), *Brassica campestris sp. perviridis, B. adpressa* and *B. nigra* were obtained from the BORDEAUX Botanical garden. *Sisymbrium irio* and *Lepidium draba* leaves were collected in a local garden. Seeds from the various rapeseed cultivars were from the INRA collection. A few plants originating from the progeny of a cross made at the INRA station in LE RHEU between a rapeseed male sterile line with OGURA radish cms and the restorer line were also analyzed.

Radish seeds were germinated on wet filter papers at 20 °C in the dark. Etiolated seedlings were harvested after 48 h; seed coats were removed manually before decontamination with calcium hypochlorite (3%) and rinsing with water. The sample was then frozen with liquid nitrogen and DNA immediately extracted. Seeds from other cruciferae were grown in a green house and leaves were collected after 3–10 weeks. They were decontaminated in the same way as seedlings and frozen in liquid nitrogen. Leaves were usually stored at −70 °C until use.

DNA extraction and analysis

Nuclear DNA was extracted from frozen radish seedlings or from frozen leaves for the other Cruciferae as previously reported (Delseny et al. 1984; Grellet et al. 1986) using a CsCl-ethidium bromide purification step. DNA was digested with restriction enzymes according to the supplier's recommendations and in the presence of an excess of enzyme. Digests were fractionated by agarose gel electrophoresis on horizontal gels run in 0.04 M tris-acetate 2 m*M* EDTA (pH 7). Size markers were Hind III, Eco RI + Hind III fragments from lambda phage DNA or Hind II fragments of a recombinant plasmid, pCa 8 (Cooke et al. 1983). Ethidium bromide stained gels were photographed and DNA was blot transferred onto nitrocellulose sheets (Delseny et al. 1984; Grellet et al. 1986). When a specific DNA fragment had to be subcloned it was electrophoretically purified on a low temperature melting agarose gel (Maniatis et al. 1982).

Hybridization was carried out overnight at 62 °C as previously described (Grellet et al. 1986) in 5×SSC (SSC is 0.15 M NaCl 0.015 M sodium citrate pH 7.0), 5× Denhardt solution and 0.1% sodium dodecyl sulphate. Filters were washed 6 times at 65 °C with 3×350 ml of 3×SSC, 0.1% sodium dodecyl sulphate and 3×350 ml of 0.3×SSC. Probes were labelled by nick tanslation using (α-^{32}P) dCTP.

Molecular cloning

A genomic library was constructed using a partial Eco RI digest (genomic DNA was digested with 1 unit enzyme for 3 µg DNA and for 5 min). The vector was λ gt λ WES (Leder et al. 1977) from which the Eco RI stuffer fragment was previously destroyed by digestion with Sst I. The Eco RI ends were dephosphorylated with bacterial alkaline phosphatase before use of the vector. After ligation, recombinant DNA was packaged with extracts from *Escherichia coli* lysogens BHB 2690 and BHB 2688 according to published procedures (Maniatis et al. 1982). The library was amplified by growing a plate stock directly from the packaging mixture. Several plates were screened for the presence of rDNA inserts in the phages (Maniatis et al. 1982) using nick translated plasmid pRE 12 as a probe (Delseny et al. 1983). This plasmid carries a radish rDNA fragment (Fig. 1). Twenty-five recombinant phages were plaque purified, their DNA was prepared according to published procedures (Maniatis et al. 1982) and restricted with various enzymes. Phage DNA fragments to be subcloned were purified by gel electrophoresis and inserted into the Eco RI site of plasmid pUC 8 (Vieira and Messing 1982). Recombinant plasmids were mapped with various restriction enzymes using single, double, and partial digestions.

Sequencing

Sequencing was carried out using the chemical method (Maxam and Gilbert 1980). Fragments to be sequenced were labelled at their 5′ ends using T4 polynucleotide kinase and γ-^{32}P ATP (5,000 Ci/mmol) (Maxam and Gilbert 1980) or at their 3′ ends using nucleotidyl terminal transferase and α-^{32}P cordycepin triphosphate (3,000 Ci/m*M*) (Tu and Cohen 1980). Alternatively they were labelled by infilling using *Escherichia coli* DNA polymerase I and α-^{32}P dCTP.

The labelled fragment was then split with an appropriate restriction enzyme and the two resulting single-end labelled fragments were recovered after gel electrophoresis in a low temperature melting agarose gel. Reactions specific for G, A+G, T+C and C were used in most cases.

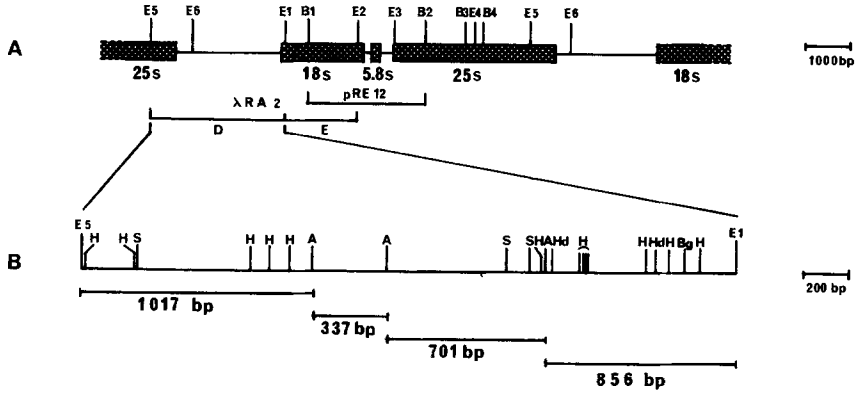

Fig. 1 A, B. General organization of the radish rDNA. **A** General organization of the rDNA units arranged in tandem arrays. Major sites for cleavage by Eco RI (*E*) and Bam HI (*B*) are indicated, as well as the sequences present in mature rRNA. Plasmid pRE 12 and phage λ RA2 represent the fragments isolated as recombinant clones. **B** Restriction map of the Eco RI fragment D. It was established by single and double digestions with various enzymes. *H:* Hpa II, *S:* Sph I, *A:* Acc I, *Hd:* Hind III, *Bg:* Bgl II. The *4 bars* drawn *below* represent the Acc I fragments and their size

Results

Isolation of a clone containing the large spacer sequence and general organization of the spacer

The general organization of radish ribosomal RNA genes has already been published (Delseny et al. 1984) and is shown in Fig. 1. Six major Eco RI sites have been located on a composite map. Some of these sites, such as E1, E2, E3 and E6, are absent in a fraction of the repeat units, and therefore multiple fragments are usually detected with a probe corresponding to a specific region of the gene. The absence of certain sites in a fraction of the genes was established by analyzing several different clones. In order to isolate fragments containing the large spacer region we built a genomic library with Eco R1 fragments, resulting from a partial digestion, inserted in the bacteriophage λ gt λ WES (Leder et al. 1977).

This library was screened with the recombinant plasmid pRE 12, previously isolated in the laboratory, encompassing about two third of the 18S RNA gene, the small transcribed spacer, and the 5′ end of the 25S RNA gene (Delseny et al. 1983). We plaque purified and characterized several recombinant phages, one of which (λ RA 2) was shown to contain two Eco R1 fragments corresponding to the region between E5 and E2 as indicated in Fig. 1 A. The D fragment, between sites E5 and E1, contains the large spacer sequence and its borders in the 25S and 18S RNA coding sequences. Its length is about 2,900 bp. It was subcloned into a pUC8 vector. Two clones, pD 12-3 and pD1-1, corresponding to the two possible orientations, were selected and used for restriction mapping, further subcloning, and sequencing. A restriction map is shown in Fig. 1 B, various fragments were subcloned as indicated, and a 337 bp Acc I fragment was sequenced (p 337).

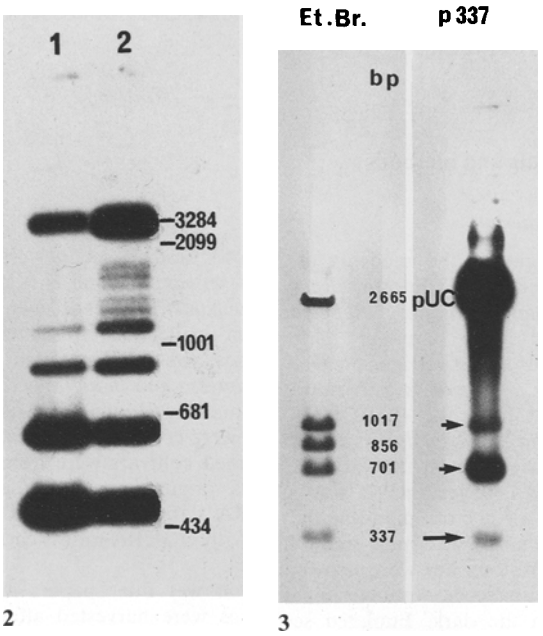

Fig. 2. Evidence for repeated Sau 3A fragments in the rDNA spacer. The Bam HI-Pvu II fragment labelled at the Bam HI end was electrophoretically purified and digested with a limiting amount of Sau 3A. *Lanes 1 and 2* correspond to two different times of incubation (1: 15 min and 2: 5 min). The resulting fragments were separated on a 1.5% agarose gel and autoradiographed. A series of fragments light up between 1,100 bp and 1,800 bp approximately every 100 bp

Fig. 3. Evidence that the Acc I 337 bp fragment contains repeated sequences. *Left:* plasmid pD 12–3 was digested with Eco RI and Acc I, run on a 1% agarose gel and then blot transferred onto a nitrocellulose sheet. *Right:* blot filter was hybridized with plasmid p337. *Long arrow* indicates hybridization with the homologous fragment and *short arrows* hybridization with adjacent fragments. Since the 337 bp fragment was cloned into a pUC 8 vector a strong signal is also observed with the vector

Evidence for a short repeated sequence in the rDNA spacer

The first evidence for a repeated sequence in the spacer region came from an attempt to map Sau 3A sites. Plasmid pD 12-3 was linearized with Bam HI (a single site in the polylinker) and was labelled with T4-polynucleotide kinase and γ^{32}P-ATP. It was restricted with Pvu II and the labelled Bam HI – Pvu II fragment was gel purified and partially digested with Sau 3A. Figure 2 shows the patterns observed for two different times of digestion. A poorly resolved ladder between 1,100 and 1,800 bp indicates a cluster of Sau 3A sites, suggesting a possible repeat.

Additional evidence that this region corresponded to repeated sequence came from hybridization studies with the 337 bp Acc I subclone (p337) (Fig. 1 B). This subclone very strongly hybridized not only to the corresponding 337 bp fragment, but also to the adjacent 701 bp and 1,017 bp Acc I fragments (Fig. 3).

Definitive evidence was obtained from direct sequencing of the Acc I subclone which contained two complete repeated units and parts of two others (Fig. 4).

Complete sequencing of the rDNA spacer (to be reported elsewhere with information on the initiation of transcription and site of pre-rRNA processing) indicates that 7 such units are arranged in tandem repeats within pD 12-3.

Since short repeats within the rDNA spacer have been described for wheat, maize, rye and broad bean (Appels and Dvorak 1982; Yakura et al. 1984; McMullen et al. 1986; Toloczyki and Feix 1986; Appels et al. 1986) we compared our data with these different

gtAGACTGTCGCGCACGTCCGTGAAGGCAAAAAAAAA
AAAAAAAAATCTCATGTACTGATCACCCACTGATCACCCACGGCTGGGTGTACTGATGGGACAGTCAGGGTGTGAAAATACTCCGCAGACTGCCGTGAACGTCCGTGAAGGCAAAAAAAAAAA
AAAAAAAAATCTCATGTACTGATCACC------TCAC--------CGGTG---------GACAGTAAGGGTGTGAAATTACCCCGCAGACTGTCGTGAACGTCCGTGAAGGCAAAAAA
AAAAAATCTCATGTACTGATCACCCACTCATCACCCACGACTGGGTGTACTGATGGGACCATCAGGTTGTGAAAATACCCCGTagac

Fig. 4. Nucleotide sequence of the 337 bp Acc I fragment. *Lower-case letters* represent the nucleotides belonging to the Acc I site but outside of the cloned fragment. Three short deletions have been introduced in the sequence (*dashes*) in order to maximize alignment between repeats

sequences. This direct comparison revealed that the radish repeat has apparently nothing in common with the other previously reported repeats.

Species specificity of the repeated sequence

In order to further infestigate the specificity of the short repeated sequence, we digested DNA from several more or less distantly related cruciferae with Eco RI and hybridized it after blot transfer with the 337 bp cloned Acc 1 fragment.

The result of this experiment is shown in Fig. 5. It shows that only *Raphanus* DNA hybridizes with this sequence in stringent conditions, indicating that these sequences diverge very rapidly. We have recently characterized the rDNA spacer short repeat from *Sisymbrium irio* and found it to be completely different from the radish one (unpublished results). However, when the same filter is hybridized again with the pD12-3 probe, a number of fragments light up on the autoradiogramme. Other cruciferae (including rapeseed) were analyzed with similar results. This latter result suggests that the two isolated probes could be used as tools in plant breeding. The next section describes preliminary results showing how they can be used.

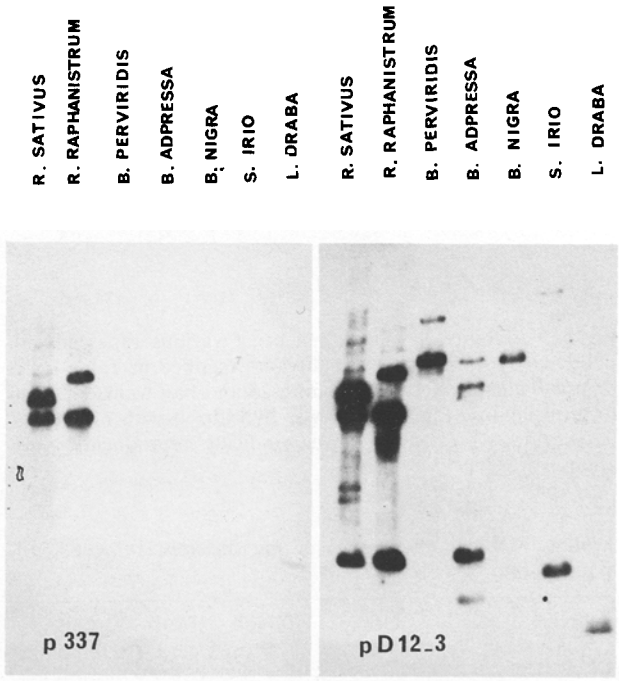

Fig. 5. Species specificity of the short repeated sequence. DNA from various cruciferae was digested with Eco RI, electrophoresed on 1% agarose gel and blot transferred onto nitrocellulose filter. The filter was initially hybridized with plasmid p337 and autoradiographed. Afterwards this first probe was removed and the same filter hybridized again with plasmid pD 12–3. Due to overexposure minor fragments are revealed on the *R. sativus* lane

Use of radish rDNA probes in rapeseed breeding

Radish is a very close relative of rapeseed. Interspecific crosses between these two species have been used to engineer cytoplasmic male sterile rapeseeds (Ogura 1968; Bannerot et al. 1974; Pelletier et al. 1983) and to restore fertility (Heyn 1976, 1978).

We first investigated the variability of the restriction pattern of rDNA spacer in well-established rapeseed cultivars. Most of them were winter oil-seeds from different geographical origins and with different characteristics (Table 1).

Figure 6 shows the results which were obtained with 13 cultivars. Obviously the Eco RI pattern of rapeseed rDNA large spacer is much more complex than that in radish, having at least six fragments. This was not unexpected, since *B. napus* is an allotetraploïd and *R. sativus* a diploid. In radish we observed a single size for rDNA units but they differed from one another by the presence or absence of restriction sites (Delseny et al. 1984). In rapeseed there are two major size classes originating presumably from each parental genome. Our interpretation is that within each class some restriction sites are also variable in the same way as in radish. The most striking point is that almost all the cultivars have the same pattern except Lituanie and Lembkes, which present an additional shorter fragment. Similar fragments are often present as minor components in other cultivars. Reproducible, discrete differences can also be noted in the relative intensity of the major fragments from different cultivars. For instance, the ratio between the two smaller fragments in Norin 9 is much lower than in the others. When these DNA were digested with Bgl II, they all seemed identical, except Lembkes which has a smaller extra fragment (Delseny et al 1987). In all these experiments DNA

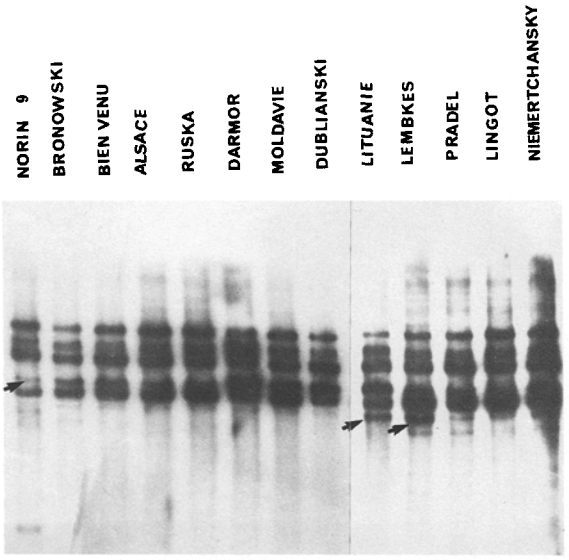

Fig. 6. Variability in rDNA spacer of various rapeseed cultivars. DNA from rapeseed cultivars described in Table 1 was digested with Eco RI, electrophoresed and blot transferred onto nitrocellulose filters. Filter was hybridized with nick translated pD 12–3 plasmid. *Arrows* indicate reproducible variations

Table 1. Main characteristics of the different rapeseed cultivars

Cultivar	Origin	Type	Erucic acid	Glucosinolates
Norin 9	Japan	Spring	+	+
Bronowski	Poland	Spring	+	0
Bienvenu	France	Winter	0	+
Alsace	France	Winter	+	+
Ruska	USSR	Winter	+	+
Darmor	France	Winter	0	0
Moldavie	USSR	Winter	+	+
Dublianski	USSR	Winter	+	+
Lituanie	USSR	Winter	+	+
Lembkes	GFR	Winter	+	+
Pradel	France	Winter	+	+
Lingot	France	Winter	0	+
Niemertchansky	USSR	Winter	+	+

were extracted from three to five plants. In another experiment (not shown), we analyzed DNA from 22 individual plants of another cultivar (Orléans) and found them all identical to the prevalent pattern shown in Fig. 6.

In another series of experiments we tried to detect rDNA sequences specific of radish in the progeny of a cross between a rapeseed cytoplasmic male sterile line (with radish OGURA cms) and its restorer line (with radish nuclear restorer genes). Although both lines have been back crossed several times with rapeseed, they might still contain some radish nuclear DNA.

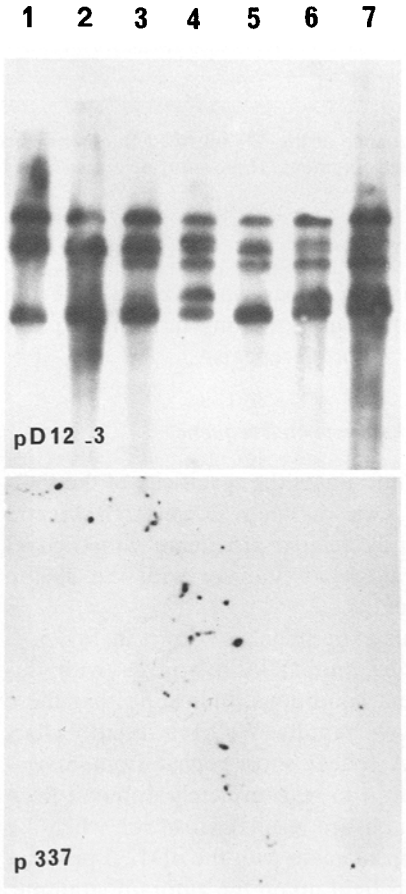

Fig. 7. Assay for radish rDNA sequences in rapeseed. DNA was extracted from seven individual plants (1–7) resulting from a cross between a male sterile rapeseed line (with OGURA radish cytoplasm) and a rapeseed restorer line (with radish nuclear restorer genes). DNA was digested with Eco RI and probed either with pD 12–3 or p 337

Results presented in Fig. 7 show that none of the seven plants analysed contained sequences hybridizing with the p 337 plasmid, specific for radish rDNA sequences. The patterns observed with probe pD12-3 are very similar to those shown in Fig. 6, although some differences can be detected between individuals.

Discussion

This paper reports the characterization of the intergenic region separating two adjacent rDNA transcription units from radish. The main structural feature of this region is the presence of seven short repeats 100 bp long. Therefore, although we did not detect length heterogeneity in the radish rDNA unit, we observed short repeats as in the other plants for which the large spacer organization is known. Accordingly, these short repeats seem to be a characteristic feature of the rDNA

large spacer in plants. The size of the radish rDNA spacer repeat is different and shorter than that of other repeats. Its sequence is also different (Appels and Dvorak 1982; Yakura et al. 1984; Flavell et al. 1986; Appels et al. 1986; Mc Mullen et al. 1986; Toloczycki and Feix 1986).

We used full length spacer radish probe to investigate variability of rDNA genes in rapeseed. In contrast to the situation in several other plants (Ellis et al. 1985; Flavell et al. 1986), we observed a remarkable conservation of the pattern from plant to plant and from one cultivar to another. A similar situation was described in soybean (Doyle and Beachy 1985). However, only a limited number of cultivars were investigated and most were winter oil rapeseed. Spring oil cultivars and forage rapeseeds remain to be investigated. Since the length of the fragments hybridizing with pD12-3 is different in most cruciferea, this spacer probe can also be used to analyse segregation of specific fragments in interspecific crosses and protoplast fusions. An analysis using this probe has recently been reported by Primard et al. (1988).

Further investigation of the repeat sequence specificity demonstrated that it specifically hybridized with rDNA from the same genus only. No cross-hybridization was detected in standard stringent conditions with rDNA from closely related species from the Brassica tribe. This suggested to us that this type of species specific sequence could be a very powerful tool to investigate for the presence of an alien sequence or chromosome within a specific crop. In a first series of experiments we used this short repeated sequence to look for radish rDNA in rapeseed, which might have kept some radish nuclear sequences following introduction of an OGURA radish cytoplasm and introgression of radish restorer genes. We failed to detect any radish rDNA in the seven plants which were analyzed. Some individual differences were noticed, illustrating the sensitivity of these probes, but a much higher number of plants should be examined if the basis for such variation is to be investigated.

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