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Molecular detection of *Lophopyrum* chromatin in wheat-*Lophopyrum* recombinants and their use in the physical mapping of chromosome 7D

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Summary. In situ hybridisation and restriction fragment length polymorphism (RFLP) analysis were used to determine the relative location of the translocation breakpoint and the size of the integrated chromatin segment in hexaploid wheat-*Lophopyrum* translocation stocks. Three $7el_2$-7D recombinant stocks were Robertsonian translocations, 7DS.7el. The remaining recombinant stock (KS10-2) was 7elS.7el-7DL and contained only the distal one-half of the long arm of 7D. The recombinant stock with $7el_1$ (K11695) could be designated 7DS.7DL-7el where approximately the distal one-half of 7DL was replaced. RFLP analysis indicated that on the 7DL RFLP map the breakpoints for K11695 and KS10-2 are in different locations and that the two recombinants contain an overlapping region (a common region) of the *Lophopyrum* chromosome 7 in which *Lr19*, a leaf-rust resistant gene, is located. RFLP analysis also indicated that RFLP markers which mapped to within 1.5 cm of the centromere of chromosome 7D are located in the distal half of the long arm.

Key words: Wheat – *Lophopyrum* – Translocations – In situ hybridisation – RFLP analysis

Introduction

Wheat, one of the most important crop species in the world, has been cultivated for more than 10,000 years. This domestication may have contributed to the limited genetic variability seen in this species. The introduction of genetic traits from alien species has been necessary in order to expand the gene pool available for wheat improvement (McFadden 1930), and many useful traits have been introduced into wheat from alien species of *Agropyron* (*Lophopyrum*), *Hordeum*, *Secale* species etc. (Larson and Atkinson 1973; Sears 1972; Knott 1989; Whelan 1988; Friebe et al. 1991; Rogowsky et al. 1991).

When alien chromatin is transferred into wheat, detection systems are essential to monitor the genetic behaviour of the alien chromosome. Banding techniques have allowed the relatively easy identification of some of the chromosomes in wheat (Endo and Gill 1984; Gill et al. 1991), but he unequivocal identification of unknown alien chromosome segments in the wheat background is sometimes limited by the paucity of bands in some of the wheat and unknown alien chromosomes. However, the identification of alien chromatin can be successfully achieved by molecular cytogenetic analyses using species-specific molecular probes and chromosome-specific markers (Sharp et al. 1989; Chao et al. 1989; Zhang and Dvorak 1990a, b; Piastuch and Bates 1990; Hu and Quiros 1991; Kim et al. 1992).

This paper describes the identification of *Lophopyrum* chromatin in wheat-*Lophopyrum* recombinants by means of a *Lophopyrum* species-specific repetitive sequence and restriction fragment length polymorphism (RFLP) markers to define the translocation breakpoints in the wheat chromosome; this information is then used in the physical mapping of chromosome 7D.

Materials and methods

Recombinant plant lines and genomic DNA extraction

Two sources of wheat-*Lophopyrum* substitution lines and recombinant stocks were used in this study: wheat-*Lophopyrum* derivatives from wheat by. *L. ponticum* (Host.) Beauv. (*Agropyron elongatum*, 2n=70) (obtained from Dr. F. X. Laubscher of the University of Stellenbosch, South Africa) were used to pro-

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duce disomic substitution lines resistant to race 15B and 56 of stem rust (Knott et al. 1977). The substitution line (K2620 = Lm28 Marquis) also showed a moderate resistance to leaf rust in mature plants. The chromosome was shown to substitute for 7D and was designated $7el_2$ (Knott et al. 1977). Caldwell et al. (1956) produced a substitution line 'Argus' from a cross between hexaploid wheat and a decaploid (2n = 70) *A. elongatum* (*L. ponticum*). Sharma and Knott (1966) backcrossed this line to Thatcher and produced addition lines that had either a complete *Agropyron* chromosome or a telosome. A substitution line (K11463) contained a chromosome that was homoeologous with wheat homoeologous group 7; this was designated $7el_1$ (Knott 1968). It has been demonstrated that $7el_1$ and $7el_2$ pair quite regularly and therefore are almost completely homologous (Dvorak 1975). Several wheat-*Lophopyrum* translocation stocks have been produced by irradiation (Sharma and Knott 1966) from the $7el_1$ (7D) substitution. The T4 translocation carrying *Lr19*, later to be referred to as Agatha (K11695), was shown to involve the replacement of chromatin in 7DL with homoeologous chromatin from the *Lophopyrum* chromosome (Dvorak and Knott 1977). The stocks KS10-2, KS23-9, KS24-1, and KS24-2 were produced by Kibirige-Sebunya and Knott (1983) using the ph1B mutant or nullisomic 5B-tetrasomic 5D to induce homoeologous recombination between $7el_2$ and 7D. These were shown to involve group 7 translocations (Knott 1988).

The protocol used to extract the plant genomic DNA was that of Dellaporta et al. (1983) as slightly modified by Kim et al. (1992).

DNA clones

The *Lophopyrum* species-specific probe, pLeUCD2 (Zhang and Dvorak 1990a), was obtained from Dr. J. Dvorak, Department of Agronomy and Range Science, University of California, Davis, California, USA. The chromosome 7-specific cDNA clones were obtained from Dr. M.D. Gale, Institute of Plant Science Research, Cambridge Laboratory, Colney Lane, Norwich, UK.

Probe labelling

For *in situ* hybridisation, the pLeUCD2 sequence was labelled with Digoxigenin according to the manufacturer's specifications (Boehringer Mannheim). For Southern hybridisation, the inserts of pLeUCD2 and homoeologous group 7 chromosome-specific cDNA clones were labelled with ^{32}P using the oligolabelling reaction, and the unincorporated nucleotides were separated using the Sephadex G50 spin column method (Maniatis et al. 1982).

In situ hybridisation

Chromosomes were prepared according to Le et al. (1989), and in situ hybridisation as well as signal detections were carried out according to the manufacturer's suggestions (DIG Nucleic Acid Labelling and Detection, Boehringer Mannheim).

Before hybridisation, the slides were treated with RNase (100 µg/ml in 2 × SSC) at 37° for 30 min, washed consecutively in 2 × SSC, 70% EtOH, and 95% EtOH for 5 min each, and air dried. Chromosomal DNA was denatured in 70% formamide in 2 × SSC at 80 °C for 3.5 min, washed in cold (−20 °C) 70% and 95% EtOH for 5 min each, and then air dried completely. Fifteen microliters of heat-denatured probe mixture (5 ng probe DNA, 50% formamide, 2 × SSC, 10% dextran sulfate, and 10 µg denatured and fragmented salmon sperm DNA) was applied to each slide, and the chromosomal DNA and probe DNA were once again denatured at 80 °C for 3 min. Hybridisation was performed at 40 °C overnight in a moisture chamber. The slides were then dipped in 2 × SSC at 40 °C for 5 min to float off the coverslips, and the unhybridised or loosely hybridised probe DNAs were washed off by washing the slides in the following solutions sequentially: 50% foramide in 2 × SSC for 5 min at 40 °C, 2 × SSC for three periods of 5 min at 40 °C, 0.5 × SSC for three periods of 5 min at 40 °C, and 0.5 × SSC for three periods of 5 min at room temperature. The ⟨Dig⟩ signals were detected at room temperature by the following procedures. The slides were dipped into Buffer I (0.1 *M* TRIS-Cl pH 7.5, 0.15 *M* NaCl) for 1 min; the slides were then transferred to Buffer II (0.5% blocking agent in buffer I) for 30 min and finally back to Buffer I for 1 min. Then 100 µl of 1:5000 diluted ⟨Dig⟩ Ap-conjugated antibody in Buffer I was applied to each slide and the slide placed into a dark moisture chamber for 2 h. The slides were once again dipped into Buffer I for three separate 10-min periods to wash off the unbound antibody and then equilibrated with Buffer III (0.1 *M* TRIS-Cl pH 9.5, 0.1 *M* NaCl, 0.05 *M* $MgCl_2$) for 2 min. The ⟨Dig⟩ signals were generated with 300 µl of colour-generating solution (35 µg NBT and 300 µg BCIP in 1.5 ml Buffer III) per slide in a moisture chamber for 3–24 h in the dark. After the appearance of maximum signals, the slides were washed in 1 × TE for a few min, dried completely in a dessicator, and mounted with Permount (Fisher).

Southern hybridisation

Plant genomic DNAs were digested with restriction enzymes (5 units/µg genomic DNA) *Hae*III, *Taq*I, *Eco*RI, and *Hin*dIII. The genomic DNAs were then electrophoresed in 1% agarose gel in 1 × TAE buffer (Maniatis et al. 1982) overnight at 35 V and transferred to nylon membrane (Hybond plus, Amersham) by the alkaline method (Maniatis et al. 1982).

Prehybridisation was performed for 6 h at 65 °C in a solution of 5 × SSPE, 5 × Denhardt, 0.5% SDS, and 500 µg of denatured and fragmented salmon sperm DNA. The membranes were transferred to hybridisation solution which was the same as the prehybridisation solution but contained labelled and denatured probe DNA. After an overnight hybridisation with constant shaking at 65 °C the membranes were washed twice for 10 min each in 2 × SSPE, 0.1% SDS at room temperature. They were then washed once in 1 × SSPE, 0.1% SDS at 65 °C for 15 min and twice in 0.1 × SSPE, 0.1% SDS at 65 °C for 10 min. The membranes were exposed to Kodak X-Omat X-ray film at −70 °C for 1–14 days depending on the remaining radioactivity in the membranes. The probes were stripped off the membranes by pouring boiling 0.5% SDS in distilled water over them, and the filters were subsequently reprobed.

Results and discussion

In situ hybridisation using Lophopyrum species-specific repetitive sequence, pLeUCD2

In situ hybridisation using the pLeUCD2 probe has shown that the pLeUCD2 sequence is distributed evenly along the chromosomes in all of the *Lophopyrum* chromosomes (Zhang and Dvorak 1990b). Our results also confirmed their findings that the fragments of *Lophopyrum* chromosomes are mainly hybridized with the pLeUCD2 sequence (Fig. 1). Although there were some minor hybridisation signals on the wheat chromosomes, this did not preclude the determination of the *Lophopyrum* chromosomes in the wheat background. In substitution lines K11463 and K2620 one pair of chromosomes in the 42 somatic chromosomes hybridised with the

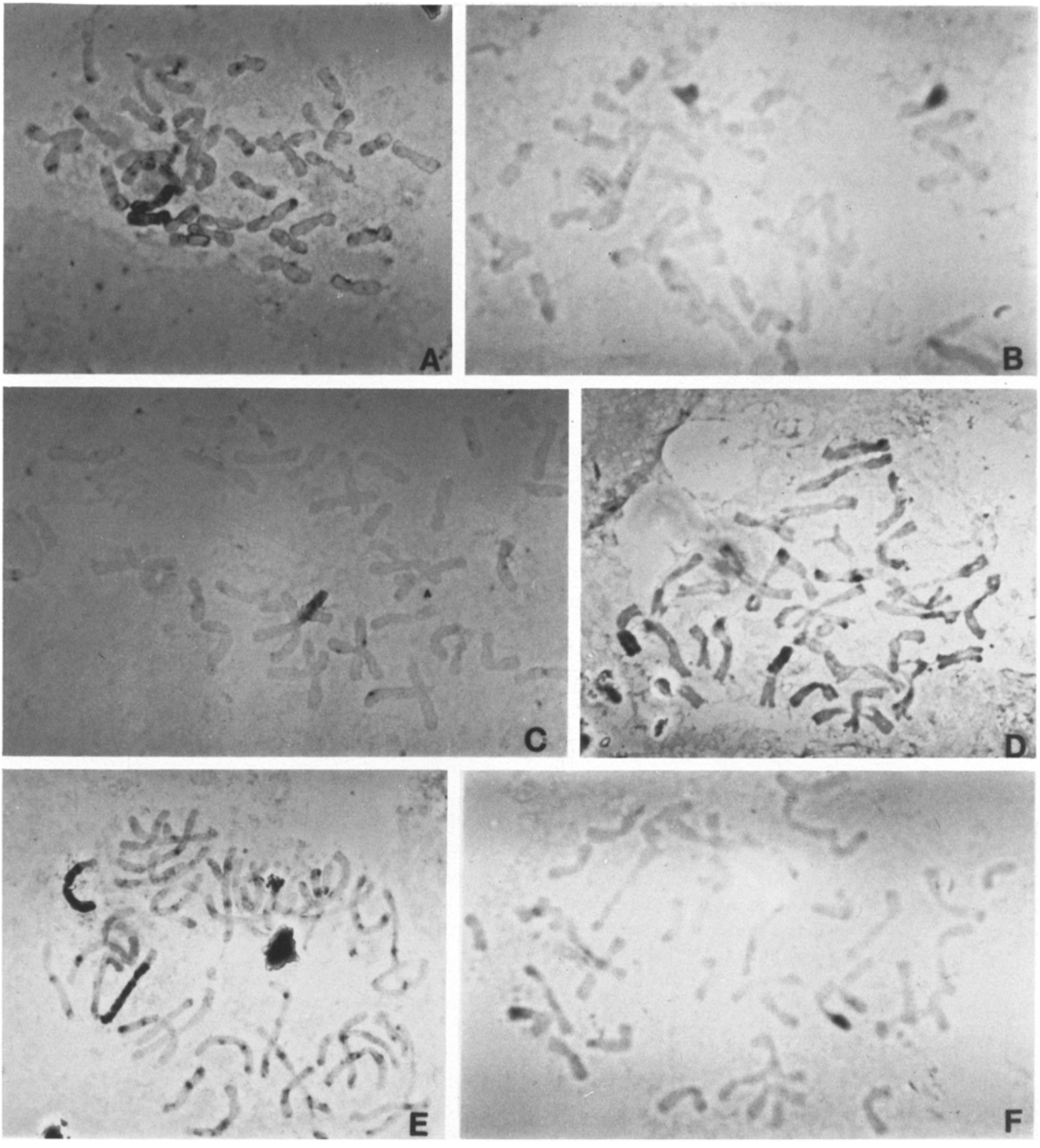

Fig. 1 A–F. In situ hybridisation using pLeUCD2 as a probe on several recombinant lines and a chromosome substitution lines. **A** Substitution line K11463; **B–F** recombinant lines KS24-1 (**B**), KS23-9 (**C**), KS24-2 (**D**), KS10-2 (**E**), K11695 (**F**)

pLeUCD2 probe (Fig. 1 A). In lines KS24-1 and KS24-2 a pair of chromosome arms were hybridised with the probe (Fig. 1 B and D), but in KS23-9 only one chromosome arm showed hybridisation (Fig. 1 C). In KS10-2 almost the whole, of each chromosome in one pair, except for a small telomeric segment, was replaced by the *Lophopyrum* chromosome (Fig. 1 E); line K11695 showed the reverse pattern to line KS10-2 (Fig. 1 F) in that approximately half of one arm of a chromosome pair was replaced with *Lophopyrum* chromatin.

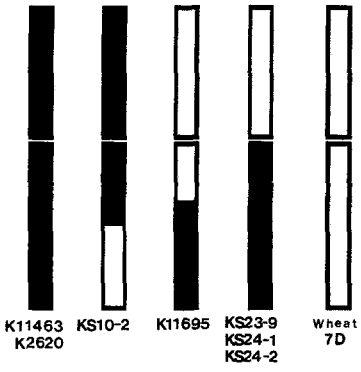

Fig. 2. Schematic drawing of recombinant chromosomes, *Lophopyrum* chromosome 7 Ag, and wheat 7D chromosome disregarding actual sizes of 7 Ag and 7D. Chromosomes painted *black* are designated as 7 Ag and *white* as 7D

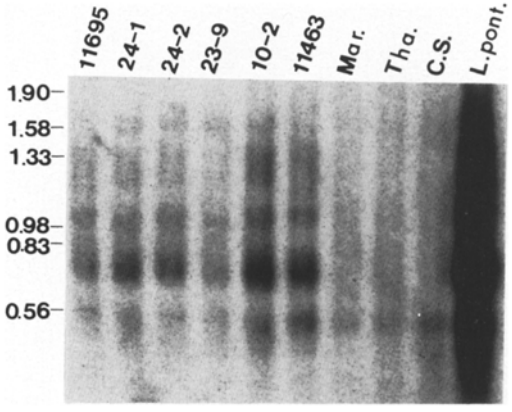

Fig. 3. Southern hybridisation using pLeUCD2 as a probe on several recombinant lines, wheat, and *Lophopyrum ponticum*. The amount of DNA loaded in each lane was 10 µg except for *L. ponticum*, which was 5 µg

Knott et al. (1977) showed that the $7el_1$ chromosome in *Lophopyrum* could compensate well for the loss of chromosome 7D in substitution lines. In subsequent studies, Dvorak and Knott (1977) localised the leaf rust resistant gene (*Lr19*) on the long arm of the chromosome $7el_1$. Therefore, in the lines in our study the translocated segment of a *Lophopyrum* chromosome should be in the long arm of chromosome 7D. The schematic drawing of the recombinant chromosomes are shown in Fig. 2.

Southern hybridisation analyses using pLeUCD2

Lophopyrum ponticum showed smear bands of sizes ranging from 0.3 to 2 Kb in the *Hae*III digest when pLeUCD2 was used as a probe in the Southern hybridisation (Fig. 3). Several bands having approximate sizes of 0.56, 0.7, 1.1, and 1.5 kb appeared in recombinant lines. The 0.56-kb band was also present in the wheat lanes, which was not observed in previous studies (Zhang and Dvorak 1990a, b). However, the remainder of the bands (0.7, 1.1, and 1.5 kb) were not present in the wheat lanes, and the intensities of each band increased according to the size of the *Lophopyrum* chromosome or segment present in the recombinant lines. The intensities of the bands in line KS24-1 and KS24-2 were approximately half those of lines of KS10-2 and K11463. The line KS23-9 showed the least intense bands, and K11695 also showed less intense bands than KS24-1 and KS24-2.

Similar results were obtained from studies using another restriction enzyme, *Taq*I (not shown). The 1.3-kb restriction fragment was common to both *Lophopyrum* and wheat. However, the substitutions and recombinants showed a few more diagnostic bands at approximate sizes of 1.1, 1.0, 0.8, and 0.7 kb. The intensity of each band also increased according to the size of the *Lophopyrum* fragment present in the recombinants. Therefore, the results obtained from Southern hybridisation corresponded well to the observations from *in situ* hybridisation.

The common band in each digest was still present after stringent hybridisation (hybridizing at 68 °C) and washing (washing twice at 65 °C for 30 min with 0.1 × SSC and 0.1% SDS). However, the presence of these common bands did not obscure the presence of the diagnostic bands for *Lophopyrum* chromatin in the recombinants.

RFLP analyses using chromosome 7-specific cDNA clones and their physical location on chromosome 7D

Chromosome 7-specific cDNA clones (Chao et al. 1989) were utilised to characterise the translocated arm in each recombinant line. When the cDNA clones, which were mapped to the short arm of 7D, were used as probes, lines K11695, KS24-1, KS24-2, and KS23-9 all showed the expected bands. However, lines KS10-2 and K11463 were missing the expected 7DS band (Figs. 3 and 4). When the cDNA clones for the long arm of 7D were used as probes, lines KS24-1 and KS24-2 lacked the expected band that corresponded to 7DL (figure not shown). Therefore, in lines KS24-1 and KS24-2, which were Robertsonian translocations, the long arm of 7D was replaced by the long arm of chromosome $7el_2$, which carries the rust resistant genes. Line KS23-9 should also be a $7DS.7el_2$, translocation heterozygote. In line KS10-2 the probes for the proximal half (X165, X72, X117) of 7DL were missing (Fig. 6), but the probes for the distal half (X129 and X121) were present (Figs. 5 and 6). However, the recombinant line K11695 showed none of the 7DL probes (Fig. 6), although the proximal half of 7DL was still present (Fig. 1F). Therefore, the breakpoint for KS10-2 and K11695 should be between X72 and X129 for line KS10-2 and between the centromere and X165 for line K11695 (Fig. 7).

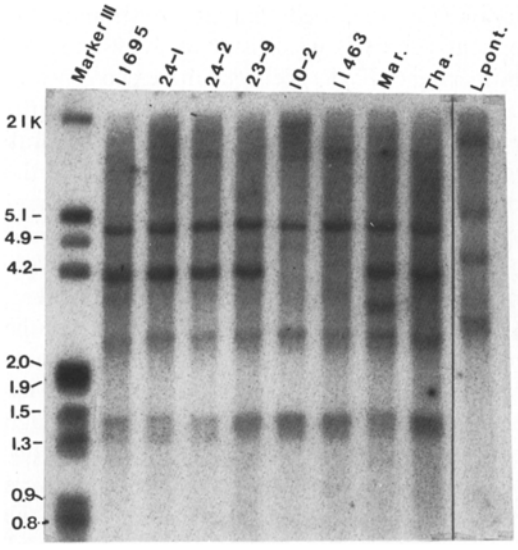

Fig. 4. Southern-hybridised autoradiogram of the *Eco*RI-digested genomic DNAs using the 7DS probe (×150). Note the polymorphism of the 7D band between wheat lines Marquis and Thatcher

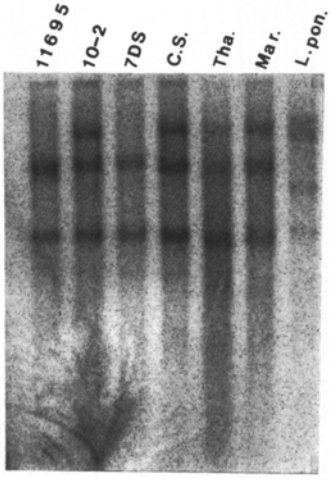

Fig. 6. Southern-hybridised autoradiogram of the *Hin*dIII-digested genomic DNAs using the 7DL probe (×129)

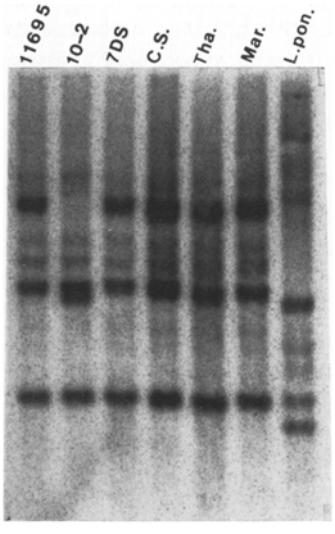

Fig. 5. Southern-hybridised autoradiogram of the *Hin*dIII-digested genomic DNAs using the same probe as in Fig. 4. Note the additional band in line 10-2, which corresponds to the *Lophopyrum* band

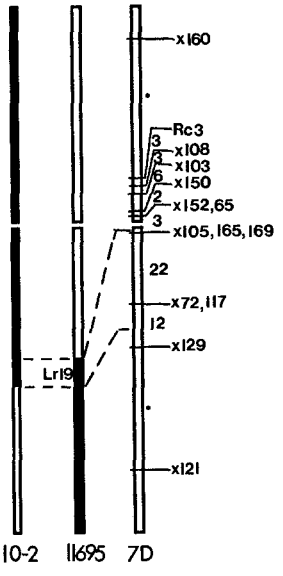

Fig. 7. The schematic drawing of the break points in chromosome 7D in lines KS10-2 and K11695. The recombinant map on the right is from Chao et al. (1989)

The physical distance from the centromere to the breakpoint for line K11695 was almost half the length of 7DL based on the measurement of eight recombinant chromosomes. The distance from the centromere to X165, which is almost half the length of the arm covers less than 1.5 cM on the recombination map (Chao et al. 1989). Similar discrepancies between the physical map and the recombination map have also been reported in chromosome 1B (Curtis and Lukaszewski 1991), 5A (Jampates and Dvorak 1986; Tsujimoto et al. 1990), and 6B (Dvorak and Chen 1984; Dvorak and Appels 1986) in wheat and 1R in rye (Baum and Appels 1990). Tsujimoto et al. (1990) showed that an approximate 13% loss of chromosome 5AL accounted for the loss of more than 85% of the genes mapped in that arm. It has also been shown that the distal part of the NOR in chromosome 1R, which is less than 30% of the arm, covers more than 140 cM of the recombination map (Baum and Appels

1991). These results suggest that either recombination in the proximal half of the chromosome is supressed or that suitable markers for this region are rare and, therefore, have not been found.

Physical location of Lr19 and Sr25 in the recombinant chromosomes

A gene for leaf rust (*Puccinia triticina*) resistance, *Lr19*, has been localised in the long arm of the recombinant chromosomes between 7D and $7el_1$ (Dvorak and Knott 1977; Sears 1977; Eizenga and Sears 1983). This localisation based on the observations of meiotic pairing, which did not permit the identification of the actual placement of the gene on 7DL. Knott (1984) reported that the recombinant lines carrying *Lr19* also carried Sr25 (information from R.A. McIntosh, Australia). In *Agropyron intermedium* (Host) P.B. a gene for leaf rust resistance appears to be distal to a gene for stem rust resistance on chromosome 7 (Friebe et al. 1992). However, it is not known if these genes would be allelic to *Lr19* and *Sr25* of *Lophopyrum ponticum*. Knott et al. (1977) also reported that the substitution lines of *Lophopyrum* $7el_2$ for wheat chromosome 7D carried a stem rust resistance gene, *Puccinia graminus tritici* Eriks. It is not known if this gene is an allele of *Sr25* or if there is an *Lr* gene linked to this unnumbered *Sr* gene (D. R. Knott, unpublished results). The inference about the location of *Lr19* and *Sr25* in Fig. 7 is an hypothesis based on two assumptions. Since both recombinant lines, K11695 and KS10-2, carried resistance to leaf rust and stem rust infection, it is assumed that the genes they carry may be alleles. If so, the physical location of the *Lr19* and *Sr25* genes, may be on the overlapping region of these two recombinant lines. Secondly, it is assumed that the breakpoints in 7DL are reflected in comparable breakpoints in the *Lophopyrum* homoeologues, $7el_1$ and $7el_2$. This is still under investigation. All 7D probes did not produce recognisable restriction fragments on *Lophopyrum* DNA, and in most cases characteristic *Lophopyrum* fragments did not replace the deleted 7DS or 7DL fragment of wheat in the recombinant lines. This suggests that, in spite of the fact that 7el compensates for 7D, the homoeology is less than complete. Similar results were obtained by Marais (1992). It has been demonstrated by means of RFLP markers that chromosomes 4R, 5R, and 7R are structurally rearranged with respect to their wheat counterparts (Liu et al. 1992). It is to be expected that structural rearrangements would also be found in *Lophopyrum*.

Future considerations and conclusions

Alien chromosome translocation lines usually carry not only the desirable gene(s) but also agronomically undesirable gene(s). For example, the recombinant lines between 7D and $7el_1$ carry a gene for yellow flour colour in addition to the genes for rust resistance (Knott 1980, 1984). Marais (1992) showed that the location of the yellow pigment gene (*Y*) was distal to *Lr19* and also located another gene (*WSP1*) between these two genes.

Several attempts have been made to dissociate the yellow gene from the rust resistance genes. Dvorak (1975) and Knott and Dvorak (1976) tried to break the linkage of the two genes by inducing pairing and crossing-over between the recombinant chromosome and a homoeologous *Lophopyrum* chromosome from *L. elongatum* ($2n=14$) that did not carry the gene for pigment. However, no crossing-over was obtained between the genes even though chromosome pairing occurred. Knott (1984) produced two low-pigment mutant plants by using ethyl methane sulfonate (EMS). However, although some lines were agronomically quite good, many contained deleterious characters such as low yield that were not present in the original recombinant lines. After the isolation of overlapping translocation lines, it may be possible to reduce the amount of *Lophopyrum* chromatin by crossingover in the overlapping region. Therefore, we crossed the recombinant line KS10-2 with K11695 and obtained a dozen F_1 seeds. Cytological analysis of the F_1 hybrid will confirm the hypothesis that K11695 and KS10-2 carry translocations that overlap. If this proves to be true, we hope to obtain hemizygous segmental translocation lines in the F_2 and subsequently obtain homozygous segmental translocation lines that carry *Lr19* on an interstitial translocation and therefore carry a reduced amount of *Lophopyrum* chromatin. The yellow flour gene will still be present on this interstitial translocated segment, and it will be necessary to induce recombination in this segment with a wheat or *Lophopyrum* segment lacking the yellow gene.

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