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Conversion of a RAPD-generated PCR product, containing a novel dispersed repetitive element, into a fast and robust assay for the presence of rye chromatin in wheat

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Abstract Bulk segregant analysis was used to obtain a random amplified polymorphic DNA (RAPD) marker specific for the rye chromosome arm of the 1BL.1RS translocation, which is common in many high-yielding bread wheat varieties. The RAPD-generated band was cloned and end-sequenced to allow the construction of a pair of oligonucleotide primers that PCR-amplify a DNA sequence only in the presence of rye chromatin. The amplified sequence shares a low level of homology to wheat and barley, as judged by the low strength of hybridization of the sequence to restriction digests of genomic DNA. Genetic analysis showed that the amplified sequence was present on every rye chromosome and not restricted to either the proximal or distal part of the 1RS arm. *In situ* hybridization studies using the amplified product as probe also showed that the sequence was dispersed throughout the rye genome, but that the copy number was greatly reduced, or the sequence was absent at both the centromere and the major sites of heterochromatin (telomere and nucleolar organizing region). The probe, using both Southern blot and *in situ* hybridization analyses, hybridized at a low level to wheat chromosomes, and no hybridizing restriction fragments could be located to individual wheat chromosomes from the restriction fragment length polymorphism (RFLP) profiles of wheat aneuploids. The disomic addition lines of rye chromosomes to wheat shared a similar RFLP profile to one another. The amplified sequence does not contain the RIS 1 sequence and therefore represents an as yet undescribed dispersed repetitive sequence. The specificity of the amplification primers is such that they will provide a useful tool for the rapid detection of rye chromatin in a wheat background. Additionally, the relatively low level of cross-hybridization to wheat chromatin should allow the sequence to be used to analyse the organization of rye euchromatin in interphase nuclei of wheat lines carrying chromosomes, chromosome segments or whole genomes derived from rye.

Key words Wheat · Rye · RAPD · PCR · *In situ* hybridization · Dispersed repeat

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Introduction

Rye (*Secale cereale*) is a widely used source of alien genes in bread wheat (*Triticum aestivum*) improvement. In particular, the translocations of the short arm of chromosome 1R to the long arms of 1A, and especially 1B, occur frequently in many selections emerging from wheat breeding programmes, as this rye chromosome arm carries genes for resistance to a number of diseases and pests, and is associated with increased yield potential across a wide range of environments (Rajaram et al. 1983).

A number of techniques are available to identify the presence of the 1BL.1RS translocation. These rely variously on polymorphism between rye and wheat at the protein level, on chromosome morphology or staining, on reactions to plant pathogens or on the use of species-specific DNA probes (reviewed in Javornik et al. 1991). These authors made a comparison of some of these techniques, and concluded that a biochemical marker such as the subtilisin inhibitor described by Koebner (1990) was the most convenient way to screen for the presence of the translocation in populations. A disadvantage of this, or any other gene product marker, is its specificity to a particular locus, while the more generalized methods that can successfully use DNA hybridization on membranes or *in situ* to detect rye chromatin are probably too time- and resource-consuming to be applied to the large numbers of individuals commonly produced in breeding populations.

With the advent of the polymerase chain reaction (PCR), it has now become possible to detect DNA polymorphism without the need for either the extensive sample purification or the use of DNA labelling that are required for South-

ern hybridization. In this study, we describe the production of oligonucleotide primers which preferentially amplify template DNA that includes rye chromatin, so that they can be used as a simple and robust PCR-based screen for the presence of 1RS, or indeed any other wheat-rye translocations. The PCR method exploited the probability of finding random amplified polymorphic DNA (RAPDs) distinguishing wheat and rye DNA, given that the frequency of polymorphism in interspecific comparisons in the Triticeae is reportedly high (King et al. 1993). The intrachromosomal localization of the resulting PCR product was elucidated by *in situ* hybridization, which represents an alternative strategy to the PRINS (primed *in situ*) technique (Gosden and Hanratty 1991), having the advantage of a more easily controllable stringency, which remains a problem when hybridizing short oligomers *in situ* for the purposes of performing PRINS.

Materials and methods

Plant selection and DNA extraction

Unreduced total protein extracts from endosperm halves of individual seeds of an F_3 from the cross bread wheat cv 'Chinese Spring'×cv 'Glennson' (carrying 1BL.1RS) were obtained and electrophoretically separated by SDS-PAGE, following Singh and Shepherd (1985). This allowed the presence of the prolamins encoded by *Gli-B1* and *Sec-1* (located on chromosome arms 1BS and 1RS, respectively) to be analysed. The presence of these markers was taken to infer the presence of the whole relevant chromosome arm, since recombination between these homoeologues is extremely rare. Ten individuals carrying both 1BS and 1RS ('++') and 10 carrying 1BS, but lacking 1RS ('+–') were selected in this way, and DNA was extracted from leaves of the resulting plants as described by Sharp et al. (1988). These DNAs were combined to form two bulk DNA samples, '++' and '+–'. DNA was similarly obtained from 12 wheat cultivars, two inbred rye cultivars, the disomic addition lines of rye cv 'Imperial' chromosomes (Driscoll and Sears 1971) and of barley (*Hordeum vulgare*) chromosome 7H (Islam et al. 1978) into wheat cv 'Chinese Spring', 21 of the nullisomic-tetrasomic set, chosen to ensure that each wheat chromosome is nullisomic in one line, of cv 'Chinese Spring' (Sears 1966), the wheat/rye recombinants which carry different segments of chromosome 1RS, as described in Rogowsky et al. (1991), and their parents cvs 'Gabo' and 'Gabo' 1DL.1RS (Shepherd 1973). Some DNA samples for PCR were extracted from endosperm tissue following the protocol of King et al. (1993).

RAPD, PCR, Southern analysis and the construction of PCR primers

A screen of random 10-mers (Operon Technologies, kits A–N, T–Z) was used to screen the '++' and the '+–' bulks. PCR conditions were as reported in King et al. (1993). The relevant PCR product, obtained from amplification from template DNA of cv 'Macvanka' (a carrier of the 1BL.1RS translocation), was excised from low melting point agarose gels, purified by Magic PCR (Promega) and cloned into pGem-T (Promega) following the manufacturer's instructions. The insert was sequenced directly from the plasmid, and the sequence data were used to design a number of oligomers. PCR, using these approximately 20-mer primers (denoted AF1 and AF4), employed the same reaction mixture as above, with 0.1 μ*M* of each of the primers, amplified for 45 cycles of 15 s at 94°C, 60 s at 55°C and 60 s at 72°C, with a 5 min extension at 72°C following the final cycle. In later experiments, this was simplified to 35 cycles of 15 s at 94°C, 45 s at 60°C and 15 s at 72°C. For Southern hybridization, the PCR-amplified product was purified from low-melting-point agarose (Magic PCR Preps – Promega), and labelled with [^{32}P], using standard techniques. Subsequent procedures followed Devos et al. (1992).

In situ hybridization

Chromosome spreads of bread wheat cv 'Beaver' (carrying 1BL.1RS) and triticosecale cv 'TC400' (a triticale with 14 rye chromosomes and 28 wheat (AB) chromosomes) were prepared using the dropping method described in Schwarzacher and Leitch (1994) for *in situ* hybridization. The probe, called AF1/AF4, was the 1.5-kb PCR amplification product generated from the cv 'Macvanka' template using the primers AF1 and AF4 (primer construction described in results), and was labelled with digoxigenin (Boehringer Mannheim) using the nick translation reaction. The *in situ* hybridization reaction was modified from Leitch et al. (1993). The hybridization mixture was prepared to a final concentration of 2.5 μg ml^{-1} labelled probe DNA, 20–40 μg ml^{-1} sheared unlabelled wheat cv 'Chinese Spring' DNA, 50% formamide, 10% dextran sulphate, 0.1% SDS (sodium dodecyl sulphate) and 2×SSC (0.3 *M* sodium chloride, 0.03 *M* sodium citrate) and denatured at 70°C for 5 min. A small volume (40 μl) of the denatured hybridization mixture was added to each slide, a coverslip applied and the slide incubated at 82°C for 10 min. The slides were then transferred to an incubator at 37°C and the probe allowed to hybridize *in situ* overnight. The slides were then given a stringent wash in 20% formamide, 0.1×SSC at 42°C for 15 min and washed in 2×SSC at 42°C for 10 min. The stringent wash allowed DNA sequences with more than about 80% sequence identity to remain hybridized if the parameters used by Meinkoth and Wahl (1984) can be extrapolated to the *in situ* situation. Sites of probe hybridization were detected using 20 μg ml^{-1} fluoresceinated anti-digoxigenin (Boehringer Mannheim) in 4×SSC containing 0.2% (v/v) Tween 20 and 5% (w/v) bovine serum albumin. Chromosomes were counterstained with 2 μg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole) in 4×SSC, mounted in Vectashield (Vector Laboratories) medium for fluorescence, examined using a Leitz Aristoplan epifluorescent microscope and photographed using Fujicolor 400 colour film.

Results

RAPD analysis

Figure 1 shows the result of amplifying the template of the '++' and '+–' bulk DNAs with a range of 10-mer primers. A total of 413 primers were screened in this way, but only 1, OPH20 (5′-GGGAGACATC), produced a polymorphism in this comparison. The OPH20-amplified profile of each bulk consisted of several bands, with strongly amplified products of size 1.9 kb and 0.5 kb common to both; a 1.5 kb product was present in the '++' bulk but absent in the '+–' bulk. This latter band was present in the profiles of each of the individual constituents of the '++' bulk and lacking in each of the individuals making up the '+–' bulk. It was also present in the profile of all the 1BL.1RS-carrying cultivars tested and absent in the non-carriers.

Sequencing, primer construction and PCR

The end-sequencing of the amplification product from cv 'Macvanka' gave 435 bp of the total 1.5 kbp sequence.

The data allowed two oligomers to be constructed: AF1 (5′-GGAGACATCATGAAACATTTG) and AF4 (5′-CTGTTGTTGGGCAGAAAG). AF1 includes bases 2–10 of the OPH20 sequence, while the AF4 sequence lies 3′ of the second OPH20 site. Figure 2A shows the PCR profile of a range of templates using these primers. The templates included individuals from the '++' and '+–' bulks, wheat cultivars with and without 1BL.1RS, two rye inbred lines and six of the set of seven wheat-rye addition lines. Clearly a single product of size 1.5 kb is made whenever the template DNA includes rye chromatin, but no 1.5 kb product is amplified when rye chromatin is absent (although there is a low level of amplification of one or more smaller products in this case). To check the distribution of the amplified sequence on chromosome 1RS, the wheat/rye recombinants and their parents were assayed; this showed that the sequence is present in all of the recombinant lines, although there appeared to be quantitative differences between them, such that those lines carrying only distal rye [I-93, WR-1, WD-1 and WD-2 (Rogowsky et al. 1991)] gave a lower intensity signal than those carrying the complete or the proximal parts of the chromosome arm (Fig. 2B). When the amplification products of the 1BL.1RS varieties and the wheat-rye addition lines were digested with a range of 4-cutter restriction enzymes, an identical restriction profile was obtained in each case (not shown), indicating that the amplified sequence is conserved across the rye genome.

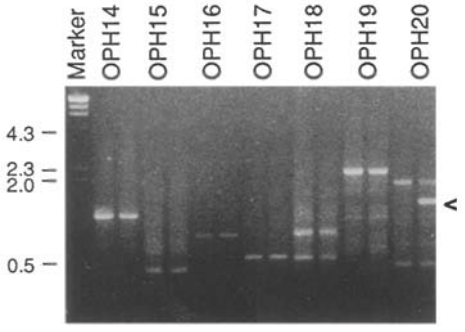
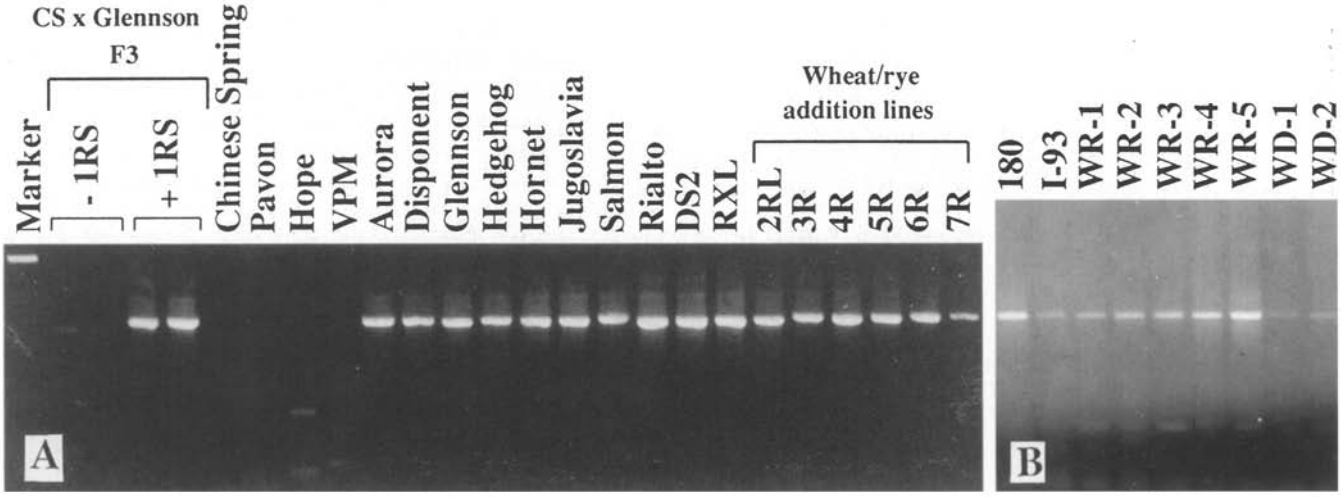


Fig. 1 PCR-RAPD profiles, generated from a range of 10-mer primers (OPH14–20), from template of +– (*left-hand lane* of each pair) and ++ (*right-hand lane* of each pair) bulk DNA obtained from F_3 individuals of the cross cv 'Chinese Spring'×cv 'Glennson'. 1RS-specific PCR product indicated by *arrow*. Size marker provided by *Hin*dIII-digested lambda, sizes in kbp marked on *left*

RFLP analysis

The amplified product from the 1BL.1RS carrying cv 'Glennson' was used as a probe to detect homologous sequences in wheat and barley and to study its chromosomal distribution in rye. A strong hybridization signal was obtained with rye DNA, as expected, while both wheat and barley DNA gave a relatively much weaker signal (Fig. 3), indicating in these species a reduced level of sequence homology to the probe and/or a far lower copy number of the sequence. The two rye inbred lines, which show a high level of restriction fragment length polymorphism (RFLP) [(sufficient to allow genetic mapping within a single cross (Devos et al. 1992)], gave identical hybridization profiles with all of the restriction digests tested; for example, with *Eco*RV, each gave a pattern of 8 equally intense bands. In *Hin*dIII digests, the individual rye chromosome additions to wheat resembled one another with respect to fragments smaller than 6 kb, with each showing an identical set of 5 fragments, clearly derived from rye, superimposed on the wheat profile, as represented by the profile of cv 'Chinese Spring' carrying barley chromosome 7H (Fig. 4). This latter profile did not differ from either that of any other of the wheat/barley addition lines or from that of cv 'Chinese Spring' itself (not shown). However, there is an indication

Fig. 2A, B PCR profiles generated using AF1 and AF4 as primers. Template DNA in **A** from wheats without 1RS ('CS'×'Glennson' F_3–1RS, cvs 'Chinese Spring', 'Pavon', 'Hope', 'VPM'), with 1RS ('CS'×'Glennson' F_3+1RS, 'Aurora', 'Disponent', 'Glennson', 'Hedgehog', 'Hornet', 'Jugoslavia', 'Salmon', 'Rialto'), rye cvs 'DS2', 'RXL', and wheat/rye addition lines. Template DNA in **B** from nine independent wheat/rye recombinants involving rye chromosome arm 1RS



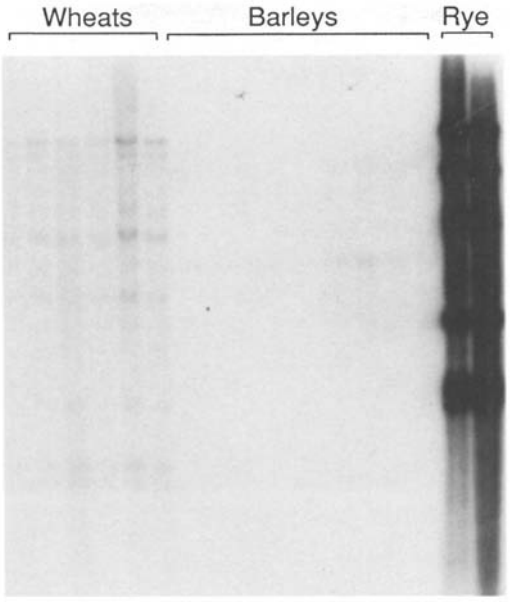
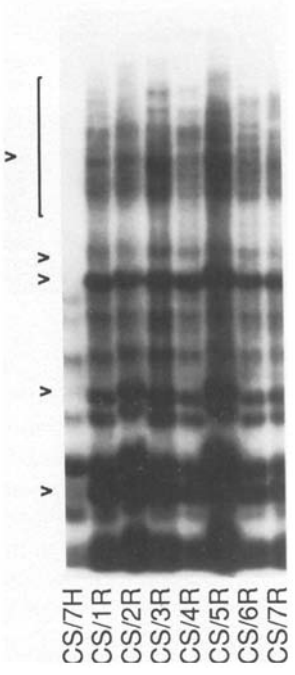


Fig. 3 *Eco*RV-digested DNA, from a range of wheat, barley and rye cultivars, probed with AF1/AF4

Fig. 4 *Hin*dIII digests, probed with AF1/AF4, of DNA from addition lines to cv 'Chinese Spring' involving barley 7H and rye 1R to 7R. Rye-specific fragments *arrowed* on *left*



of RFLP between the rye-derived fragments larger than 6 kb. The cv 'Chinese Spring' *Eco*RV profile consists of 8 bands, but none of these is absent in any of the 21 nullisomic-tetrasomic lines (not shown), thereby indicating the presence of the hybridizing sequence on more than a single wheat chromosome.

In situ hybridization analysis

The AF1/AF4 probe hybridized strongly *in situ* to all 14 rye chromosomes in the triticale and to the pair of 1RS chromosome arms in cv 'Beaver', with the sites of probe hybridization fluorescing strongly (Fig. 5B, D). The target sequence is in great abundance, occurring as a dispersed sequence across the euchromatic regions of the rye genome. However, the signal was reduced or absent in the subtelomeric heterochromatin regions, being identifiable in both metaphase and interphase nuclei by a bright DAPI fluorescence signal (Fig. 5A, C and Leitch et al. 1991a). Fluorescence is also reduced or absent in the centromeric region of the rye chromosomes. The rye nucleolar organizing region (NOR) could also be identified by a reduced level of fluorescence in some, but not all metaphases in both cv 'Beaver' and in the triticale. In these cases, the area of reduced fluorescence appeared less than the area expected to be occupied by condensed rRNA genes on rye chromosome 1R.

In both the triticale and the 1BL.1RS translocation, many wheat chromosomes were labelled, although to a much lesser degree than were the rye chromosomes. The extent and repeatability of this signal was too great to have been merely a background effect. A number of equal-sized *in situ* hybridization signals were observed on the wheat chromosomes at the same location on both chromatids, an event extremely unlikely to have been produced by chance background labelling. Indeed this phenomenon is usually used as a sign that low- or single-copy sequences have been successfully localized *in situ* (Leitch and Heslop-Harrison 1993).

An examination of interphase cells reveals that rye chromosome arms occurred in elongate domains with very little lateral dispersion of the chromatin (Fig. 5F,H). In cv 'Beaver', the domains were such that the centromeric ends of the arm occurred at one pole and the telomeric end of the arm at the other (as determined by bright DAPI fluorescence of the sub-telomeric heterochromatin, Fig. 5G). In triticale, the arms also occurred in elongate domains, appearing to lie in the same orientation all around the nucleus (Fig. 5E, F).

Discussion

The AF1/AF4 sequence is distributed across all of the rye chromosomes and is only reduced or absent at the subtelomeric heterochromatin, at the centromere and around the NOR. Its localization and a failure to find homology with any known sequence from data-base searches, show that it is unrelated to any of the major known rye heterochromatic sequences. The commonality between the seven rye chromosomes of restriction fragments containing the sequence, as seen by the identical Southern blot profiles of the individual wheat/rye addition lines, indicates that the sequence is part of a repetitive unit that occurs throughout the genome. The apparent length polymorphism for larger restriction fragments may originate from chromosome to chromosome differences in the position of the junctions between chromatin with and chromatin without the sequence. These junctions are visible in the *in situ* prepara-

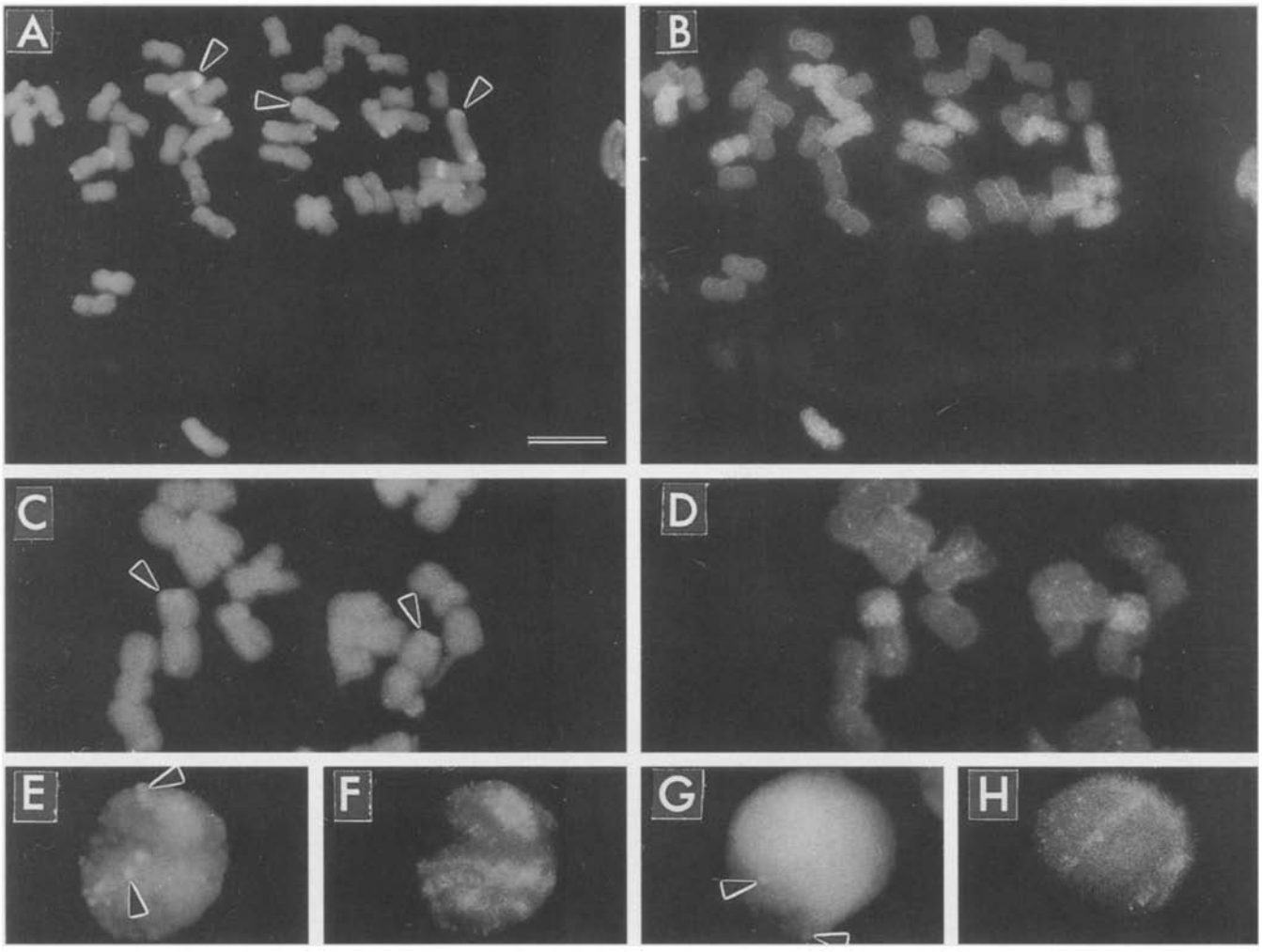


Fig. 5 Fluorescent micrographs of root-tip nuclei and chromosomes of triticale cv 'TC400' (**A, B, E, F**) and wheat cv 'Beaver' (**C, D, G, H**). **A, C, E, G** show DAPI staining of chromatin, while **B, D, F, H** show sites of *in situ* hybridization of digoxigenin-labelled probe AF1/AF4 detected by FITC-antidigoxigenin. Chromatin of rye origin can be identified by bright DAPI positive subtelomeric heterochromatin (e.g. *arrowed* in **A, C**) and by *in situ* signal dispersed across rye euchromatin (**B, D**). Interphase nuclei show rye subtelomeric heterochromatin clustered at one pole (bright spots in **E, G**, some *arrowed*), while rye euchromatin occurs in elongate domains (**F, H**). *Bar*: 8 μm (**A, B, E–H**) and 5 μm (**C, D**)

tions, and their closeness, particularly to the telomeric heterochromatin, clearly varies between the rye chromosomes. The intrachromosomal distribution of the sequence *in situ* closely mirrors that of the RIS 1 sequence (Moore et al. 1993), although nucleotide sequence comparisons of the 500-bp RIS 1 element and the end sequences of the AF1/AF4 amplification product show no homology (unpublished data). Furthermore, in PCR reactions using as primers one or another of AF1 and AF4 together with one or another of the two oligonucleotides used to PCR-amplify the RIS 1 element (Koebner 1994a), no product is amplified (unpublished data). Thus, the likelihood is that the AF1/AF4 repetitive sequence occurs in conjunction with other dispersed repetitive sequences (including RIS 1) that are amplified together as a unit within the rye genome (Flavell et al. 1993). The *in situ* signal strength appears greater than that found with RIS 1 (unpublished data) and is probably similar to that found with the BIS 1 repetitive element in the barley genome. BIS 1 has been considered to represent at least 5% of the barley genome (Moore et al. 1991), and thus the AF1/AF4 sequence must be present in many thousands of copies in the rye genome.

Although little or no amplification occurs from the wheat (lacking 1BL.1RS) or barley (not shown) template when AF1 and AF4 are used as primers, a low level of sequence homology is nonetheless revealed between the amplified rye sequence and these related cereal genomes, leading to a low level of cross-hybridization signal in *in situ* and Southern blot hybridization experiments. However, the lack of any identifiable specific chromosome effects in wheat, the conserved size of the amplification product across all rye chromosomes and its strong hybridization signal against rye DNA all provide good evidence that the sequence has diverged between the cereal species. Nevertheless, it is present in many copies [as is a high proportion of all cereal DNA (Flavell et al. 1977)] and thus the divergence probably preceded a substantial amplification process, which may itself be a cause of speciation. However, the divergence could also have occurred following

speciation, where it would be conserved as a result of the genetic isolation existing between distinct species.

The appearance of the *in situ*-labelled interphases is consistent with that shown by Heslop-Harrison et al. (1990) who used total genomic rye DNA as a probe to localize rye chromatin in a wheat background. This technique also labels the subtelomeric heterochromatin at interphase, which is unlabelled by the AF1/AF4 probe. Thus, AF1/AF4 provides a probe that could be used in parallel with genomic probes for examining the distribution of rye euchromatin separately from heterochromatin (labelled with, for example, pSC119.2, Leitch et al. 1991a). An advantage of a species-specific probe over the use of total genomic DNA is that a definable sequence is being localized rather than unknown sequences that are able to hybridize in the presence of a large excess of blocking DNA. In triticale the rye chromosome arms occurred in elongate domains (as did the rye chromosome arm in cv 'Beaver'), with the subtelomeric heterochromatin located at one pole of the nucleus. The elongate rye chromosome domains in the triticale appeared to occur all around the nucleus. It would be interesting to see if there was a concentric spatial separation of the rye chromosomes from the wheat chromosomes as occurs in barley × rye hybrid nuclei (Leitch et al. 1991b).

The value of the RAPD technique has been illustrated recently for a number of plant species (e.g. Martin et al. 1991; Paran et al. 1991; Reiter et al. 1992), but its application to wheat genetics and breeding has been questioned, following doubts about reproducibility, both from run to run, but more especially from laboratory to laboratory (Penner et al. 1993). However, the system's advantages in terms of its practicality, safety and speed are such that there is a continuing interest in its use. The level of polymorphism within wheat itself has been reported to be low (Devos and Gale 1992), although an improvement appears to be possible by restriction of the template prior to PCR (Koebner 1994b). A higher level of success has been shown when comparing RAPD profiles of wheat with those of one of its wild relatives (King et al. 1993). It was surprising therefore to find that only 1 primer out of over 400 tested detected a polymorphism between sequences on wheat and those on rye chromosome 1RS. However, a similarly low level has been found in comparisons between wheat and a chromosome of wild rye, *S. montanum* (unpublished). Nevertheless, once established, conversion of the primers by the methods described here is effective in improving both the robustness and the ease of interpretation of the assay. The preferential amplification of rye sequences obtained with these primers may lend itself to the conversion of the assay from an electrophoretic one to a microtitre plate ELISA format by incorporation of biotinylated nucleotides in the PCR reaction. In combination with the simple extraction of PCR-grade DNA from endosperm tissue, the primers described will provide a rapid screening system for carriers of 1RS or indeed any introgressed segment of rye other than one involving an extremely distal translocation breakpoint. Such a segment, involving only the subtelomeric heterochromatin, is likely to contain few, if any, genes.

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