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Generation of PCR-based markers for the detection of rye chromatin in a wheat background

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Abstract Oligonucleotide primers were developed to detect the presence of four rye sequences using a PCR assay. These assays give a rye-specific signal from wheat DNA template which contains various rye chromosomes or chromosome segments. The sequences identified were associated with the nucleolar organiser region, the *5S-Rrna-R1* locus, the telomere, and a widely dispersed, rye-specific repetitive element Ris-1. The primers amplified from the well-established loci *Nor-R1* and *5S-Rrna-R1* on rye chromosome arm 1RS, and also located a *5s-Rrna* locus on chromosome 3R. The telomere-associated sequence was present on every rye chromosome, and was also present, at a low copy number, in both wheat and barley. These assays will be particularly useful for introgression programmes aimed at reducing the rye content of the 1BL.1RS wheat-rye translocation. When multiplexed, the primers will enable a rapid, simultaneous assay for a number of distinct rye loci, which can be derived from a small portion of mature endosperm tissue.

Key words PCR · Wheat · Rye · NOR · 5S-rDNA Telomere

Introduction

The development of molecular markers has opened up numerous possibilities for application to plant breeding. Much effort is currently being expended on the creation of RFLP-based genetic maps in many crop species, including wheat and rye, but the use of RFLP technology in a real breeding environment is restricted both by the complexity and the cost of the procedures, particularly in wheat and barley, where non-radioactive methods have not yet been generally established. In contrast, PCR offers a less technically demanding and more rapid methodology, and the direction of genetic mapping programmes is therefore tending to be focussed on the conversion of an RFLP-based to a PCR-based assay (Olson et al. 1989).

The introduction of rye chromatin into a wheat background has been a goal of wheat cytogeneticists for many years, as rye offers a range of adaptation not readily accessible within the wheat gene pool (Zeller and Hsam 1983), and a wealth of cytogenetic material which allows for the manipulation of individual rye chromosomes is available (listed in Shepherd and Islam 1988). The major hurdle for introgressing rye genes into wheat is the low level of homoeologous recombination which can be achieved between wheat and rye chromosomes (Koebner and Shepherd 1985, 1986). Effective selection of infrequent recombinants therefore necessitates markers which are both efficient, polymorphic and plentiful. Biochemical (proteins and isozymes) and morphological characters are both suitable for this purpose, but few are available, and the RFLP assay, while providing many markers, is not readily adaptable to large-scale screening. A particular advantage of the PCR assay in this context is that it does allow for the efficient screening of large populations and, in principle, it can be developed for any targeted part of the genome where nucleotide-sequence information is either available or can be readily obtained from RFLP probes. The present study is aimed at the development of such assays, specifically in relation to the agronomically important wheat-rye translocation 1BL.1RS, which appears to give a yield advantage over normal 1B carriers, but commonly suffers from a dough-quality defect (Dhaliwal et al. 1988).

Materials and methods

DNA was extracted from single seeds, using the CTAB method described in King et al. (1993), or from leaf tissue using phenol/chloroform (Sharp et al. 1988), from the following lines: wheat cvs 'Chinese Spring', 'Gabo', 'Frontana', 'Favorits', 'Pavon', 'Glennson', 'Licanka', 'Macvanka 1', 'Kavkaz' and 'Dean' (the latter five are

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known carriers of the wheat-rye translocation 1BL.1RS); rye cv 'DS2'; disomic addition lines carrying rye cv 'Imperial' chromosomes 1R, 2RL, 3R, 4R, 5R, 6R and 7R in wheat cv 'Chinese Spring' (Driscoll and Sears 1971); the wheat-rye translocation 1DL.1RS (Shepherd 1973), and its derived wheat-rye recombinants 180, I-93 (Koebner and Shepherd 1986) and WR-1 (Rogowsky et al. 1991). The DNA was diluted to 25 ng/μl and 1 μl was used as template for PCR. For some assays, template DNA was obtained by first immersing an Eppendorf tube containing a small piece (approximately 5 mg) of crushed endosperm in 20 μl of 1×TE in a sonicating waterbath for 10 min, followed by a 2-h incubation at 37°C. After this the tube was centrifuged and 1 μl of the resultant supernatant was taken as template.

Four pairs of oligonucleotides to be used as primers for PCR were synthesised; these were directed at the loci *Nor-R1* and *5S-Rrna-R1*, at the telomere-associated 350 family (Appels and McIntyre 1985), and at the widely dispersed rye-specific repetitive sequence Ris-1 (Moore et al. 1993). These will be referred to hereafter as, respectively, NOR, 5*S*, TEL and RIS. For NOR, the sequences of the intergenic spacers between the ribosomal repeats of wheat (Barker et al. 1988) and rye (Appels et al. 1986 b) were compared, and the region of least homology (approximately 600 bp, starting 30 bp downstream from the 3′ end of the coding region of the 25*S*-RNA gene) of the rye sequence was used to design primers. These correspond to bases 1383–1404, and 1768–1751 of the sequence given in Appels et al. (1986 b). For 5*S*, the 349-bp intergenic spacer sequence between the 120-bp repeat units of the rye 'short lineage' (Reddy and Appels 1989), which lie at bases 153–172 and 259–241, was used to design primers. For the TEL assay, primers were designed from the consensus 380-bp sequence of the repeating unit of the 350 family [bases 139–158 and 261–242, Appels et al. (1986 a)]. For RIS, the Ris-1 element was sequenced (unpublished data) and this sequence of 497 bp was used to design appropriate primers (positioned at bases 33–53 and 143–124).

PCR conditions were similar for each primer. The reactions were carried out in a 25-μl volume, containing 25 ng of template, 0.4 U of *Taq* polymerase, 2 nmol of dNTP and an optimized quantity of primer (0.1–1 pmol). The reaction buffer was made to 12% w/v sucrose, 0.2 mmol cresol red, as recommended by Hoppe et al. (1992), to allow direct loading of the PCR reaction into the agarose gel. The PCR was carried out over 25–35 cycles consisting of 15 s at 94°C, 45 s at 65°C and 45 s at 72°C with a minimum transition time between each temperature, and the run was completed with a 5-min final extension at 72°C. In later experiments, the extension step was omitted to give a two-step cycle (15 s at 94°C and 45 s at 65°C). PCR products were separated on 1% or 2% agarose gels containing 10 μg/ml of ethidium bromide, and visualised under UV light.

Results

NOR

The amplification profile obtained from rye DNA template consisted of four products, of approximate sizes 400 bp, 600 bp, 700 bp and 800 bp. Wheat DNA template was not amplified. Three of the four (400 bp, 600 bp, 700 bp) rye-specific products were also amplified from template where the entire rye chromosome 1R was present, but not from template DNA of wheat lines carrying any other rye chromosome (Fig. 1A). The 800-bp product was not produced from any of the addition line templates. No amplification was observed from a range of barley cultivars (data not shown). The expected amplification product size was 386 bp, and this was generally the most efficiently amplified product of the profile; however, inspection of the DNA sequence of the rye intergenic spacer shows that the reverse primer sequence recurs at bases 2003–1986 and again, with only one base mismatch at the 5′ end, at bases 2136–2119. These two primer sites generate additional amplification products of sizes 621 bp and 754 bp, respectively. Analysis of the wheat-rye recombinants showed that the three priming sites present on chromosome 1R were also present on the 1DL.1RS translocation, and on the proximal rye recombinant 180, but not in either of the two distal rye recombinants I-93 or WR-1 (Fig. 2A).

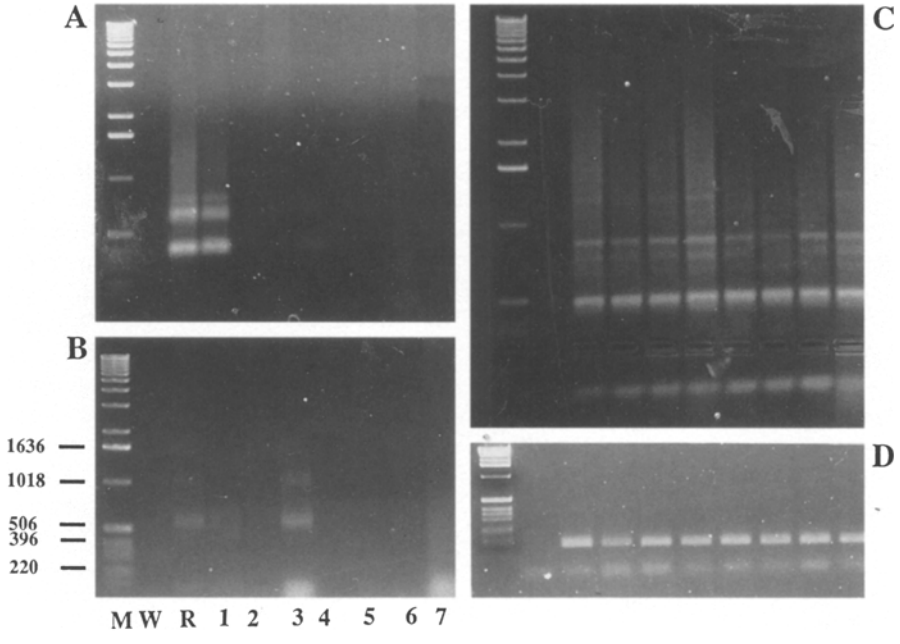

Fig. 1 Amplification profiles of wheat cv 'Chinese Spring'(*W*), rye cv. 'DS2'(*R*), and the Chinese Spring/Imperial rye disomic addition line set (1=1R, 2=2RL, ..., 7=7R) using primers for **A** NOR, **B** 5*S*, **C** TEL, **D** RIS. *M*: kilobase ladder molecular-weight marker (sizes in bp marked on the left of **B**). The same sequence of template DNAs is used in each figure

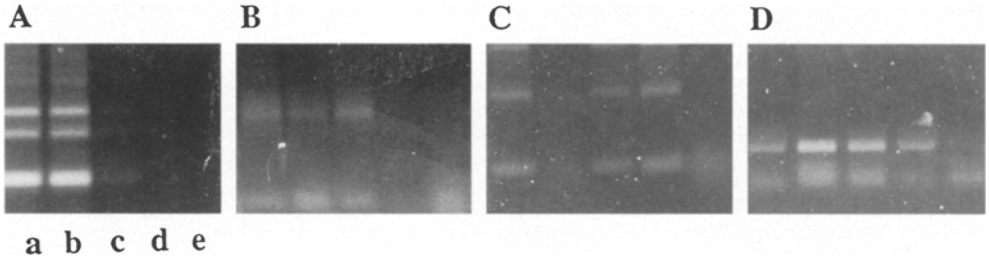

Fig. 2 Amplification profiles of wheat-rye translocation 1DL.1RS (*a*), three of its derived recombinants (*b*: 180, *c*: I-93, *d*: WR-1) and wheat cv 'Gabo' (*e*) using primers for **A** NOR, **B** 5*S*, **C** TEL, **D** RIS

Table 1 Sequence of oligonucleotides used as primers for assaying rye *Nor-1* (NOR), 5*s-Rrna* (5*s*), Telomere-associated sequence (TEL) and dispersed repeat Ris-1 (RIS)

NOR	F: GCATGTAGCGACTAACTCATCG	R: CCCAGTTTTCCATGTCGC
5*S*	F: TTTTTGCGTCTCGTGACAAG	R: ACCGGCCTAAAACGTATCG
TEL	F: CCAACGCCTATGAAAACGAT	R: GCCAACTCTCGCAAAGAAAC
RIS	F: TAATTTCTGCTTGCTCCATGC	R: ACTGGGGTGCACTGGATTAG

5*S*

The amplification profile obtained from either rye DNA template, or from wheat DNA template containing the entire rye chromosomes 1R or 3R, consisted of two products of approximate sizes 100 bp and 600 bp (Fig. 1A). In some runs, a further amplification product of approximate size 1000 bp was faintly visible. Wheat or barley template was not amplified. The expected size of amplification product was 106 bp. The same rye-specific amplification products were produced from 1DL.1RS and from the wheat-rye recombinants 180 and I-93, but not from WR-1 (Fig. 2B).

TEL

At least four distinct amplification products were produced from templates of rye or wheat containing the telomere of any one of the seven rye chromosomes. These were of approximate sizes 100 bp, 500 bp, 900 bp and 1300 bp. In addition the profile always included a smear of higher-molecular-weight DNA (Fig. 1C). Wheat and barley DNA template produced a similar, but much fainter profile when the number of PCR cycles was increased to 40, but was not visibly amplified after 35 cycles. The expected PCR product size was 123 bp. The rye profile was present in 1DL.1RS and the recombinants I-93 and WR-1, but not in the proximal recombinant 180 (Fig. 2C).

RIS

The RIS primers amplified a single product of approximately 100 bp from all templates containing rye DNA, but not from templates lacking rye DNA (Figs. 1 D and 2 D). The expected PCR product size was 111 bp. The assay also worked satisfactorily when template was obtained directly from a small section of mature endosperm, without any prior DNA purification procedure, as described above (Fig. 3).

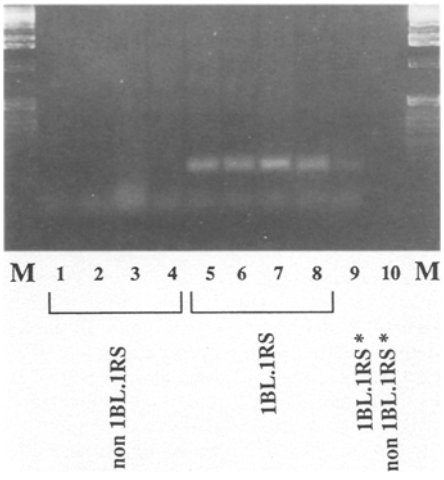

Fig. 3 RIS-generated amplification profiles obtained by priming template derived from varieties without (*tracks 1–4, 10*), and with (*tracks 5–9*) the 1BL.1RS translocation. * denotes template obtained by sonication/incubation. *Tracks 1–4*: cvs 'Chinese Spring', 'Pavon', 'Frontana', 'Favorits'; *tracks 5–8*: cvs 'Licanka', 'Macvanka 1', 'Kavkaz', 'Dean'; *track 9*: cv 'Glennson'; *track 10*: cv 'Gabo'

Triplex PCR for NOR, 5*S*, and TEL

When all three primer pairs corresponding to NOR, 5*s* and TEL were included in the PCR reaction mixture, all the expected PCR products were obtained from template of a wheat (cv 'Licanka') containing the wheat-rye translocation 1BL.1RS (Fig. 4). However the balance of primer concentrations to obtain this result was delicate. To obtain a simultaneous assay for all three loci, the concentrations of the primers used were 0.5 fM (NOR), 0.07 fM (5*S*) and 0.05 fM (TEL). When any one of the primer pairs was present in excess, amplification from at least one, and sometimes from both, of the other target loci was suppressed. The primer sequences were aligned to uncover any base-pair complementarity between the various combinations. This analysis showed that only between four and

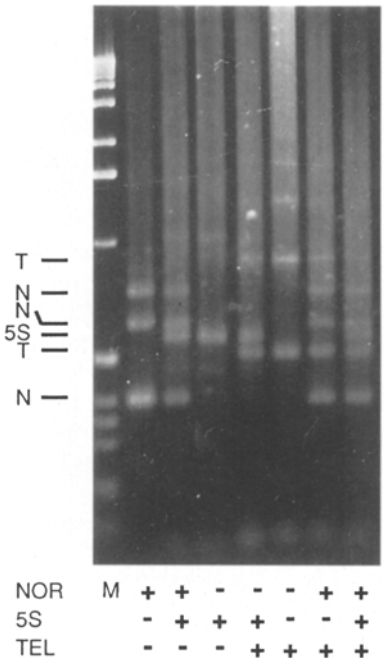

Fig. 4 Multiplex PCR amplification profiles from cv 'Licanka' template (carries wheat-rye translocation 1BL.1RS). *N*, *5S*, *T* indicate position of PCR products amplified by, respectively, primers NOR, *5S* and TEL. Primers used in each reaction indicated by + at the base of the figure. *M:* kilobase ladder molecular-weight marker

seven bases could be aligned between any two of the primer sequences, while four (TEL) or five (NOR, *5S*) bases were complementary when comparing the individual forward and reverse primer sequences. Thus it was not thought likely that primer complementation interfered with the efficiency of the multiplex reactions.

Discussion

The conversion of the RFLP assay into a PCR-based one is being widely pursued as a means of improving the applicability of molecular markers as diagnostic and selection tools. A necessary step in this process is a check that the locus being assayed by the primers is identical to that assayed by the RFLP probe. A number of ways of confirming this identity are possible, including linkage analysis and hybridisation between the PCR product and the RFLP probe. In the present case, the availability of a number of wheat-rye recombinants involving distinct segments of the short arm of rye chromosome 1R allows a simple check. These recombinants have been previously classified for genotype at *Nor-R1*, *5S-Rrna-R1* and the telomere (Koebner et al. 1986; Rogowsky et al. 1991), and these classifications fitted exactly with those obtained by the present PCR assay, both for the lines illustrated in Fig. 2, and for the remainder of the 1RS recombinant lines (data not shown). The PCR products could now be used as probes for *in situ* hybridisation studies to further confirm their provenance, as the physical location of the three loci on chromosome 1R is well established (Appels et al. 1980, 1981). The Ris-1 element is known to be widely dispersed throughout the rye genome, except near the centromeres and telomeres of the chromosomes (Moore et al. 1993), and thus was expected to give a positive signal for all the recombinant lines.

Whereas the origin of the multiple PCR products using the NOR primers was due to repetition of the reverse primer sequence at two other closely located positions, the multiple bands in the *5S* and TEL profiles have a different origin. The *5S-Rrna-R1* locus consists of multiple repeats of a 120-bp coding unit separated by about 400 bp of non-coding spacer DNA (Reddy and Appels 1989), in which the primer annealing sequences lie. The primary amplification product is expected to be 106 bp in length, but for any one forward priming site, there will be reverse priming sites in each of the adjacent spacer segments, giving rise to amplification products of lengths 610 bp and 1 120 bp (and higher), fully consistent with the observed data (Fig. 1B). The 610-bp product appears to be preferentially amplified over the other two, and those larger than 1 120 bp (1 630 bp, 2 140 bp etc.) are probably too large to be amplified efficiently under the reaction conditions used. In an attempt to promote the production of the 106-bp product, the extension phase of the PCR cycle was omitted, but the resulting profile was similar to that achieved earlier. It remains unclear why the 610-bp product is preferred over the 106-bp product.

A similar situation pertains with respect to the TEL PCR profile. The primary product length is 123 bp, but those of approximate lengths 500 bp, 900 bp and 1 300 bp were usually distinguishable, as well as other even higher-molecular-weight products and a background high-molecular-weight smear. These results are readily explicable by assuming that the 350 sequence occurs as blocks of multiple copies in a tandem array as suggested by Appels and McIntyre (1985). In this case, a series of PCR products with a size periodicity of 380 bp (the length of the consensus sequence) will be amplified, i.e., 123 bp, 503 bp, 883 bp, 1 263 bp ..., which matches the PCR profile exactly (Fig. 1C). Once again, the smallest PCR product is not the most efficiently amplified, and the profile was not modified by omission of the extension step.

Sequences related to the 350 family have been isolated in a range of grass species (Xin and Appels 1988; McNeil et al. 1994), and the postulation is that sequence 'specificity' reflects dominance, rather than exclusive presence of that particular sequence in a genome. The observation that higher numbers of PCR cycles produced a signal from both barley and wheat template, and particularly that the PCR profile was identical to that of rye, is consistent with the presence of 350-like sequences in these other species, but at a level magnitudes lower than is present in rye. Comparison of the target sites for annealing of the TEL primers between the rye sites and those in tetraploid wheat and other grass species [Fig. 7 in McNeil et al. (1994)] shows just one mismatch in each of the two

primer sequences in the tetraploid wheat AB1 350 sequence, with more mismatches in the other comparisons. The expectation would therefore be that only a subset of the wheat 350 family sequences would amplify from the TEL primers. Since the frequency of the 350 family in wheat is below the hybridisation threshold (McNeil et al. 1994), the target for the TEL primers from a wheat template is reduced to a subset of a small number, which explains why only higher numbers of amplification cycles generate a visible PCR product.

The PCR assay for *5S-Rrna-R1* identified a site on chromosome 3R, in addition to the one on 1R, the location of the major rye site (Appels et al. 1980). A 3R site has recently also been identified in rye using *in situ* hybridisation, although not on the cv 'Imperial' 3R present in the addition line used in this study (Cuadrado et al. 1995). As quantitative variation in the number of repeat units at the *5S-Rrna* loci has been observed in rye (Appels et al. 1989), this apparent anomaly is readily explicable as a copy number effect, with the higher sensitivity of PCR enabling the identification of a locus at which the copy number is below the level of detection with the *in situ* hybridisation technique. A similar PCR-based assay has also located a site on barley chromosome 3H and shown polymorphism within the species for copy number (Kanazin et al. 1993). A further site, *5S-Rrna-R2*, on chromosome 5R was identified by Reddy and Appels (1989), but was not detected in the present study. This is probably both because the site is virtually non-existant in rye cv 'Imperial' (Appels et al. 1989), and because effective priming from the few remaining sites may be prevented because the oligonucleotide primers were designed from the sequence of the 'short' variant, which preferentially hybridises *in situ* to the 1R, rather than to the 5R, site (Reddy and Appels 1989). A comparison of the nucleotide sequences of the primer sites between the long and short variants shows three mismatches in the forward primer sequence and four in the reverse, probably a sufficient difference to inhibit adequate primer annealing. A similar situation exists in barley, where primers can be selected which amplify exclusively from either the site with the 'long' spacer (3H), or from the 'short' spacer (2H) (Kanazin et al. 1993).

These PCR-based markers are ideally suited to programmes aimed at the introgression of rye genes into wheat. Even in the absence of the *Ph1* gene, which normally suppresses non-homologous recombination in wheat, the frequency of wheat-rye recombination is low (Koebner and Shepherd 1985, 1986), and therefore recombinant individuals are rare. There is therefore a need to use markers which can be readily applied to large numbers of individuals and, in particular, to be able to screen seeds rather than growing plants. Although RFLP markers are efficient at distinguishing rye from wheat loci, they are not readily applicable to this situation, both because the assay requires more DNA than can be extracted from a single seed [generally about 1 μg (King et al. 1993)] and because the procedure is too time-consuming and expensive for large populations. In contrast, the PCR can be scaled down to a 10-μl reaction which requires minimal reagents and, using the multiplex approach, a single reaction can be used to assay for multiple loci simultaneously. The DNA extraction system used in this study produced sufficient template from a single seed for 500 PCR reactions – clearly a less efficient, but more rapid, system would be more applicable, and the simple sonication/incubation described here is adequate for this purpose. A similar approach has recently been described by Chunwongse et al. (1993).

The loci targeted in the present study are aimed at generating markers to aid breaking up the agronomically important wheat-rye translocation 1BL.1RS; nevertheless, any rye chromosome, or indeed non-wheat chromosome, can be targeted in a similar way by judicious choice of loci on the basis of the well-developed RFLP maps of the Triticeae genomes. Furthermore, the RIS primers can be used as an initial screen, to eliminate segregants lacking any rye chromatin, thereby limiting the number of individuals which need to be screened with the more specific primers.

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