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A repeated sequence probe for the C genome in *Avena* (Oats)

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Summary. The genus *Avena* consists of at least 23 species composed of three ploidy levels. Cytogenetic analysis has characterised four distinct karyotypes. These are the A, B, C and D genomes. We have isolated a repeated sequence clone that can be used for the detection of the C genome in *Avena* by filter hybridization techniques. This clone, termed RS-1, is a genomic DNA clone containing at least one highly repeated sequence that is abundant in *Avena* species containing the C genome. This sequence or a related sequence is also present, but at much reduced levels, in species that do not contain the C genome. Because of its abundance and the characteristic Southern blot pattern, we have termed this clone a C genome-specific clone. We have also done similar analysis of the *Avena* genus using a rDNA clone from wheat. The results of these experiments demonstrate that clearly definable C genome-specific markers can be identified with both probes. These molecular probes can be useful in studying the genomic relationships of *Avena* and can provide some clues as to the origin of the cultivated *Avena* species. These results can, therefore, provide breeders with directions for the efficient transfer of desirable traits of wild *Avena* species into commerical varieties.

Key words: Repeated DNA – Ribosomal DNA – *Avena* – C genome – Speciation

Introduction

The genus *Avena* contains up to 23 identified species that span three ploidy levels (Rajhathy and Thomas 1974; Baum et al. 1974; Baum 1977; Baum and Fedak 1985a, b). The phylogenetic relationships of the various species have been elucidated using a variety of morphological, biochemical and genetic tests (Bhatti 1972; Baum 1975). The origin of and the relationships between the various genomic karyotypes have not been explored at the molecular level. For example, the origin of the D genome in the hexaploid oats is not known, nor is the origin of some of the tetraploid species, which could be autotetraploid or allotetraploid in genetic constitution. The molecular similarities of these different species or genomic karyotypes could provide clues as to the identify of some of these different genomes. The genomic relationship of wheat, e.g. has been extensively studied using such techniques (Flavell et al. 1979a). By using cloned repeated sequences such as those used in these other cereal systems (Flavell et al. 1976, 1977, 1979b; Bedbrook et al. 1980; Jones and Flavell 1982a, b), we have been able to isolate a repeated sequence probe that is related to the C genome in *Avena*.

Materials and methods

Plant material

The individual species karyotypes and their accession number from the Canadian Avena Collection (CAV numbers) were as follows: *A. sativa* cv Hinoat (AACCDD), *A. byzantina* (AACCDD) (2787), *A. sterilis* (AACCDD) (2614), *A. fatua* (AACCDD) (2794), *A. magna* (AACC) (4078), *A. murphyi* (AACC) (2832), *A. abyssinica* (AABB) (2847), *A. vaviloviana* (AABB) (2736), *A. barbata* (AABB) (2384), *A. strigosa* (AsAs) (2845), *A. hirtula* (AsAs) (2223), *A. wiestii* (AsAs) (5227), *A. damascena* (A_dA_d) (0257), *A. longiglumis* (A_1A_1) (2215), *A. canariensis* (AcAc) (3862), *A. clauda* (C_pC_p) (4908), *A. pilosa* (C_pC_p) (4910), *A. ventricosa* (C_vC_v) (2208), *A. macrostachya* (unkown) (5264), *A. atlantica* (unknown at present) (M95), *A. agadiriana* (unknown at present) (M60). Plants were grown in potted soil under a 16-h photoperiod at 20 °C. Five plants from each accession were grown, and material collected from all five plants of each accession was pooled.

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DNA isolation

Young green leaf material was collected from healthy plants and stored at −70 °C. Leaf material (5 g) was ground to a fine powder in a coffee grinder while maintained at −70 °C with dry ice. This powder was added to 50 ml of buffer made of 0.35 *M* sucrose, 0.05 *M* *Tris*-HCl (pH 7.6), 0.025 *M* KCl, 0.005 *M* $MgCl_2$ and 400 µg/ml ethidium bromide, and was homogenized with the medium tip of a polytron set at 5 for 60 s while the temperature was maintained at 4 °C. The homogenate was filtered through cheesecloth and centrifuged for 10 min at 3,000 × *g*. The pellet was resuspended in a minimal volume of 0.05 *M* *Tris*-HCl (pH 8.0), 0.1 *M* EDTA and 0.015 *M* NaCl, and added dropwise to 10 vol of a preheated solution of 0.1 *M* *Tris*-HCl (pH 9.5) and 0.3 *M* EDTA at 65 °C. The solution was cooled to 50 °C and SDS was added to 0.5% followed by Proteinase K, to a final concentration of 100 µg/ml. The digestion was allowed to proceed for 3 h at 50 °C. The solution was extracted with phenol-chloroform twice, then with chloroform twice. The aqueous phase was dialyzed against TE (0.01 *M* *Tris*-HCl, 0.001 *M* EDTA, pH 7.5). The DNA was ethanol precipitated, collected by spooling with a glass rod and resuspended in TE at 2 µg/ml. Gel electrophoresis revealed most of the DNA to be greater than 40 kb.

DNA restriction, gel electrophoresis, blotting, labelling and filter hybridization

These operations were carried out using standard techniques (Maniatis et al. 1982).

Generation of DNA probes

The repeated sequence probes used in this study were isolated from a partial genomic library of *A. sativa* DNA constructed in Ch4A. The library was constructed by partial EcoRI digestion of *A. sativa* (cv Hinoat) nuclear DNA, followed by recovery of fragments in the 14- to 20-kb range. These fragments were ligated to EcoRI-cut Ch4A arms and packaged in vitro (Maniatis et al. 1982). The clones were identified as containing repeated sequences based on strong hybridization to labelled *Avena* nuclear DNA. This clone isolation procedure involved labelling nuclear *A. sativa* DNA by nick translation to a specific activity of 107 cpm/µg. This probe was used in 50 ml of hybridization mixture to screen 20,000 pfu of the library. Hybridization was for 24 h. Strongly hybridizing plaques were chosen and plaque purified. One of these clones was referred to as RS-1. The other probe used in this study was pTa-71, a rDNA clone from wheat (Gerlach and Bedbrook 1979), kindly supplied by Dr. R. B. Flavell.

Results

Characterization of the RS-1 repeated sequence clone

A number of clones that contain repeated sequences were isolated. One of these clones, RS-1, is a genomic clone containing about 14 kb of inserted DNA. It was important to determine the portion of cloned DNA that represented repeated DNA. The RS-1 clone (5 µg) was restricted with four different enzymes, separated on agarose gels, blotted to nitrocellulose and hybridized to nick-translated *Avena* DNA for short periods of time. There were a number of repeated fragments that showed hybridization indicative of repeated DNA (Fig. 1). However, not all of the cloned DNA exhibited this strong hybridization, therefore, we believe that the repeated DNA contained in this RS-1 clone may be interspersed with DNA not repeated. It should also be noted at this point that these repeated sequences did not show homology to rDNA (not shown).

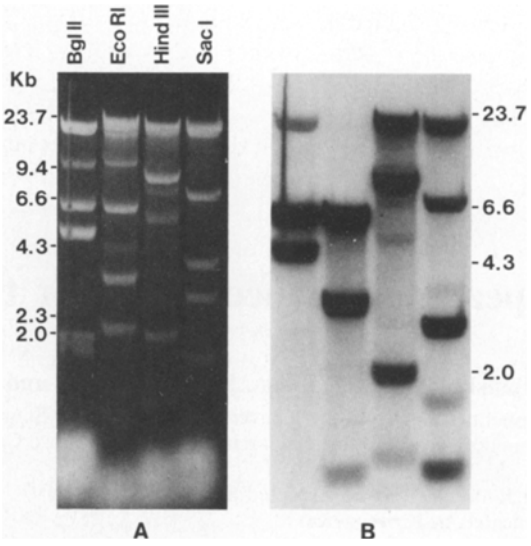

Fig. 1 A and B. Hybridization of labelled oat genomic DNA to restriction fragments of RS-1. RS-1 DNA (5 µg) was restricted with the indicated restriction enzyme, and the resultant fragments (consisting of both vector and DNA) were separated on 0.8% agarose gels (**A**). The fragments were transferred to nitrocellulose and hybridized to labelled oat genomic DNA. After washing, the blot was exposed to X-ray film for 24 h (**B**). The fragments that show high levels of radioactivity represent those fragments that contain repeated DNA, the intensity being proportional to the degree of repetition. The *Eco*RI fragments of 6.6- and 3.0-kb show strong hybridization, while the 0.8-, 2.3- and 4.5-kb fragments show weaker hybridization, suggesting most of the cloned DNA represents repeated sequences. This is also seen in the *Sac*I lane, where only one of the fragments shows hybridization at the levels of single copy sequences

The location of these repeated elements in this clone is unknown at present, but it is likely that they are present along the length of the cloned segment, since all of the restriction fragments that correspond to the ends of the clone, such as the 25-kb HindIII fragment and the 8.5-kb HindIII fragment, showed hybridization indicative of repeated DNA. These two fragments correspond to the left and right ends of the cloned DNA. In addition to these fragments, strong hybridization was also seen with an internal 2.2-kb HindIII fragment. This hybridization pattern was also seen with the other enzymes shown. Of particular interest was that of two EcoRI fragments, the 6.6- and 3.0-kb bands which represent only inserted DNA showed strong hybridization, while the three smaller EcoRI fragments did not. This implied that the amount of repeated sequence in this clone is substantial,

but that there was some non-repeated DNA present. In the SacI digest there were three bands that were similar to the bands seen on Southern blots of SacI-digested *Avena* DNA. These were the 0.8-, 1.9- and 3.0-kb bands that are the most diagnostic markers for the C genome (see below).

Genome analysis using repeated sequence clones

In order to obtain some information on the degree of repetition of these repeated elements in various *Avena* species, we performed hybridization experiments to compare copy numbers of RS-1 and pTa-71. RS-1 and pTa-71 were hybridized to slot-blotted DNA from the various *Avena* species (Fig. 2). It was clear that all of the species contained these sequences and that the signal from RS-1 was stronger than that seen for pTa-71. This implies that RS-1 represents a highly repeated sequence.

On a percentage basis, the species that contain the C genome had more of the RS-1 sequence. For example, the C-genome diploids had about ten times stronger a signal than the other diploids. The tetraploid and hexaploid species carrying the C genome contained between four and ten times the number of these C genome sequences than the A-genome diploids. There was variation among the hexaploids with respect to copy number of this clone. It was clear that there were significant differences in the levels of these repeated sequences in these species, and that C genome-containing species contained considerably more than the non-C genome species.

The exact number of DNA sequences in the chromosomal DNA that these clones represent was not determined. However, if one assumes a value of between 5,000–15,000 copies of the rDNA gene in oats as in wheat (Flavell and O'Dell 1976), one can roughly determine copy number by comparing hybridization signals. The RS-1 clone was present at five to ten times the abundance of pTa-71, based on the hybridization signal obtained when similar amounts of genomic DNA and specific activities were used to compare the hybridization of the two clones (Fig. 2). This estimate would place the sequences of RS-1 in the highly repeated category.

There was much less variation in copy number of the rDNA genes in *Avena* than seen with sequences homologous to RS-1. In fact, the variation was only two- to five-fold in the rDNA gene copy number. Since equal amounts of DNA were applied to the filter paper both for pTa-71 and RS-1, the results clearly indicate that the variation seen in the RS-1 hybridization was based on different numbers of RS-1 related sequences. Although these results were based on the hybridization of this repeated sequence to equal amounts of genomic DNA from the different species, one must take into consideration that many of these species of oats are tetra- or hexaploid

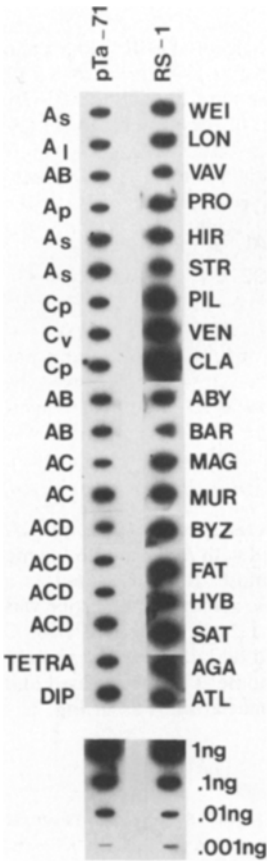

Fig. 2. Slot blot analysis of *Avena* DNA using RS-1 and pTa-71. Denatured *Avena* DNA was applied to nitrocellulose using a slot blot apparatus (BRL) and the blot hybridized to the indicated clones, both of which were at the same specific activity (5×10 cpm/μg), for 24 h. Exposure was for 24 h. The different species and their genomic karyotypes are listed. In addition, the indicated amounts of both RS-1 and pTa-71 were slot-blotted and hybridized at the same time. The abbreviations used for the species are derived from the first three letters of the species name. For a complete listing of the species name, refer to the 'Materials and methods' section

species and have more chromosomes per nucleus than the diploid species. The actual number of repeated sequences per nucleus may, therefore, be higher in the polyploid species than the diploid species since, e.g. only one-third the amount of C-genome DNA would be loaded for a hexaploid that contains the C genome when compared to a diploid C-genome DNA sample.

By including the cloned DNA as standards in this experiment, it is possible to estimate the degree of repetition of these sequences in the genomes. By our analysis (Fig. 2), 2 μg of genomic DNA from the different species contained between 0.5 and 1 ng of RS-1 related sequences, while pTa-71 related sequences are present at about 0.1 ng per 2 μg of nuclear DNA. This suggests that RS-1 and pTa-71 represented 0.025%–0.05% and 0.005% of the total DNA, respectively.

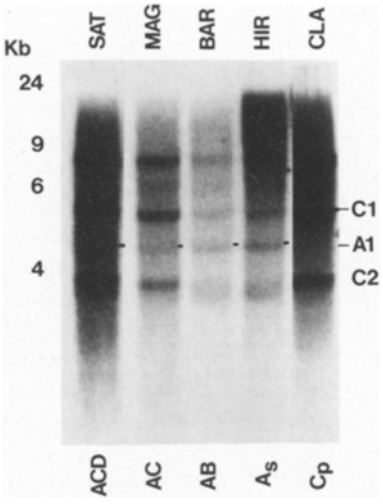

Fig. 3. Southern blot analysis of selected species of *Avena* DNA using RS-1. DNA (10 μg) was digested with *Bgl*II and, following electrophoresis, blotted to nitrocellulose. RS-1 was used as a probe. Exposure was 24 h and specific activity of the probe was 5×10^7 cpm/μg. The bands C1 and C2 are associated with C genome-containing species. The band designated as A1 is associated with the presence of the A genome. The species used and their karyotypes are indicated. Nomenclature is as in Fig. 2

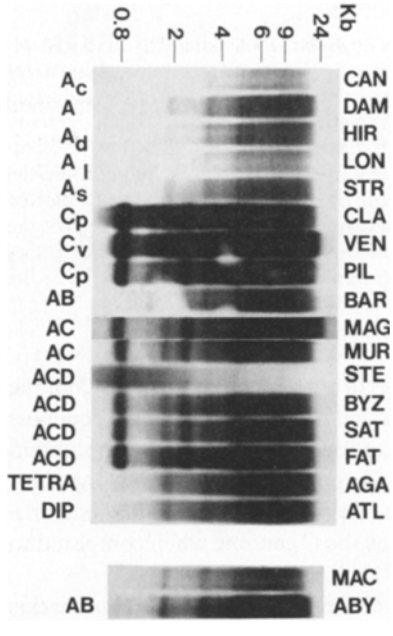

Fig. 4. Southern blot analysis of *Avena* species using RS-1. Genomic DNA (10 μg) was restricted with *Sac*I and the fragments were separated by gel electrophoresis. Following transfer to nitrocellulose, the blots were hybridized to labelled RS-1 (specific activity 5×10^7 cpm/μg). In C genome-containing species, there is a very strongly hybridizing 0.8-kb band, as well as less visible bands at 1.9 and 3.0 kb. Low level hybridization is seen in species that do not contain the C genome. Nomenclature is as in Fig. 2

Southern blot analysis using repeated sequence clones

The RS-1 clone was used as a probe on Southern blots of DNA from various *Avena* species to determine if they had different arrangements of these repeated sequences. A number of species was chosen to represent the various genomic constitutions (di-, tetra- and hexaploid) before all known species were screened with this probe. When RS-1 was hybridized to DNA that had been digested with Bq1II, the different genomic karyotypes displayed different banding patterns (Fig. 3). Moreover, RS-1 showed a different hybridization pattern in the *Avena* species that have been designated to contain the C genome. This distinctive banding pattern was evident even when other genomes were in combination with the C genome. The 7.0-kb and 4.8-kb bands identified as C1 and C2 are indicative of the C genome. The A genome-containing species also showed a distinctive banding pattern as shown by the absence of C2 and the presence of a band identified as A1. However, both A1 and C2 are present in species containing the A and C genome.

Species-specific Southern blot patterns

A variety of different restriction enzymes were used to look for variation among the different genomes. One enzyme, SacI, gave a very diagnostic pattern with RS-1 for the presence of the C genome. Most of the *Avena* species were examined, including two recently described species, *A. agadiriana* and *A. atlantica* (Baum and Fedak 1985a, b). The *Avena* species that contained the C genome in their karyotype had a diagnostic 0.8-kb fragment that showed a very strong hybridization signal (Fig. 4). This band was most likely the same as a 0.8-kb band seen in Fig. 1 that was present in the SacI digest of the RS-1 clone itself. In addition to this 0.8-kb band, bands of approximately 2.0 and 3.1 kb are also visible (again, these bands are most likely similar to those seen in the SacI digest in Fig. 1). All of the other species showed very weak hybridization to this clone, mainly in three bands and some background hybridization. These results support those seen in Fig. 2, where it was apparent that the C-genome species had higher copy number of these repeated sequences. Of interest was that the two newly identified *Avena* species, *A. agadiriana* and *A. atlantica*, do not appear to contain the C genome.

The hybridization pattern of *Avena* species DNA restricted with SacI and probed with pTa-71 showed a number of different patterns (Fig. 5). The most obvious feature was that there was a 5.5-kb major band that was common to all of the species tested. This band was present in similar quantities or intensity in the diploid, tetraploid and hexaploid species (on a percent of total DNA basis). The C-genome diploid species had an 8.6-kb band that was a diagnostic marker. However, these

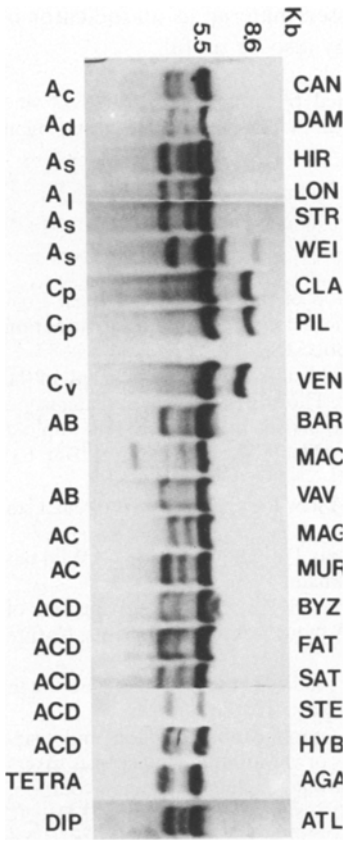

Fig. 5. Southern blot analysis of *Avena* DNA using pTa-71. Genomic DNA (10 μg) was restricted with *Sac*I and the resultant fragments were separated by gel electrophoresis. Following transfer to nitrocellulose, the blot was hybridized with pTa-71 (specific activity 5×10^7 cpm/μg). A common band at 5.5 kb is seen in all of the species, and an 8.6-kb band is diagnostic for the C-genome diploids. Similar patterns are seen for all of the *Avena* C diploids and for all of the A diploids. Nomenclature is as in Fig. 2

bands did not appear in the species that contained other genomes in addition to the C genome, such as *A. magna, A. murphyi* or the hexaploid species. This indicates that the addition of the C genome in the evolution of the polyploids was accompanied by the loss of a portion or all of the sequence arrangement of the C-genome rDNA genes. Therefore, the simple additive principle of the generation of new species is not true in this case. The variation seen in the other bands in the different species does not readily give rise to observable relationships, with many of the bands very similar in the various species.

The two newly described species, *A. atlantica* and *A. agadiriana,* do not show distinctive C-genome hybridization patterns with RS-1 or pTa-71. This suggests that *A. atlantica* is not a C-genome diploid, and *A. agadiriana* is not a C genome-containing tetraploid. These species are similar to the other A-genome and AB-genome tetraploid *Avena* species that were analyzed with these probes.

Discussion

A repeated sequence clone was isolated that can be used as a C genome-specific clone for *Avena*. The RS-1 probe contains fragments showing hybridization intensities consistent with being repeated sequences. The relative copy number of sequences homologous to this clone are much higher in C genome-containing species than those that are not. The chromosomal location or function of these repeated sequences is unknown. The fact that the different cytological karyotypes also exhibit distinctive molecular characteristics suggests that changes in repetitive sequences have accompanied the differentiation of the various genomes. The degree of repetition of the RS-1 sequences in the chromosomal DNA is estimated to be about 100,000 copies per C genome (assuming that the complement of rDNA in the *Avena* species is similar to that of wheat). This level of repetition could be involved in the formation of heterochromatin regions on the chromosomes, making these sequences interesting candidates for in situ studies.

Using probes that represent repeated sequences, such as RS-1 and pTa-71, we are able to demonstrate that the arrangement of these repeated sequences within species can be used to define, at least in some instances, the contributions of the various species to the evolution of the complex hexaploid species.

In terms of karyotype analysis, the use of these repeated sequence probes can be particularly useful in rapid screening of different species and actual assignment of karyotype in instances where the cytological or morphological data are inconclusive. The RS-1 probe can be used to differentiate the presence from the absence of the C genome within the genomic combination. This was shown by the presence of the 0.8-kb SacI band on the genomic Southern blots when using this enzyme and the RS-1 probe. The pTa-71 probe can also be useful for the detection of C-genome species in the diploid state. The characteristic banding pattern in the C-genome diploids was lost when the C genome was in combination with other genomes such as the A or D genome. The formation of the tetra- or hexaploid species from C genome species was accompanied by the loss of this particular restriction fragment site or the loss of the C-genome rDNA genes.

In situ hybridization of pTa-71 to tetra- and hexaploid wheats revealed two major hybridization sites on chromosomes 1B and 6B (Flavell and Smith 1974; Flavell and O'Dell 1976; Hutchinson and Miller 1982; Miller et al. 1980). The total number of sites does not equal the sum of those present in the diploid progenitors, suggesting elimination or severe diminution. In the tetra- and hexaploid *Avena* species the number of satellited chromosomes does not equal the summation of those

present in the potential diploid progenitors (Rajhathy and Thomas 1974). Either inactivation or elimination (or severe diminution) of the rDNA sites must have occurred in some of the genomes. Studies with in situ hybridization are required in *Avena* to attempt to distinguish between the possible mechanisms.

In addition, the origin of the third pair of satellites in tetraploid *A. magna* and the hexaploids is unclear. The RS-1 probe indicated that these species contain a C genome, but the C-genome characteristic rRNA is not present in these species. Further research is required to explain the absence of the C-genome rDNA pattern in these polyploids.

The origin of the D genome is unclear. However, it should be noted that hybridization of RS-1 to hexaploid species is approximately as strong or stronger than that seen in C genome-containing tetraploids (e.g. Fig. 2). This is not what is expected if the D genome in the hexaploids is totally unlike the C genome, since the addition of the D genome should dilute the signal for a C-genome probe such as RS-1. If the C genome is completely distinct from the other genomes, tetraploids should give one-half the signal seen with C-genome diploids and hexaploids should give one-third the signal. Since this is not seen, it may be entirely possible that the D genome bears some resemblance to the C genome. Perhaps a re-examination of the similarities at the molecular level between the diploid, tetraploid and hexaploid species is in order, with the emphasis on finding similarities between the genomes of these species and the D genome in the hexaploid.

The two newly described species, *A. atlantica* and *A. agadiriana* (Baum and Fedak 1985a, b), were examined in this study as well. It was found that neither of these two species contains the C genome and, based on the results, neither contains any unique genome or distinctively different genome that could be considered a new karyotype. *Avena atlantica* is probably an A-genome diploid, and *A. agadiriana* an AB tetraploid.

The results of these studies demonstrate the usefulness of repeated sequence probes for the study of species relationships within the genus *Avena*. Repeated sequences have been implicated in the divergence of genomes and may play a role in genomic architecture, chromosome pairing, gene expression or cross compatibility (Flavell 1982, 1983). At present we do not know whether the RS-1 sequences are present in all of the chromosomes of C genomes or only in a few. Since there are no chromosome substitution lines available for the C-genome diploids in *Avena*, only in situ hybridization can determine this at the present.

In summary, repeated sequence probes have been shown to be useful for identifying specific genomic karyotypes. The use of these repeated sequence probes in analysis of different crossed material as an indicator of chromosome transfer may also be useful.

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