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Use of the polymerase chain reaction to detect spacer size heterogeneity in plant 5S-rRNA gene clusters and to locate such clusters in wheat (*Triticum aestivum* L.)

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Summary. We have used the polymerase chain reaction to analyse variation in the size of individual 5S-ribosomal gene spacer sequences. This reaction can be used to demonstrate inter- and intraspecific variation in spacer size, and combined with DNA sequencing it may thus be a valuable taxonomic tool. Two sets of nested polymerase chain reaction primers were designed to amplify the non-transcribed spacer DNA between repeated 5S-rRNA genes. These "universal" primers were used to generate fragments from the genomic DNA from several unrelated monocotyledonous plants. Ribosomal RNA spacer sequences generated in these experiments could also be used to locate 5S-rRNA gene clusters on specific chromosomes in hexaploid wheat (*Triticum aestivum*). Three distinct spacer sizes were observed after amplification. These were assigned locations on chromosomes by analysing amplification products of genomic DNA from nullisomic/tetrasomic and ditelosomic wheat stocks. "Large" 508-bp 5S repeats are located on the short arm of chromosome 5B and "short" 416-bp and 425-bp repeat unit variants are located on the short arms of chromosomes 1B and 1D, respectively. No other loci were detected. The spacer fragments were cloned, sequenced, and shown to be homologous to wheat 5S-rRNA spacers previously identified. Spacers of uniform size but with some sequence heterogeneity were shown to be located at each locus.

Key words: PCR – 5S-ribosomal RNA - Non-transcribed spacer – *Triticum aestivum* – Chromosome location

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Introduction

Determination of DNA sequence heterogeneity can provide useful taxonomic information on intra- (Rogers et al. 1986) and inter- (Jorgensen et al. 1986) specific genetic variation in plants. In particular, RFLP analysis, based on changes in the location of restriction enzyme sites, has enabled workers to construct finely detailed genetic maps of various nuclear (Sharp et al. 1988a) and extranuclear DNAs (Palmer and Stein 1986). Additional resolution may be added by sequencing specific regions of the DNA, although there are upper limits on the size of DNA sequences that can be determined routinely. Successful RFLP analysis depends upon the identification of reliable restriction enzyme and gene marker systems. When detailed analysis is required, such a system must be sufficiently sensitive to distinguish between individual species and also to detect any variation within species.

In order to develop a rapid, taxonomically useful system to define intra- and inter-specific DNA sequence variation we have used the polymerase chain reaction to amplify DNA fragments displaying sequence heterogeneity. The polymerase chain reaction (PCR) has been used to isolate and sequence specific regions of genetic variation from within the genome (Wrischnik et al. 1987). The great selectivity of the PCR allows such sequences to be preferentially amplified (Saiki et al. 1988). This is a great advantage over other more lengthy methods for their isolation such as by random cloning of similar genomic sequences. PCR-generated DNA fragments may be analysed using restriction enzymes or by directly determining their sequence. A disadvantage of the PCR is that sequence data are required as a pre-requisite for the construction of specific primers.

The clusters of 5S-rRNA genes are particularly suitable for the analysis of genetic variation using PCR. This

is because the genes occur in all of the eukaryote organisms investigated to date in tandemly repeated units comprised of a 120-bp coding region with a non-transcribed spacer of variable length between them (Dvorak et al., 1989). Whilst the genes themselves show a very high degree of sequence conservation (Long and David 1980), the non-transcribed spacer sequence can vary widely as it is apparently not under the same rigorous selection pressure. PCR primers based on a consensus sequence of the conserved 5S-rRNA coding can be used to selectively amplify the much more variable non-transcribed spacer. The amplified spacers can also be cloned easily for sequence determination so providing and additional level of resolution. We used the PCR to examine 5S-rRNA gene clusters and their spacers in several plant species, to assess variation, and to judge its value in identifying plants from their DNA.

Materials and methods

Plant material

The wheat material used was *Triticum aestivum* L. (2n = 6x = 42) cv 'Chinese Spring' and its derived Group 1 and Group 6 aneuploid nullisomic-tetrasomic (N/T) and long and short arm ditelosomic (DTL and DTS) stocks. These were N1B/T1A, N1D/T1B, DT1BS, DT1BL, DT1DS, DT1DL, N5B/T5A, N5D/T5B, DT5BL, N5D/T5A, N5A/T5D, N1A/T1B, N1A/T1D. Genomic DNA was extracted as described by Sharp et al. (1988b). Genomic DNA from the grass species *Brachypodium arbuscula* Gay ex. St. Yves, *B. sylvaticum* (Huds.) P. Beauv. and *B. distachyon* (L.) P. Beauv. was kindly donated by Xing Chi and John Draper (University of Leicester). *Gibasis karwinskyana* (Rafin.) (Commelinaceae, 2n = 20) genomic DNA was extracted according to Saghai-Maroof et al. (1984).

DNA amplification

Templates for PCR consisted of total genomic DNA. The standard amplification mixture comprised 100 ng genomic template DNA, 1 ng each primer, 250 μ*M* each dATP, dCTP, dGTP, and dTTP (Pharmacia/LKB), 5 μl reaction buffer (50 m*M* KCl, 2.5 m*M* $MgCl_2$, 10 m*M* TRIS-HCl, pH 8.2), and 2.5 units Taq polymerase (Perkin Elmer/Cetus). Twenty-seven amplification cycles were performed, each consisting of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and primer extension at 72 °C for 2 min. Based on a comparison of compiled plant 5S-rRNA sequences (Wolters and Erdmann 1988), four slightly redundant "universal" primers were synthesized, which it was hoped would facilitate the amplification of the intergenic spacer region of as many different types of plant as possible (Fig. 1). The sequences of these primers were as follows: PI: 5′-TGGGAAGTCCT(C/T)GTGTTGCA-3′(20mer); PII: 5′(T/G)T(A/C)G(T/C)GCTGGTATGATCGCA-3′(20mer); PIII: 5′-GAGAGTAGTACATCGATGGG-3′(20-mer); PIV: 5′-GGAGTTCTGACGGGATCCGG-3′(20 mer). PI and PII were used to assign chromosomal locations. Primers PIII and PIV contain ClaI and BamHI restriction sites, respectively, and were used when the amplification products were to be cloned. A BamHI site was obtained by priming from a sequence overlapping the natural BamHI site in the 5S-rRNA genes. The ClaI site was generated by including a 2-bp mismatch in the primer sequence when compared to the 5S-rRNA consensus sequence.

Fractionation of PCR products

Separation of amplification products was by electrophoresis through thin-section (2–3 mm) 4% agarose gels [(3% NuSeive agarose (ICN Biomedicals) 1% agarose (BioRad)] made up in 1 × TBE (89 m*M* TRIS-Borate, 89 m*M* Boric acid, 2 m*M* EDTA). The gels were loaded with 10 μl of PCR mixture and electrophoresed for 2 h at 10 V cm^{-1}; they were then stained with ethidium bromide and visualized using an ultraviolet transilluminator.

Cloning of amplification products

Attempts to blunt-end ligate PCR fragments generated by primers PI and PII (which contain no restriction sites) to plasmid vectors proved unsuccessful. DNA was therefore amplified using primers PIII and PIV. Total PCR mixtures (amplified using conditions identical to those described above) were extracted with phenol/chloroform and ethanol precipitated. The DNA was washed twice in 70% ethanol and dried briefly under vacuum before being resuspended in 30 μl TE buffer (10 m*M* TRIS-HCl, pH 8, 0.1 m*M* EDTA, pH 8.0). A 4-μl aliquot of 10 × TA buffer (1 × TA: 33 m*M* TRIS/acetate, pH 7.9, 66 m*M* KAc, 10 m*M* MgAc, 4 m*M* spermidine, 0.5 m*M* DTT) was added, and the volume made up to 38 μl with water. One microlitre each of BamHI and ClaI (10 U/μl) was then added and the digest incubated overnight at 37 °C. PCR primers and fragments were removed by spin dialysis through Sepharose CL-6B (Pharmacia) equilibrated with TE. Fragments were ligated to the sequencing plasmid pUBS.3 (G. Murphy, personal communica-

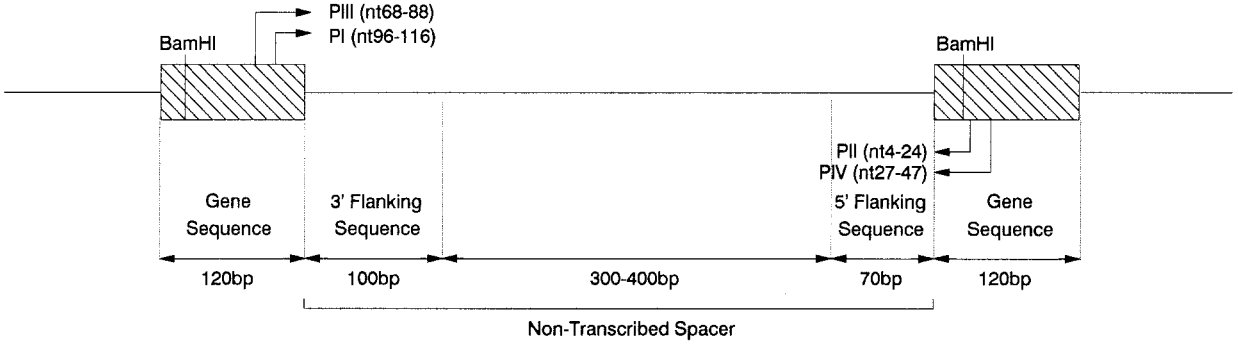

Fig. 1. The nature of the repeating units in 5S-rDNA gene clusters in wheat. The positions in the genes from which the primers initiate DNA synthesis are indicated

tion) restricted with BamHI and ClaI and transformed into *E. coli*. Recombinant clones were plated onto selective media and the colonies replica plated onto nitrocellulose filters [0.45 μm pore size, Schleicher and Schuell, (Anderman)]. Colonies were screened by colony hybridization (Maniatis et al. 1989) using as probes PCR amplified fragments labeled with ^{32}P-dCTP by random hexamer priming (Maniatis et al. 1989). Mini preparations of positive clones were prepared, and insert sizes were checked by PCR with the PI/PII primer combination before being sequenced using the improved dideoxy chain terminating method described by Murphy and Kavanagh (1988).

The Sequence Analysis Software of the Genetics Group of the University of Wisconsin was used to compare the spacer sequences.

Results

Characteristics of PCR-amplified rRNA spacers

PCR was used to amplify 5S-rRNA spacers from three species of *Brachypodium*. The results of the amplification are shown in Fig. 2. There appear to be at least three different sizes of rRNA repeats, all less than 500 bp in length, and a pattern of bands characteristic of each species is produced. However, when DNA from *Gibasis karwinskyana* was amplified a single band of approximately 250 bp was produced. This indicates that the spacer sequences between 5S-rRNA genes in *G. karwinskyana* is only 80 bp if the 5′ and 3′ flanking regions are similar in length to those of wheat (170 bp – see Fig. 1). Fragments produced from amplification of the *Brachypodium* DNA were as small as 200 bp. This shows that some *Brachypodium* intergenic regions could be only 30 bp long. This is therefore likely to be near the smallest size that can be sustained if there is to be transcription of the adjacent 5S-rRNA genes. A *Brachypodium* spacer of comparable size has recently been sequenced (Xing Chi and J. Draper, unpublished results).

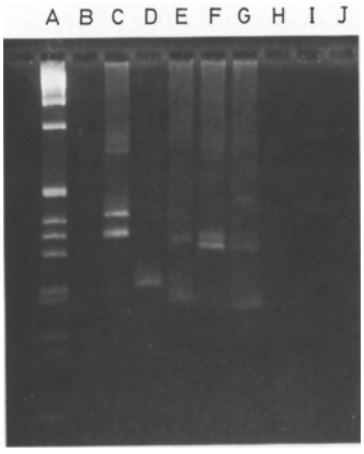

Fig. 2. Gel-fractionated products of the PCR reactions in which the spacer sequences of 5S-rRNA gene clusters have been amplified. *A* 1Kb ladder; *B* negative control; *C Triticum aestivum* cv. Chinese Spring; *D Gibasis karwinskyana*; *E Brachypodium arbuscula*; *F B. sylvaticum*; *G B. distachyon*

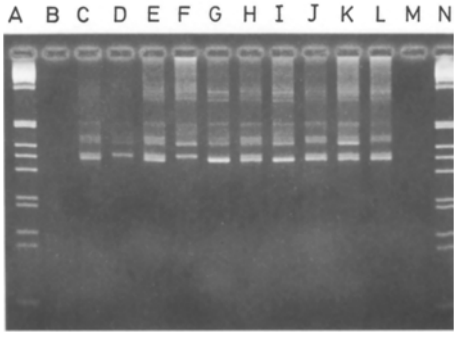

Fig. 3. Gel-fractionated products of the PCR reactions in which the spacer sequences of wheat 5S-rRNA gene clusters have been amplified. *A* 1Kb ladder; *B* blank; *C Triticum aestivum* cv Chinese Spring; *D* N1B/T1A; *E* DT1BS; *F* DT1BL; *G* N1D/T1B; *H* DT1DL; *H* DT1DS; *I* N5B/T5A; *K* N5D/T5B; *L* DT5BL; *M* blank; *N* 1Kb ladder

Numbers and location of rRNA loci in Triticum aestivum

Amplification of sequences in total genomic DNA from 'Chinese Spring' using primers PI and PII yielded three fragments (Fig. 3). Only amplification of genomic DNA from group 1 and group 6 nullisomic/tetrasomic and ditelosomic wheat stocks yielded amplification products that lacked one or other of these three fragments. It can therefore be concluded that only these chromosome groups posses 5S-rRNA template sequences. Figure 2 shows that the smallest fragment was absent in amplifications of nulli-1B stock DNA, but was increased in amount in tetra-1B stock DNA. The band was also absent in amplifications from the ditelosomic 1BL line indicating that the template 5S-rRNA sequence for this band was present on the short arm of chromosome 1B. A similar analysis of the middle-sized band located its sequence on the short arm of chromosome 1D. The sequence represented by the largest band could be placed on the short arm of chromosome 5B. No other loci were detected.

Amplifications using primers PIII and PIV gave identical results (data not shown). Examples of all three fragments were sequenced and their sequences are shown in Figs. 4 and 5. These sequences display a 98% homology with those previously determined for wheat 5S-rRNA spacers (Appels et al., 1980; Gerlach and Dyer 1980; Scoles et al. 1987). Knowledge of the nucleotide sequence of the spacers and of the position of amplification primers permits calculation of the exact length of each non-gene sequence from the three 5S-rRNA repeat units, namely:1BL:296 bp, 1DL:305 bp, 5BL:388 bp. Interestingly, considerable variation was found between different isolates of the same sequence. It is possible that some of

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1                   20                  40                  60
----T-A------TCGCGCCGC-T-----------------T-TAA-TAA-ATA------ 1Bi
----*-T------TGCGACCGC-T-----------------T-TAA-TAA-ACA------ 1Bii
----T-A------*GCGCGGCG-*-----------------T-TAA-TAA-ATA------ 1Di
----T-A------TGCGCGGCG-T-----------------A-CGC-ACG-TTG------ 1Dii
TTTT.A.TTTTTT.........T.GCAAAACAAAACGCACG.G...G...T...TTTACC CONS

61                  80                  100                 120
GT------*---*----G----AGT-CG------C------G-*---TCACG------G- 1Bi
GT------*---*----T----AGT-CG------C------G-C---TCCCG------T- 1Bii
GT------A---A----G----AGT-CG------C------G-G---TCACG------G- 1Di
AC------*---*----G----*T*-AC------T------C-C---AAATT------T- 1Dii
..GTTTTA.TTA.TTTT.CACG...G..GTAAGT.ATAGCT.G.TGC.....ATTCAC.G CONS

121                 140                 160                 180
---C-*GC*-*--CG--G---------------------------------------------- 1Bi
---C-*GCG-C--CG--G---------------------------------------------- 1Bii
---C-GGCG-*--CG--G---------------------------------------------- 1Di
---T-*CGC-C--AA--A---------------------------------------------- 1Dii
GTC.A....T.GG..TT.TGGCGCGGCAAGCGTGCACTGGTGCGGTTGAGAGGGAGGGGT CONS

181                 200                 220                 240
--------------A------*-*---------------CG*CCC--A**GC-CAAGCGA 1Bi
--------------A------T-C---------------C*GGCC--TGTAG-CAAGCGA 1Bii
--------------*------*-C---------------CGGGCC--A**GC-ATGTGCA 1Di
--------------A------T-C---------------C*GGCC--A**GC-CAAGCGA 1Dii
--------------*------*-C---------------G*****--AC*GC-CAAGCGA 1Diii
--------------*------*-C---------------G***CC--***GC-CAAGCGA 1Div
--------------*------*-C---------------C*GGCC--AG*GC-CAAGCGA 1Dv
GGAAACCGCGTTAA.CTCGTC.C.GTAGTTGAGAGGGAG......AA.....A....... CONS

241                 260                 280                 300
---**--------*********-----------------------------*-------- 1Bi
---**--------*********-----------------------------G-------- 1Bii
---**--------AGTGGAGCT-----------------------------*-------- 1Di
---GT--------AGTGGAGCT-----------------------------*-------- 1Dii
ATC..GTCTTTGT.........GGGAGGGGCAAGGATAAGGGACGAAGACC.GGGGTAAC CONS

301
----- 1Bi
----- 1Bii
----- 1Di
----- 1Dii
ATGTC CONS
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Fig. 4. Sequences of some cloned spacer regions from wheat 5S-rRNA gene clusters on chromosomes 1B and 1D. Only those bases differing from the consensus sequence are shown; (–) bases in common with the consensus sequence, (*) gaps, (•) positions of heterogeneity

this could have been generated by the PCR reaction itself, which lacks absolute fidelity since Taq polymerase has no 3′–5′ proof-reading activity (see Bloch 1991). However, the results probably indicate that there is a greater constraint on spacer length than composition.

Discussion

One of the challenges of trying to use molecular approaches to improve plant systematics is to find molecular system that give just the right level of discrimination between genotypes when comparisons are made within or between taxa. For a system to be useful it should not be too sensitive, otherwise it is difficult to interpret the results because of the difficulties in recognizing homologies. Alternatively, if it is not sensitive enough, then it may not produce sufficient numbers of useful markers. Consequently, different systems are suitable for different purposes. For instance, the use of ribosomal RNA sequences is appropriate for the study of relationships between kingdoms and phyla (Pace et al. 1986). RFLPs of chloroplast DNAs have been very useful in the study of relationships between and within angiosperm genera (Jansen et al. 1990), and for studies in man the use of RFLPs of hypervariable minisatellites (Wong et al. 1986) is now widely used in forensic science (Lander 1989). As yet, no equivalent hypervariable DNA has been detected in plants. We therefore sought a highly variable sequence that might at least be useful in distinguishing between closely related species and populations, if not between individuals.

In general it seems that spacer sequences vary much more than gene sequences, presumably because there are many fewer functional constraints conferring a selective disadvantage upon them. We therefore examined the spacer sequences between the tandemly repeated 5S-rRNA genes for useful variation in length and composition. To facilitate this comparison we used a PCR method to amplify the spacers, so simplifying the analysis by circumventing the need to do restriction enzyme digests, blots, and the preparation of probes.

```
1                   20                  40                  60
**-TTAT---A----TT**----------*-*-------------------------- 5Bi
**-**TT---A----TT**----------T-A-------------------------- 5Bii
TT-TTTT---T----****----------T-A-------------------------- 5Biii
*T-TTTG---A----TTTT----------T-A-------------------------- 5Biv
 .T....ATT.TTTT....GCGCGCCTTGT.G.CAAACGTGTCGCACGTGCGCGATATAT CONS

61                  80                  100                 120
-----TC-C*--T-T*-TTAA-G---T-*--G----C----------*---*-*---- 5Bi
-----TC-CT--T-AA-TTAA-G---T-C--G----C----------*---G-T---- 5Bii
-----*G-AC--C-AC-CA**-T---*-*--A----T----------C---G-G---- 5Biii
-----TC-CT--T-AA-TTAA-G---T-*--G----C----------*---G-G---- 5Biv
ATTAA..C..GT.A..A....T.TTT.G.AC.TTTG.GATATGTTTAAG.TCG.A.GCTC CONS

121                 140                 160                 180
-----------------------------------------------------------*- 5Bi
----------------------------------------------------------G- 5Bii
ATTGCTCGCGCGTCCTGGGGCGGCTTTGTGGCGCGAAGAGCCTGTGCTGAGAGGGGTG.G CONS

181                 200                 220                 240
------------------------*----*-------A-A*------*---------C- 5Bi
------------------------A----A-------T-GC------T---------G- 5Bii
AAAAAACTCGTGGTTGCTGCGGTAT.GGAG.GGAGGGG.G..AAACCG.GGAAAACTC.T CONS

241                 260                 280                 300
-----AT---*-*--G----------------------------*--G---------G-- 5Bi
-----TG---G-G--A----------------------------A--A---------A-- 5Bii
CTCCG..ATT.A.CG.GAGAATAAGTAGTATAGGTCATTATCCA.TT.TTAGGGAAC.GT CONS

301                 320                 340                 360
------------GG--------G-AT-------GG------*-C-**-------C----- 5Bi
------------AA--------C-GC-------AT------T-*-TT-------G----- 5Bii
TGTAATGGTAGT..AGAATGTA.A..CGTCTTT..AGCGGA.C.G..GGAGTGG.AAGCA CONS

361                 380
--------------*------------ 5Bi
--------------C------------ 5Bii
--------------G------------ 5Biii
TAAGGGACGAAGAC.GGGGAAACATGTC CONS
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Fig. 5. Sequences of some cloned spacer regions from wheat 5S-rRNA gene clusters on chromosome 5B. Identity of symbols as shown in Fig. 4

Our results clearly show that the spacer region between the 5S-rRNA genes differs in size between related species and can therefore be used as a diagnostic tool. Figure 2 clearly shows the differences in size between spacer sequences of three different *Brachypodium* species. Furthermore, we show that the spacers at different loci in the genome of hexaploid wheat can be distinguished from one another (Fig. 3), thus permitting us to map their locations.

As 5S-rRNA gene spacers are fairly short, they can be sequenced comparatively easily, which is an additional advantage in their study. This refines the analysis further as changes in sequence composition need not necessarily be reflected in changes in sequence length. In fact, our results show that in wheat, while the spacer sequences at each of the three loci are uniform in size, there is heterogeneity in composition within each size group. This is indicated by the minor differences in the sequence of individual cloned molecules of each type (Figs. 4, 5).

Our results concerning the location of 5S-rRNA gene clusters in wheat are consistent with those found by in situ hybridization by Scoles et al. (1988) and by Reddy and Appels (1989). However, using the same method in conjunction with a probe derived from rye, but with increased sensitivity, Mukai et al. (1990) also found additional hybridization to chromosomes 1A, 5A, and 5D. Despite the sensitivity of the PCR method in detecting single and low copy number sequences, we could find no evidence of the tandem repeats at these additional loci. We therefore suggest that the extra hybridization signals found by Mukai et al. (1990) could have been given by vestigial 5S-rRNA gene loci that were inactivated following polyploidization, possibly caused by spacer deletion. We do find some large amplified material in our PCR reaction mixtures whose origins are as yet unresolved. Since these products occurred as regular sizes, they may have been amplification products of more than one 5S-rRNA repeat unit. This could occur during amplification when primers are not able to saturate all of the homologous binding sites at the rRNA loci. Some of these products could also be derived from vestigial sequences. Clearly, further analysis of the 5S-rRNA repeat sequences is required to resolve this question.

Comparisons between the two short and the long spacer sequences reveal considerable homology between them (Fig. 6) and show that the main differences are the result of two insertion/deletion events. The extra DNA in the longer forms show little homology with the remaining

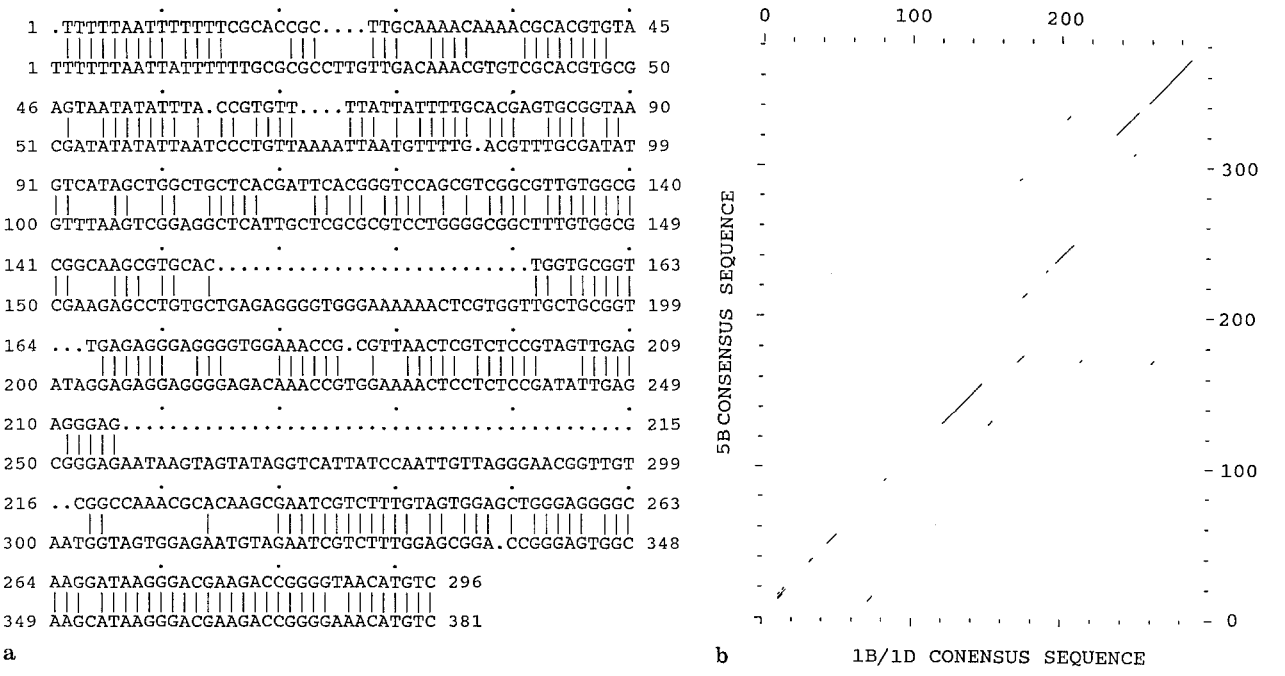

Fig. 6a,b. Comparison of the short and long spacer sequences of th 5S-rRNA gene clusters of wheat. **a** GAP alignment. **b** DOTPLOT made by aligning 21 base segments of sequence and registering those with homology between 14 or more of these

sequences, so presumably it did not arise by the duplication of segments of the shorter sequences. Thus, it seems more probable that the shorter spacers were derived from the longer ones, as a result of deletions rather than vice versa, through insertions or duplications.

Interestingly, the spacer fragment in the *Brachypodium* species is particularly short (200 bp) compared with those of most other organisms in which similar arrays of 5S-DNA have been studied. As the 5′-flanking region is involved in transcription factor and RNA polymerase III binding (Kassavetis et al. 1990) and the 3′-flanking region is involved in termination (Geiduschek and Tocchini-Valentino 1988), there is perhaps little non-essential 5S-spacer DNA in such organisms.

The present work has demonstrated the usefulness of the PCR method for amplifying variable DNA sequences flanked by conserved sequences to which "universal" primers can be made. The tandem repeats containing the 5S-rRNA gene sequences are particularly amenable to this treatment, but others such as the spacer sequence in chloroplast and mitochondrial DNA may also be suitable for examination in a similar way. Cloned rRNA spacers make ideal probes for the localization of rRNA clusters by in situ hybridization where nullisomic/tetrasomic stocks are not available. PCR carried out in conjunction with DNA sequencing can be of considerable additional use in obtaining quantitative data. It seems likely that such techniques will soon have a major impact on plant systematics (Fukuda 1990).

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