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MOLECULAR PROBES FOR DETECTION OF WHEAT CHROMOSOMAL FRAGMENTS IN RYE

ABSTRACT

Twelve DNA fragments amplified on templates of *Triticum urartu*, *Aegilops speltoides* and *Aegilops squarrosa* were separated, reamplified and used as digoxigenin-labelled probes. The species and genome specificity of probes was evaluated by Southern dot-blot hybridisation to Triticum durum, the donors of wheat genomes, rye and rye with introgressed wheat chromosome fragments. In comparison to labelled genomic DNA of T. durum, the probes obtained by labelling PCR-amplified fragments exhibited higher specificity to wheat genomes. Under the applied hybridisation conditions all probes showed different degree of cross-hybridisation to rye DNA. Some probes indicated quantitative difference in their specificity to wheat genomes, but generally the intensity of hybridisation to the genomes A, S and D was independent of the origin of an amplified fragment. Selected probes, used in dot-blot hybridisation system together with genomic DNA of T. durum, may increase the sensitivity of screening wheat chromosomal fragments introgressed to rye.

Key words: introgression, PCR, rye, Southern blotting, *Triticum durum* probe, wheat, wheat specific probes.

INTRODUCTION

Chromosomal engineering i. e. transferring of alien chromosomes or chromosome fragments from related species into crops has been used for introgressions of useful genes. Rye (*Secale cereale* L.) has proved to be a reservoir of genes for wheat. On the other hand, breeding programs for rye reach the border of limited genetical variability. Thus, some efforts have been made to exploit of related species in breeding of rye. The genetical variability and some favourable characters, such as for example resistance to pathogens, could be introduced to rye also from wheat.

Schlegel (1982) demonstrated first, that rye is able to tolerate single wheat chromosomes in the addition lines. Several addition lines of rye with wheat chromosomes from A and B genomes were documented by Thiele *et al*. (1988). The alien chromosomes were identified by C- and

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N-banding and by izoenzyme markers (Melz and Thiele 1990). It was observed by Melz and Thiele (1990) that the wheat chromosome 3B added to rye genome caused resistance to powdery mildew of rye. Despite of spontaneous loss of the whole chromosome, both the gene of resistance and its isoenzymatic marker appeared to be transferred into rye genome. However, the production of addition lines was not followed by successes in chromosomal engineering such as in wheat.

The comparative RFLP mapping of genera and species within the tribe *Triticeae* with the use of sets of homeologic probes indicated, that only 1R chromosome of rye shows complete colinearity with its wheat homologues (Devos et al. 1995). The substitution of other chromosomes from rye to wheat disturb the chromosomal balance and negatively affects its agronomic performance. Such disturbances are more detrimental on the diploid level. For this reason, only the presence of small wheat chromosomal fragments can be tolerated in the rye genome. The minimalization of the wheat chromosome fragment, containing *Glu-D1* locus was the essential problem in breeding rye with improved breadmaking quality (Łukaszewski and Brzeziński 1996). The presence of such small chromosome fragments could not be detected by traditional cytological methods. In rye samples investigated by Łapiński et al. (1999) the presence of small wheat DNA fragments, detectable with the use of *Triticum durum* genomic probe by Southern dot-blot hybridisation, could not be confirmed cytogenetically even by the genomic in situ hybridisation approach.

The development of molecular techniques increases the possibility of identification and characterisation of introgressed alien chromosomes or chromosomal fragments. The hybridisation methods widely used for identification of alien chromatin in wheat utilise labelled genomic DNA of introgressed species as probes (Le et al. 1989, Anamthawat-Jonsson et al. 1990, Heslop-Harrison et al. 1990, Schwarzacher et al.1992), probes from rye specific dispersed repetitive sequences (Rogowsky et al. 1991, Talbert and Clack 1991, McIntyre et al. 1990) or single-copy clones localised on specific chromosomes or chromosome regions (Sharp et al. 1989, Rogowsky et al. 1993, Bomminieni et al. 1997). It was also found that when the fragment used as a probe contains species-specific repetitive sequence dispersed throughout the genome, then only the introgressed alien fragments showed hybridisation signal. The data of Guidet et al. (1991) documented, that the hybridisation signal reflected the amount of rye genetic material introgressed to wheat genome. Such probes can be used in Southern blots and for *in situ* hybridisation. Chromosome preparation for in situ hybridisation is more difficult and time consuming than Southern blotting, therefore it can be applied for characterisation of a limited number of plants. For screening of a large number of engineered plants it is recommended to use dot blots or slot blots hybridisation system.

For screening plants with unidentified fragments of alien material, the probes used should hybridise to sequences uniformly dispersed throughout the genome. The probes from amplified single copy sequences can not be used for such screening because they may be located to specific chromosome or chromosome regions. Although the large homology between species of the tribe of *Triticeae* limited the possibility of selection of DNA fragments which are species-specific or genome-specific, it was possible to isolate repeated sequences which are rye-specific or wheat- specific. Among five repetitive sequences described in rye (Bedbrook et al. 1980) only two are species-specific and are not located in telomeric regions of chromosomes (Appels et al. 1986, Guidet et al. 1991).

The aim of presented study was the evaluation of suitability of digoxigenin-labelled DNA fragments for detection of wheat chromosomal fragments introgressed to rye. These fragments were amplified by PCR method on genomic DNA templates from *Triticum urartu,* and *Aegilops squarrosa,* the proposed donors of A and D genomes of hexaploid wheat. *Aegilops speltoides,* carrying genome S, was included as a representative of the *Sitopsis* section of *Triticeae* which gave rise to the B genome of wheat. The PCR primers complementary to the wheat specific dispersed repetitive sequence (Mettzlaf et al. 1986) were used alone or in combination with primers targeting intron-exon junction sequence (Rafalski et al. 1997).

MATERIAL AND METHODS

The seed samples of rye were chosen from Dr Łapiński program of crosses between rye and tetraploid triticale. The samples of seeds of *T. urartu, Ae. speltoides* and *Ae. squarrosa* were obtained from National Centre of Plant Genetic Resources, Plant Breeding and Acclimatization Institute, Radzików.

The DNA was prepared from single 6-10 days old rye seedlings with introgressed wheat chromosome segments or bulks of 8-10 seedlings (*T. durum*, rye cv. Dańkowskie Złote, *T. urartu, Ae. speltoides. Ae. squarrosa*) according to procedure of Davis et al. (1986). The concentration of DNA was evaluated fluorometrically according to the procedure described in TKO minifluorimeter booklet (Hoefer Sci. Instruments, S. Francisco, USA).

The polymerase chain reactions were carried out in 20 µl volume containing about 10 ng of DNA template, 1 x buffer (Promega), 2 mM $MgCl_2$, 0.01% of gelatin, 200 mM dATP, dGTP, dCTP and dTTP, 0.7-0.8 mM of each primer and 0.8 unit of Taq polymerase (Promega). The reactions were carried out in UNOII Thermal Cycler (Biometra). The PCR reaction consisted of 7 cycles, each of 40 sec at 95°C, 1 min. at 60°C and 2 min. at 72°C followed by 33 cycles, each of 40 sec. at 94°C, 1 min. at 64°C and 2 min. at 72°C. The final step was 10 min. at 72°C. During the secondary

amplification the reaction mixture was as above and the PCR reaction consisted of 35 cycles, each of 40 sec. at 94°C, 1 min. at 64°C and 2 min. at 72°C followed by final steep for 10 min. at 72°C. Five µl of solution of a single fragment isolated from agarose gel (2 – 3 ng of DNA) was used as the template.

DNA fragments were separated on 1.5% agarose gel and visualised by staining with ethidium bromide. Single DNA fragments were cut out from the gel, the ethidium bromide stained DNA was washed out from the gel by centrifugation, liquid nitrogen treatment and washing with TE buffer. The fragments were purified with the use of Wizard PCR Preps DNA Purification System (Promega) according to the procedure proposed by producer and eluted with 50 µl of TE buffer. The DNA fragments obtained during the second amplification step were washed out from the gel and purified as above. The DNA was usually isolated and purified from 4 – 8 electrophoresis wells (40-80 µl of PCR reaction solution), precipitated with ethanol, centrifuged and diluted in 50-100 ml of water.

The DNA fragments were labelled with digoxigenin-dUTP with the use of random primed system according to the procedure described in the booklet for DIG DNA Labeling and Detection Kit (Boehringer Mannheim). The 20 µl reaction mixture contained about 200 ng of genomic DNA from *T. durum* (fragmented mechanically to about 10000 bp) or 60-100 ng of an isolated fragment of DNA. The incubation time was 18-20 hours.

The denatured samples of DNA were blotted onto nylon membranes with the use of Bio-Rad dot-blot gasket (96 wells) and Vacu-Gene (Pharmacia-LKB) equipment. For hybridisation with total genomic DNA from *T. durum* 25, 12.5 and 6.25 ng of each sample were blotted, for hybridisation with PCR generated probes the amounts of blotted DNA were 100, 50 and 25 ng.

Hybridisation, immunological detection and colour development were performed according to the instruction of producer (Boeringer, Mannheim). The only modification of the procedure was that the standard hybridisation buffer was supplemented with sonicated rye cv. Dańkowskie Złote DNA (about 300 bp) at the concentration of 5 µg/ml and the concentration of blocking reagent was decreased to 0.5%. The prehybridisation temperature was 60°C, the incubation with hybridisation buffer was 16 hours at 68°C. For 10 ml of hybridisation buffer DIG-labelled DNA from *T. durum* was added in amount of 40 ng, in the case of probes from PCR amplified fragments the amount of DNA was about 20 ng. During the post-hybridisation washes we followed the procedure described by Metzlaff et al. (1986).

RESULTS AND DISCUSSION

For detection of wheat chromatin in rye we developed the approach with the use of probes derived from species-specific repetitive sequences dispersed uniformly in a genome. In comparison to rye, the amount of repetitive sequences in wheat is significantly lower, but it was possible to isolate clones, which indicated high degree of wheat specificity and can be used for construction of wheat specific probes. Metzlaff et al. (1986) described wheat specific clones containing the fragments of repeated sequences. The primers flanking repeated sequence were used alone or in combination with primers targeting intron-exon junction of genes (Weining and Langridge 1991). The semiconservative sequences of intron-exon junction occurred frequently throughout the genome and are likely to be evenly dispersed. Because of some degree of degeneracy of this consensus sequence only a sub-set of splice junction will be targeted by selected primers.

The primers used for amplification of DNA fragments are presented in Table 1. The primers Rep.1 and Rep.2 were constructed on the basis of sequence data of wheat specific clone pTa8 (Metzlaff et al. 1986). The sequence of primer R1 is based on consensus sequence for the intron-exon junction. This semirandom exon targeting primer was used by Weining and Langridge (1991) in combination with random primers for study of polymorphism of wheat, barley and rye. The primer IT5 (intron targeting)

Table 1

The primers used for amplification of DNA fragments. The bases that match the intron-exon consensus sequence are shown in bold type

Primer	Sequence (5' - 3')
IT5	GGTGCGGGA**CAGGTAAGT**
R1	TCGTGGCTG**ACTTACCTG**
Rep1	GATCTCCTCCCTTGCAACCG
Rep2	ACCATGAGACTGTAGAGGCT

belongs also to the group of primers targeting intron-exon junction (Rafalski et al. 1997). In both primers the 9 bases of consensus sequences for the splice junction were supplemented with 9 bases selected at random to extend the length of primers and to generate their variability.

The primers used amplified some DNA fragments on rye genomic template and usually higher number of fragments on templates *T. urartu, Ae. speltoides* and *Ae. squarrosa*. After separation of PCR products on agarose gel, for reamplification and labelling were chosen bands amplified on wheat genomes donor templates, but not on template of rye genomic DNA. Altogether twelve DNA fragments were selected for second amplification. The second amplification step with the use of DNA fragments as templates gave possibility to obtain DNA fragments in

amounts sufficient for labelling. The amount of DNA isolated from 4-8 lanes varied from 80 to 250 ng. For one labelling procedure from 30 to 75 ng of each DNA fragment was used and after prolonged incubation the amount of synthesised DIG-labelled DNA varied from 100 to 220 ng.

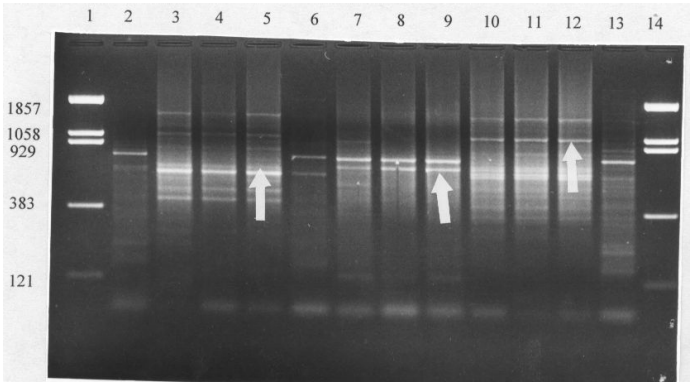

Fig.1. Amplification of DNA with the use of Rep1 primer in combination with intron targeting primer IT1. Lanes 1 and 14, DNA size marker; lanes 2, 6 and 13, rye cv. Dańkowskie Złote; lanes 3-5 *Aegilops squarrosa*; lanes 7-9, *Aegilops speltoides*; lanes 10-12 *Triticum urartu*. Arrows indicated the position of bands selected for reamplification.

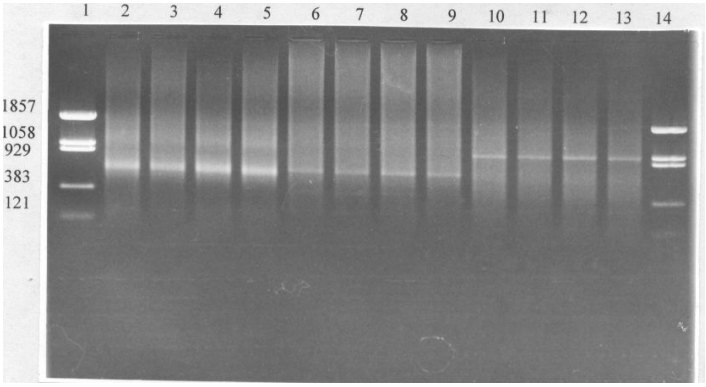

Fig.2. Reamplification of PCR fragments with the use of primers Rep1 and IT1.
Lanes 1 and 14, DNA size marker; lanes 2-5, 631 bp fragment (probe 3); lanes 6-9, 682 bp fragment (probe 2); lanes 10-13, 1077 bp fragment (probe 1).

As an example, Fig. 1 shows the results of amplification with the use of Rep.1 primer in combination with IT5 primer. After isolation and purification the fragments selected were used as templates during the second amplification step (Fig. 2). The data characterising probes are presented in Table 2.

The specificity of probes in comparison to DIG-labelled total genomic DNA from *T. durum* was evaluated by hybridisation to eight DNA samples. Denatured samples of rye DNA, rye with introgressed wheat chromosomal fragment, *T. urartu*, *Ae. speltoides, Ae. squarrosa* and *T. durum* were blotted on nylon membranes. These control blots were supplemented

Table 2

The characters of PCR-amplified fragments used as probes.

Probe	Origin (template)	Primer	Size (bp)
1.	*T. urartu*	IT 5 + Rep1	1077
2.	*Ae. speltoides*	IT 5 + Rep1	682
3.	*Ae. squarrosa*	IT 5 + Rep1	631
4.	*T. urartu*	IT 5 + Rep2	683
5.	*Ae. Speltoides*	IT 5 + Rep2	452
6.	*T. urartu*	R1 + Rep2	956
7.	*Ae. speltoides*	R1 + Rep2	669
8.	*Ae. speltoides*	R1 + Rep2	450
9.	*Ae. squarrosa*	R1 + Rep2	823
10.	*Ae. squarrosa*	IT5 + Rep2	452
11.	*T. urartu*	Rep1	1106
12.	*Ae. speltoides*	Rep1	449

with two samples of engineered rye, one indicating morphological characters of rye and one with some morphological characters of wheat.

It was found by Anamthawat-Jonsson et al. (1990) that the discrimination between related species was increased by the use of high concentration of blocking DNA. The excess of unlabelled DNA from rye appeared to be essential for blocking cross-hybridisation between wheat and rye when *T. durum* genomic DNA was used as a probe. For this reason, we used the excess of rye DNA as blocking reagent both in hybridisation with the use of genomic DNA from *T. durum* and in the case of probes from PCR amplified fragments.

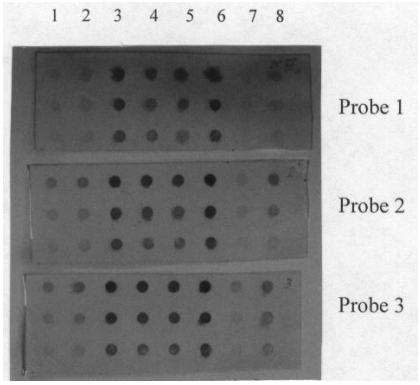

Fig.3. Dot-blot hybridisation of DNA to probes 1 – 3.
1, rye cv. Dańkowskie Złote; 2, rye with introgressed wheat fragment; 3, *Triticum urartu*; 4, *Aegilops speltoides*; 5, *Aegilops squarrosa*; 6, *Triticum durum*; 7, engineered rye –negative sample; 8, engineered rye – positive sample. DNA blotted in amount 100, 50 and 25 ng.

The results of hybridisation with the use of successive probes are presented on Fig. 3-6.

In comparison to genomic DNA isolated from *T. durum,* all PCR amplified fragment used as probes, showed lower specificity to rye and higher specificity to all three possible donors of wheat genomes. As in the case of *T. durum* genomic probe, all the PCR amplified probes hybridized slightly to rye DNA. The primers Rep1 and Rep2 used for amplification were complementary to the flanking regions of the short (about 130 bp) clone which did not show any cross-hybridisation to rye DNA (Metzlaff et al. 1986). The longer PCR-amplified fragments used as the probes contain probably wheat sequences with partial homology to rye DNA.

Independently on the origin of a PCR amplified fragment used as a probe only quantitative differences in genome specificity was observed. The labelled fragment amplified on template from *T. urartu* (probe 1) indicated the highest specificity to the genome A and the lower hybridisation signals with genomes S and D (*Ae. speltoides* and *Ae. squarrosa*). However, the probes 2 and 3 which originated from *Ae. speltoides* and *Ae. squarrosa* indicated hybridisation signal of similar strength to the donors of wheat genomes (Fig. 3).

Despite of small genome specificity or lack of it, the probes 1– 3 indicated relatively high specificity to wheat DNA. The hybridisation signal to both samples of rye with introgressed wheat fragment was markedly higher than to normal rye, indicating that these probes might be useful in screening rye with introgressed wheat chromosome fragments. The probes 4 and 5 are not useful for distinguishing introgressed rye, the differences in hybridisation signals to positive and negative rye sam-

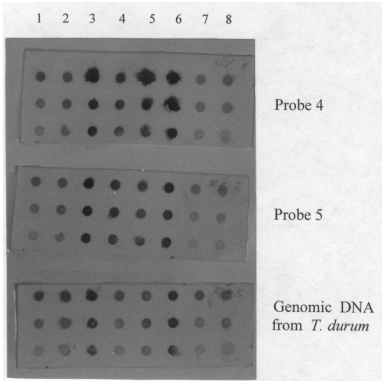

Fig. 4. Dot-blot hybridisation of DNA to probes 4, 5 and genomic DNA of *Triticum durum*.
1, rye cv. Dańkowskie Złote; 2, rye with introgressed wheat fragment; 3, *Triticum urartu; 4, Aegilops speltoides*; 5, *Aegilops squarrosa*; 6, *Triticum durum*; 7, engineered rye –negative sample; 8, engineered rye – positive sample.

ples were rather low and did not exceed the differences observed in hybridisation with genomic DNA of *T. durum* (Fig. 4). Probe 4 amplified on

DNA template from *T. urartu* indicated enhanced specificity to genomes A and D and lower to genome S.

Fig. 5 shows the results of hybridisation with the use of probes 6 – 9. The probes 6-8 indicated similar specificity to DNA of all donors of wheat genome.

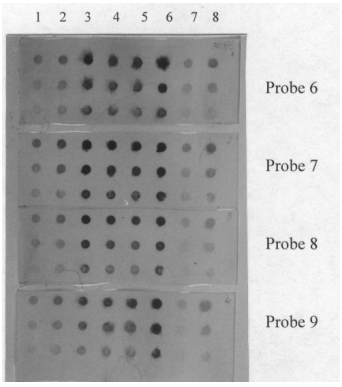

Fig.5. Dot-blot hybridisation of DNA to probes 6 – 9.
1, rye cv. Dańkowskie Złote; 2, rye with introgressed wheat fragment; 3, *Triticum urartu*; 4, *Aegilops speltoides*; 5, *Aegilops squarrosa*; 6, *Triticum durum*; 7, engineered rye –negative sample; 8, engineered rye – positive sample. DNA blotted in amount 100, 50 and 25 ng.

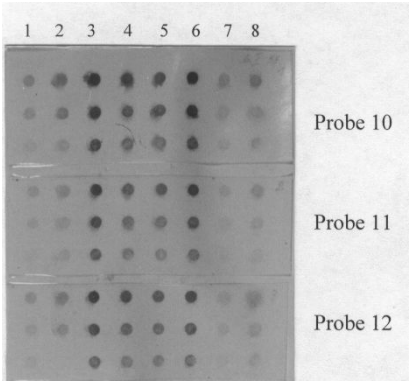

Fig.6. Dot-blot hybridisation of DNA to probes 10-12.
1, rye cv. Dańkowskie Złote; 2, rye with introgressed wheat fragment; 3, *Triticum urartu*; 4, *Aegilops speltoides*; 5, *Aegilops squarrosa*; 6, *Triticum durum*; 7, engineered rye –negative sample; 8, engineered rye – positive sample. DNA blotted in amount 100, 50 and 25 ng.

The probe 9 (*Ae. squarrosa*) hybridised a bit more strongly to DNA of *Ae. squarrosa* than to the genomes A and S. All probes (especially 7 and 9) allow discrimination between positive and negative samples of engineered rye and appear to be useful for screening rye with introgressed wheat chromosome fragments.

The probes 10-12 indicated similar characters (Fig.6). The probe 10 prepared from the fragment amplified on DNA template from *Ae. squarrosa* hybridised with similar intensity to the genomes A, S and D. The probe 11 hybridised strongly to the genome A and the probe 12 to the

genomes A and S. The probe 12 seems to be the best for screening of engineered rye.

The DNA fragments for probes 11 and 12 were amplified with the use of single primer Rep1. The amplification of some bands with the use of single primer suggest the existence of tandem arrays of this repeat organised in head to head orientation.

The results presented in this paper indicated the possibility of selection of DNA fragments that are species or genome specific. Selected probes can be used for screening of introgressed rye produced as a result of single backcross of F_1 hybrids between tetraploid triticale and rye. Tetraploid triticales have the wheat cytoplasm and contained the mixture of A and B genome chromosomes of wheat. Because of the possibility that wheat DNA fragment introgressed to rye may be derived from A and B genomes of wheat, we used DNA of *T. durum* as a genomic probe.

The use of some probes obtained by labelling PCR amplified fragments may increase the sensitivity of screening of engineered rye and limit the number of unclear results. Some probes indicated quantitative difference in their specificity to genomes A and B. It can not be excluded that some differences in hybridisation signals may be attributed to some chromosomes or chromosomal fragments. Thus, to avoid mistakes, the detection of unknown wheat fragments in rye genome can be accomplished by dot-blot hybridisation with the use of both labelled genomic DNA from *T. durum* and the selected probe from PCR amplified fragment.

CONCLUSIONS

1. In comparison to genomic DNA of *T. durum* PCR amplified fragments used as probes indicated higher generic specificity in distinguishing between rye and wheat group species (*T. durum* and presumable donors of wheat genomes) however, under applied conditions all probes showed slight cross-hybridisation with rye DNA.

2. Some DNA fragments used as probes indicated quantitative differences in their specificity to genomes A, S and D. Generally these differences are not necessarily related to the origin of an amplified fragment.

3. Selected probes used in Southern dot-blot system may improve reliability of screening for detection of wheat DNA introgressed to rye genome.

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