Identification of a 5S rDNA spacer type specific to *Triticum urartu* and wheats containing the *T. urartu* genome

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Abstract: A PCR system was designed to amplify 5S spacer rDNA specifically from homeologous chromosome 1 in a variety of species representative of the *Aegilops* and *Triticum* genera. Two polymerase chain reaction (PCR) primer combinations were used, one of which appears to be apomorphic in nature and specific to chromosome 1A in *Triticum urartu* and tetraploid and hexaploid wheats containing the AA genome donated by *T. urartu*. The value of studying single repeat types to investigate the molecular evolution of 5S-rDNA arrays is considered.

Key words: 5S rDNA, polymerase chain reaction, *Triticum*, wheat.

Résumé : Un système d'amplification PCR (« polymerase chain reaction ») a été mis au point pour amplifier spécifiquement l'espaceur d'ADNr 5S situé sur les chromosomes homéologues du groupe 1 chez diverses espèces appartenant aux genres *Aegilops* et *Triticum*. Deux combinaisons d'amorces ont été utilisées dont l'une est apomorphe et spécifique du chromosome 1A chez le *Triticum urartu* ainsi que chez les blé tétraploïdes et hexaploïdes possédant le génome AA provenant du *T. urartu*. Les auteurs discutent de l'intérêt que présente l'étude de types uniques de ces séquences répétées en vue de mieux comprendre l'évolution moléculaire des amas d'ADNr 5S.

Mots clés : ADNr 5S, réaction de polymérisation en chaîne, *Triticum*, blé.

[Traduit par la Rédaction]

Introduction

In eukaryotes, the 5S rRNA gene encodes a highly conserved 120-bp molecule (Erdmann and Wolters 1986) that complexes within the large subunit of the ribosome with the 28S ribosomal RNA and ribosomal proteins. In animals and plants, the 5S rRNA gene is separate from the main rDNA units, often at multiple loci (e.g., Goldsborough et al. 1981 for *Linum*; Dvorák et al. 1989 for *Triticum*; Reddy and Appels 1989 for *Secale*), in tandem arrays made up of varying numbers of repeating units (e.g., Lagudah et al. 1989). The repeating unit consists of the gene and spacer, the latter varying from 100–700 bp at different loci (Sastri et al. 1992), with a few exceptions, such as the 30-bp spacer of *Brachypodium* (Cox et al. 1992). The precise function of the spacer is not known, though it has been suggested that the 5′ region contains a transcription initiation signal (Gerlach and Dyer 1980) and that the 3′ region is involved in transcription termination (Scoles et al. 1988).

The 5S-rDNA spacer evolves rapidly with apparently little homogenization between paralogous (Scoles et al. 1988) or orthologous loci (Dvorák et al. 1989). This has led to hopes that the spacer might be useful for phylogenetic purposes, particularly between closely related species such as those of the Triticeae. In Triticeae, two principal lineages of 5S rDNA have been identified, *5S-DNA-1* and *5S-DNA-2*, most readily defined by the lengths and sequence compositions of their spacers (Gerlach and Dyer 1980; Dvorák et al.1989; Appels et al. 1992; Baum and Appels 1992). In *Triticum*, the *5S-DNA-1* and *5S-DNA-2* spacers are 200–349 bp and 350–380 bp, respectively (Appels et al. 1992), the size difference being due to an insertion–deletion in the mid-spacer region. The *5S-DNA-1* and *5S-DNA-2* loci occur on different chromosomes but the identities of these chromosomes vary among the Triticeae. In *Triticum* and *Secale*, the *5S-DNA-1* units are on chromosome 1 and the *5S-DNA-2* units are on chromosome 5 (Baum and Appels 1992), but in *Hordeum* the smaller units are on chromosome 2 (Kolchinsky et al. 1990) and the larger units on chromosome 3 (Kanazin et al. 1993). This could indicate that smaller 5S-rDNA arrays are present in *Triticum* and *Secale* on chromosomes other than 1 and 5, there being precedents for the existence of minor rDNA arrays (Dubcovsky and Dvorák 1995).

It appears that the *5S-DNA-1* lineage can be further subdivided. Dvorák et al. (1989) found that on homeologous chromosomes 1B and 1D of hexaploid wheats, the 5S-rDNA repeating units have a periodicity of 410 bp, but failed to detect any locus on chromosome 1A. Diploid species with A genomes (e.g., *T. monococcum*) do have a locus on chromosome 1, but with a repeating periodicity of 360 bp, corresponding to a spacer length of 240 bp. Sequences of this approximate length have been obtained from *T. aestivum* cv. Chinese Spring (Appels et al. 1992), implying the possible

Corresponding Editor: P.B. Moens.

Received May 17, 1999. Accepted October 7, 1999.

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existence of a locus on chromosome 1A of cultivated wheats, although no such suggestion was made by the authors.

In this study, we designed a polymerase chain reaction (PCR) system that would amplify a target specifically from the spacer region of the 360-bp repeating units to determine if a 5S-rDNA locus is present on chromosome 1A of cultivated wheats. The PCR system was then used to survey a wide range of *Triticum* and *Aegilops* accessions.

Materials and methods

Wheats were obtained from the Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany, and the Institute of Plant Science Research Collection of Wheat and Related Species, Norwich, U.K. One wheat was obtained from Dr. Glynis Jones, University of Sheffield, U.K., and one sample of DNA from Dr. Marcus Hamer, University of Manchester, U.K. Nullisomic and ditelosomic stocks of *T. aestivum* 'Chinese Spring' were obtained from Mr. Terry Miller, John Innes Centre, Norwich, U.K.

Nucleic acids were extracted from grains of wheat using a modification of the CTAB (cetyltrimethylammonium bromide) protocol (Rogers and Bendich 1985) as described by Sallares et al. (1995). To design PCR primers, the sequences published by Appels et al. (1992) were aligned (Fig. 1). Two upstream primers were used, each with a 5′-extension of 10 bp containing a *Bam*HI site. The first upstream primer (primer A) targeted a sequence present in both the 360-bp and the 410-bp repeating units; the second upstream primer (primer B) was specific for the 360-bp repeating unit. Neither primer A nor primer B had annealing sites for the longer repeating units associated with the *5S-DNA-2* lineage on chromosome 5 as shown in published sequences. The downstream primer (primer C) spanned the *Bam*HI site within the 5S gene (see Fig. 1). PCRs were 100 μL in volume and contained 300 ng of each primer, 150 μM each dNTP, 10× buffer (Promega), 10–100 ng template DNA, and 2.5 units of Taq DNA polymerase (Promega). Cycling conditions were: 2 min at 94°C; 2× 2 min at 58°C, 1 min at 74°C, 1 min at 94°C; 2× 2 min at 57°C, 1 min at 74°C, 1 min at 94°C; 30× 2 min at 56°C, 1 min at 74°C, 1 min at 94°C; 2 min at 56°C, 8 min at 74°C. Electrophoresis was carried out at 3.33 V/cm in 3% NuSieve (FMC BioProducts, Rockland, Maine).

Results

The available sequence data indicated that PCRs with primers A and C should have given two products: one of 242 bp corresponding to the *5S-DNA-A1* locus, and one of 326 bp corresponding to the *5S-DNA-B1* and *5S-DNA-D1* loci. Primers B and C should have produced just a single product of 241 bp corresponding to the *5S-DNA-A1* locus.

The results of PCRs with diploid species are summarized in Table 1A. We examined seven cultivated einkorns, three wild einkorns and eight *Aegilops* species spanning a range of genome types. All of the AA diploids gave the expected product, approximately 242 bp, after PCR with primers A and C. Unexpected products of about 400 bp were obtained with some diploid accessions, and *T. urartu* gave an additional product of 370 bp. All of the *Aegilops* species gave a product of 326 bp with primers A and C, this being the predicted size for the *5S-DNA-D1* locus, except *Ae. speltoides*, whose products were 370 and 400 bp. Of the diploids, only *T. urartu* gave a product (of approximately 241 bp) after PCR with primers B and C.

Tetraploid wheats of both the AABB and AAGG lineages were examined, as well as *Ae. ovata*, whose genomes are described as UUM^oM^o (Table 1B). Amplifications using the B–C primer combination gave the expected product of approximately 241 bp for all the tetraploids tested with the exception of *Ae. ovata*. PCRs with primers A and C gave 326-bp products with the AABB tetraploids and with *Ae. ovata*, sometimes accompanied by a 400-bp product. The AAGG species gave only the 400-bp product.

All hexaploid wheats studied, including both the AABBDD and AAAAGG lineages, gave a 241-bp product after PCR with the B–C primer combination (Table 1C). Results with primers A and C were similar to those obtained with tetraploids; all AABBDD wheats gave a 326-bp product not seen with the AAAAGG species *Triticum zhukovskyi*. The latter gave a 400-bp product that was occasionally obtained with AABBDD wheats. *T. zhukovskyi* also gave a 242-bp product with primers A and C.

Several nullisomic and ditelosomic preparations of *T. aestivum* 'Chinese Spring' were tested with the B–C primer combination (Table 1D). Stocks in which chromosome 1A was absent (N1AT1B and N1AT1D) did not yield any PCR product. The ditelosomic stock in which the short arm of chromosome 1A was present (DT1AS) and the long arm of 1A was absent gave a 241-bp product, while the stock in which the long arm of 1A was present (DT1AL) and the short arm absent not did not give any product.

Discussion

The results summarized in Table 1 show that the two primer pairs are specific to different 5S-rDNA repeat types, with the B–C combination only amplifying sequences from chromosome 1A of *Triticum urartu* and tetraploid and hexaploid wheats containing the AA genome donated by *T. urartu*. This is indicated by the absence of products when primers B and C were used with diploids other than *T. urartu*, together with the uniform occurrence of the 241-bp product in these PCRs with tetraploids and hexaploids other than *Ae. ovata*. PCRs with nullisomic and ditelosomic stocks of *T. aestivum* 'Chinese Spring' show that primers B and C amplify sequences from the short arm of chromosome 1A in 'Chinese Spring,' demonstrating the existence of an rDNA locus on chromosome 1A in cultivated wheats. The consistency of the sizes of the products suggests that this sequence type is a recent apomorphism. The results support the proposal that *T. urartu* is the A-genome donor of both the B- and G-genome lineages of tetraploid and hexaploid wheats (Dvorák et al. 1993).

The A–C primer pair gave a 326-bp product with all the tetraploids except *T. araraticum* and *T. timopheevi* (AAGG wheats), and all the hexaploids except *T. zhukovskyi* (AAAAGG). This product is presumably due to amplification of the *5S-DNA-B1* and (or) *5S-DNA-D1* loci, so its absence in the G-lineage wheats was anticipated. The A–C combination also gave 242-bp products with all the AA diploid wheats, although with *T. urartu* only a faint band could be seen after electrophoresis. The wide range of species, including members of both the *Aegilops* and *Triticum* genera, yielding this product suggests that the target sequence is an

Fig. 1. Alignment of 360-bp *5S-DNA-1* units of wheat and *Hordeum* (Appels et al. 1992). EMBL accession numbers are Z11449, Z11450, Z11416, and Z11417 for the four *T. aestivum* sequences, Z411461 for *T. monococcum*, and Z11436 for *Hordeum*. Sequences were aligned with CLUSTAL W (Thompson et al. 1994). Dots show identities with the consensus sequence and dashes represent insertions–deletions. The 5S gene region is shown in bold type and the priming sites are underlined. Two variant primers (A and B) were designed to anneal to the upstream region, these primers also including a 10-nucleotide 5′ extension containing a *Bam*HI restriction site. The site for primer A is shown in the upper sequence and the site for primer B in the second sequence from the top. The downstream primer (C) spans a naturally occurring *Bam*HI site in the 5S-rDNA gene region. The predicted product sizes are 242 bp for primers A and C and 241 bp for primers B and C. According to the published sequence information (not shown), primers A and C should also amplify a 326-bp product from the 410-bp repeat units at the *5S-DNA-B1* and *5S-DNA-D1* loci.

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T. aestivum    -TTTTTAA-T-TTTTTTCGCGCCGCTTGCAAAACAAAACGCACGTGTAAGTAATATATTTACCGTGTTTTATTATTTTACACTGGT
T. aestivum    -.......A.-......T..ATG..A.......G.....T..T.GCCGT.AC..........GAC............G...--..
T. aestivum    -.......-.-.....................................................................
T. aestivum    -.......A.-......T..ATG..A.......G.....T..T.GCCGT.AC..........GAC............G...--..
T. monococcum  -.......-.-.A....T...TGA.G.....................CGC.GC...........AC....C........G...--..
Hordeum        C.......A.A.A....T.....A.G..AC..GG.TG....GG.A.CGT.ATC.....G-...TCA...AC.......TG..-.A.

T. aestivum    CGGTTGAGAGGGAGGAGTGGAAACCGCGTTAAACTCGTCTCCGTAGTTAAGAGGGAGCAGCCAAAGCAATGTACAATCGTCTTTGT
T. aestivum    TT.C--------.GTA...TTT.G.T-....G--G..A.TATT-C.CGCGT.TA.CG..G.G........A.............
T. aestivum    ................................................................................
T. aestivum    TT.C--------.GTA...TTT.G.T-....G--G..A.TATT-C.CGCGT.TA.CG..G.G........A.............
T. monococcum  TT.C--------.GTA...TT-.G.T-....G--...A.GATT-C.CGCGT.TA-CG..G.G........A..G...C...G.G...
Hordeum        T-AC.-------.GT..C.TT---------------..-C.A...CGC..G-------A..C....--.G.C---T.G.A..A---..

T. aestivum    AGTGGAGCTGGGAGGGGCAAGGATAAGGACCGAAGCCCG-GGGTGCCATGTCGGATGCGATCATACCAGCACTAAATCACCGGATC
T. aestivum    ..................C......CA.....A.G.-....AA..C.........T.........................
T. aestivum    ..........................................-.....................................
T. aestivum    ..................C......CA.....A.G.-....AA..C.........T.........................
T. monococcum  ......-.............C......GA.........A-....AA.....................G.........
Hordeum        ...A.---.A.T.........C.......A.A..--TA.ATA..TG.....................................

T. aestivum    CCATCAGAACTCCGAAGCTAAGCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAAGTCCTCGTGTTGCATTCCC
T. aestivum    ..................T.....................G.....................A.....................
T. aestivum    ................................................................................
T. aestivum    ..................T.....................G.....................A.....................
T. monococcum  ..................T.....A..............................................................
Hordeum        ..................T.............T.....................................................
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ancient, hence plesiomorphic, repeat type. The 242-bp product was also amplified from *T. zhukovskyi*, as expected because this wheat results from hybridization between *T. timopheevi* (an AAGG tetraploid) and a diploid AA species, probably *T. monococcum* (Dvorák et al. 1988; Dvorák et al. 1993). The absence of the 242-bp product after PCRs with other tetraploids and hexaploids was unexpected, especially because the primer pair had been designed to amplify products of this length from the sequences of the 360-bp repeat units reported from *T. aestivum* 'Chinese Spring' by Appels et al. (1992). A possible explanation is indicated by the results of A–C PCRs with diploid species. According to these PCRs, the short 5S-rDNA repeat is wholly or predominantly of the A–C primer type for all AA diploids except *T. urartu*, the latter containing short repeats of both the A–C and B–C types, though judging from the intensities of the PCR products, the A–C type has a lower copy number. The absence of a 242-bp product after A–C PCRs with tetraploid and hexaploid wheats suggests that in polyploids, the copy number of the A–C repeat type has been further diminished beyond the detecting ability of this PCR system. However, the sequences obtained by Appels et al. (1992) show that the A–C repeat type has not been completely eradicated from the array. Its replacement with the B–C repeat type provides an opportunity to study locus homogenization, and hence concerted evolution (e.g., Dover 1982).

PCRs with primers A and C occasionally gave products of 300, 370, and (or) 400 bp. The sporadic occurrence of these products implies that they derive from 5S-rDNA loci that are amplified inefficiently by these primers. This is either because their arrays are relatively short, or because there are sequence variations within one or both of the priming sites. When PCRs were carried out with *Triticum* species, these minor products were always accompanied by a product of the expected size (either 242 or 326 bp) except for the two A^uA^uGG tetraploids, which gave only the 400-bp product. This result could suggest that the minor array that gives rise to the 400-bp product in *T. urartu* has become the predominant 5S-rDNA locus in the G-series tetraploids. According to this hypothesis, the 400-bp product obtained after PCR of the $A^uA^uA^mA^mGG$ hexaploid *T. zhukovskyi* derives from the tetraploid lineage, and the 242-bp product from the *T. monococcum* partner in the hybridization that led to this hexaploid.

Relevance to studies of 5S rDNA evolution

A principal objective of comparative biology is to identify homologous structures in different organisms. In molecular evolutionary studies, the prevalence of paralogy through duplicating mechanisms, as during 5S rDNA evolution (Scoles et al. 1988), can make this objective difficult to achieve. It was noted by Gerlach and Dyer (1980), and later by Appels et al. (1992), that in *Triticum* the sequences of spacers in a single 5S-rDNA array are heterogeneous, varying by 2–13% with an average heterogeneity of 5%. Kellogg and Appels (1995) showed that within a single array the genes are as variable as the spacers, and that 5S rDNA variation within a species is equal to or greater than that between species, al-

Table 1. Results of PCRs.

Accession[a]	Source[b]	Genomes	PCR product sizes (bp)[c] A and C	PCR product sizes (bp)[c] B and C
(A) Diploids				
T. boeoticum Boiss. subsp. *aegilopoides*	IPSR 1030001	A^m	242	none
T. boeoticum Boiss. subsp. *thaoudar*	IPSR 1020009	A^m	242	none
T. monococcum L.	G. Jones	A^m	242, 400	none
T. monococcum L. subsp. *atriaristatum*	IPSR 1040023	A^m	242	none
T. monococcum L. subsp. *flavescens*	Gat 432475	A^m	242, 400	none
T. monococcum L. subsp. *macedonicum*	IPSR 1040022	A^m	242	none
T. monococcum L. subsp. *nigricultum*	Gat Schg 3 17	A^m	242	none
T. monococcum L. subsp. *vulgare*	Gat Schg Z 16	A^m	242	none
T. sinskajae A. Filat and Kurk.	IPSR 1050001	A^m	242, 400	none
T. urartu Tum.	IPSR 1010011	A^u	242, 370, 400	241
Ae. bicornis (Forsk.) Jaub. and Spach.	IPSR 2190001	S^b	326	none
Ae. caudata L.	IPSR 2090001	C	326	none
Ae. comosa Sibth. and Sm.	IPSR 2110001	M	326	none
Ae. longissima Schweinf. and Muschl.	IPSR 2150001	S^l	326	none
Ae. mutica Boiss.	IPSR 2130001	Mt	326	none
Ae. squarrosa L.	IPSR 2220001	D	326	none
Ae. speltoides Tausch	IPSR 2140001	S	370, 400	none
Ae. sharonensis Eig.	IPSR 2180001	Sl	326	none
(B) Tetraploids				
T. araraticum Jakubz. subsp. *nachitchevanicum*	IPSR 1150002	A^uG	400	241
T. carthlicum Nevski subsp. *persicum*	IPSR 1090001	A^uB	326, 400	241
T. dicoccoides (Korn) Schweinf.	IPSR 1060017	A^uB	326, 400	241
T. dicoccum (Schrank.) Schulb.	IPSR 1070004	A^uB	326, 400	241
T. dicoccum (Schrank.) Schulb. subsp. *aethiopicum*	IPSR 1140001	A^uB	326	241
T. dicoccum (Schrank.) Schulb. subsp. *farrum*	Gat Schg 5 21	A^uB	326	241
T. timopheevi Zhuk.	IPSR 1160001	A^uG	400	241
Ae. ovata L.	IPSR 2020001	UM^o	326	none
(C) Hexaploids				
T. aestivum L.	IPSR 1190291	A^uBD	300, 326, 400	241
T. aestivum L.	IPSR 1190293	A^uBD	326, 400	241
T. aestivum L.	IPSR 1190305	A^uBD	300, 326	241
T. aestivum L.	IPSR 1190638	A^uBD	300, 326	241
T. aestivum L. 'Galahad'	M. Hamer	A^uBD	326, 400	241
T. compactum Host.	IPSR 1200012	A^uBD	326	241
T. macha Dek. and Men.	IPSR 1190291	A^uBD	326	241
T. sphaerococcum Perc.	IPSR 1210001	A^uBD	326	241
T. vavilovi (Tum.) Jakubz.	IPSR 1230001	A^uBD	326	241
T. zhukovskyi Men. and Er.	IPSR 1260001	A^uA^mG	242, 400	241
(D) Nullisomic and ditelosomic stocks				
T. aestivum L. 'Chinese Spring' (parent)	T. Miller	A^uBD	not tested	241
N1AT1B	T. Miller		lacks chromosome 1A	not tested
N1AT1D	T. Miller	lacks chromosome 1A	not tested	none
DT1AS	T. Miller	lacks chromosome 1A long arm	not tested	241
DT1AL	T. Miller	lacks chromosome 1A short arm	not tested	none

[a]The nomenclature and taxonomy of Miller (1987) is followed here. *Triticum* and *Aegilops* are retained as two separate genera for the sake of convenience.

[b]Gat, Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben; IPSR, Institute of Plant Science Research Collection of Wheat and Related Species, Norwich, U.K.

[c]Longer products were sometimes seen, presumably due to amplification of adjacent repeating units, but are not listed.

though between-species variations in the gene are generally fixed at a much lower frequency than in the spacer. Consequently, 5S-rDNA coding regions are phylogenetically uninformative and considered to be unresolvable. Appels et al. (1992) assigned spacer sequences to loci on the basis of similarity to sequences from wild progenitors, but this can not

be done for all sequences, such as those obtained by Cox et al. (1992), due to high instances of homoplasy. Currently it is unclear how spacers could be employed for phylogenetic purposes, or indeed how evolution progresses in a 5S-rDNA array. Kellogg and Appels (1995) suggest a mechanism of 5S rDNA evolution in which selection acts on the rDNA array as a whole, a minimum number of functional gene copies being required for survival, as has been postulated for *Drosophila* (Schlötterer and Tautz 1994). To understand better the process of 5S rDNA evolution, it might be useful to reassess the rationale for selection of sequences for comparison. Any single organism may have several 5S-rDNA arrays, each of which is made up of multiple repeat types. Different repeat types are essentially paralogous in relation to each other, but may also pre-date the species in which they occur. Therefore, in phylogenetic analyses it might be more informative to consider single 5S-rDNA repeat types, separate from the other repeat types within an array, to increase the probability of comparing orthologs. By studying nucleotide diversity of the spacers and genes within a particular repeat type it might be possible to obtain a more accurate idea of how a 5S-rDNA array changes with time. The design of PCR systems that enable amplification products derived from different repeat types to be identified, because they are either the only products obtained with a particular pair of primers, or because the sizes of the products are diagnostic, is the first step towards this goal.

Acknowledgements

This work was supported by a grant from the Natural Environment Research Council. We thank Terry Miller, John Innes Centre, Norwich, and Glynis Jones, University of Sheffield, for providing wheat specimens, and Marcus Hamer, University of Manchester, for providing a sample of DNA.

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