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The isolation, characterization and application in the Triticeae of a set of wheat RFLP probes identifying each homoeologous chromosome arm

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Summary: To investigate the use of RFLP analysis in the Triticeae, a set of low copy number probes has been isolated from a wheat cDNA library. The probes identify each of the 14 homoeologous chromosome arms of wheat as determined by analysis of DNA fragments hybridizing to the probes in aneuploid lines of Chinese Spring. These probes can be used in RFLP analyses both for the assignment of homoeology of alien chromosomes or arms added to wheat, and for the determination of chromosome dosage in wheat aneuploids. Different chromosomes from various Triticeae species can therefore be followed in a wheat genetic background using a single technique. The potential uses of the set in facilitating the transfer of alien segments into wheat are outlined.

Key words: Hexaploid wheat – RFLPs – Homoeology – Chromosome arm – Alien additions

Introduction

The introduction of genetic material from relatives of wheat into wheat itself has long been practised (see Gale and Miller 1987, for review), and many intentionally constructed, or fortuitously isolated wheat-alien chromosome addition, substitution and translocation lines exist (Shepherd and Islam 1988). Some of these lines have been important parents in the development of varieties which retain segments of alien chromosomes conferring desirable characteristics, mainly disease resistances (Johnson and Lupton 1987). The isolation and the subsequent manipulation and characterization of such lines is greatly aided by the use of marker genes, such as isozymes and morphological markers. These also enable an assessment of chromosome homoeology to be made (Gale and Miller 1987; Miller and Reader 1987). However, the use of many of the morphological markers requires considerable experience, since these are subject to variable expression in different genetic backgrounds, while the various isozymes and proteins which are available each require separate, and different, electrophoretic and staining techniques. In addition, extensive intercrossing between the addition lines, followed by examination of meiosis in the hybrids, is necessary to identify and classify the different additions produced. The assignment of homoeologies within a single addition series requires, therefore, the use of a large number of techniques. For example, Forster et al. (1987) used both chromosome and plant morphology, as well as analysis of ten isozyme and protein markers in extracts of two tissues, separated in four different types of gel, stained by seven different methods, in their study of the homoeology of six *Agropyron intermedium* addition lines. The advantages of being able to use a single technique to achieve this type of resolution are obvious. DNA hybridization technology offers this prospect through the detection of restriction fragment length polymorphisms (RFLPs) between the wheat and alien chromosomal segments.

Materials and methods

cDNA library construction

A cDNA library was constructed using mRNA extracted from mature leaves of the cultivar Chinese Spring (CS), using an "Amersham" cDNA synthesis kit as recommended by the manufacturer. Colony hybridization (Maniatis et al. 1982) was used to identify clones containing cDNAs for the small subunit of ribulose-1,5-bisphosphate carboxylase (SSU), and the major chlorophyll a/b binding protein (CAB), using probes for these genes (Broglie et al. 1983; Lamppa et al. 1985).

Table 1. Homoeologous relationships of the three series of alien addition lines analysed

Additions			
Wheat homoeologous group	*Hordeum vulgare*[a] cv Betzes	*Secale cereale*[b] cv Imperial	*Aegilops umbellulata*[c]
1	–	1R	B
2	E	2R	D
3	F	3R	–
4	A	4R	F
5	B	5R	C
6	C	6R	A
7	D	7R	G

[a] Islam et al. 1981; Gale and Miller 1987
[b] Driscoll and Sears 1971; Miller 1984
[c] Kimber 1967; Chapman et al. 1975

Genetic material

DNA was isolated from CS nullisomic-tetrasomic (NT) and ditelosomic (DT) lines from three sets of alien addition lines to CS: *Hordeum vulgare* cv Betzes (Islam et al. 1981), *Secale cereale* cv Imperial (Driscoll and Sears 1971) and *Aegilops umbellulata* (Kimber 1967), and from Betzes and the CS/Imperial and CS/*Ae. umbellulata* amphiploids. The assumed homoeologous relationships of the alien chromosomes present in these addition lines are shown in Table 1. The chromosome nomenclature used here is that agreed upon at the 7th International Wheat Genetics Symposium, Cambridge, in July 1988. The chromosomes previously designated 4A and 4B are now designated 4B and 4A, respectively.

All seed came from IPSR stocks maintained by T. E. Miller and S. M. Reader and, where appropriate, plants were examined cytologically by these workers for correct chromosome constitution before DNA isolation.

RFLP procedures

All methods of DNA extraction, restriction enzyme digestion, agarose gel electrophoresis, alkaline "Southern" blotting to nylon membranes, probe preparation, hybridization and fragment size determination were as described by Sharp et al. (1988), with the modification that Gene Screen Plus (NEN) membranes were used.

Results and discussion

Initial screening of the cDNA library

Approximately 600 cDNA clones were picked and grown individually. Plasmid mini-preparations were made of clones not hybridizing to the cloned SSU and CAB genes, and the inserts were excised by a double digestion with EcoRI and HindIII. Following agarose gel electrophoresis and ethidium bromide staining, 140 clones were selected for further study, as these had inserts larger than approximately 300 bp.

Each of these probes were hybridized individually to Southern blots of HindIII-digested DNA from the *H. vulgare* cv Betzes, *S. cereale* cv Imperial and *Ae. umbellulata* addition line series (Table 1). Unique DNA fragments, contributed by the alien parents and not present in CS but present in at least one of the addition lines, enabled the DNA fragments hybridizing to the probes to be located to homoeologous chromosome groups. In most cases, assignment to homoeologous group was unequivocal, as the addition lines carrying the same homoeologous group chromosome had the alien DNA fragments. In those cases where the assignments based on each of the addition line series did not agree, the assignment based on the *H. vulgare* cv Betzes series was taken as the basis for further analysis. The patterns of DNA fragments revealed by each clone also enabled duplicates of previously analysed clones to be identified. Of the 140 clones analysed, 73 were unique. The distribution of these to homoeologous groups is shown in Table 2, with 67 clones detecting fragments from one homoeologous group only, whilst six clones hybridize to fragments located on two groups.

Table 2. The location to homoeologous group of fragments identified by 73 unique cDNA clones

No. of homoeologous group	Homoeologous group							Total
	1	2	3	4	5	6	7	
One only	5	20	8	10	7	9	8	67
Two groups	1	3	1	1	2	–	4	12

The clones are distributed over all seven homoeologous groups. The total assigned to group 1 is lowest, while group 2 has the largest number. This is the reverse of the situation with respect to isozyme and protein markers (mostly from grain samples), where group 1 has the most, and group 2 has the fewest assigned markers (McIntosh 1988). Further work will be needed to assess whether this result is a feature of using mature leaf cDNA clones.

Isolation of a set of probes to identify each homoeologous chromosome arm

In order to isolate a set of probes which identify a set of loci on each of the 14 wheat homoeologous chromosome arms, probes for each homoeologous chromosome group were hybridized individually to filters containing HindIII- and BamHI-digested DNA of both the CS NT and DT lines, and the appropriate homoeologous group alien addition lines. This enabled the probes to be classified on the basis of detecting DNA fragments located to individual wheat chromosome arms, and reconfirmed the previous locations in the alien addition sets.

Table 3 lists the probes chosen with their insert lengths, while Tables 4 and 5 give their chromosomal location and the sizes of the fragments detected. Figure 1 shows the hybridization of the two group 5 probes chosen, 118 and 128, to HindIII- and BamHI-digested

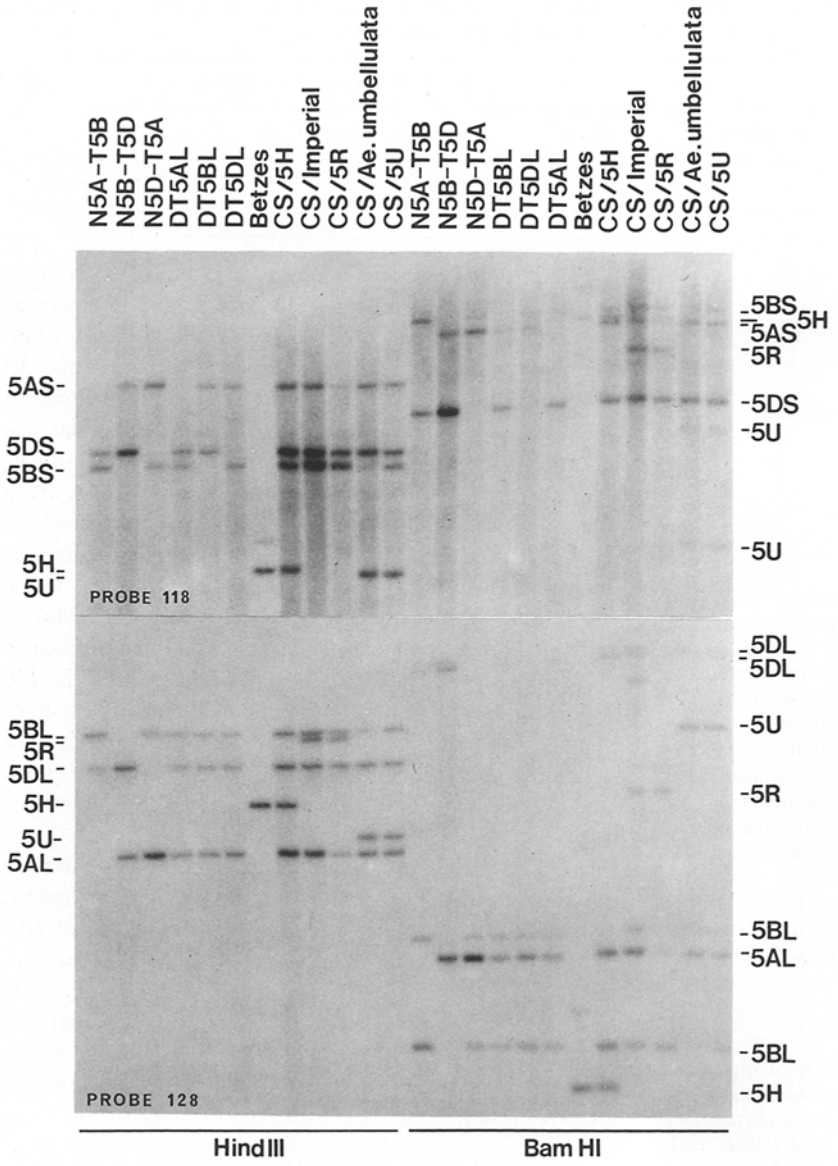

Fig. 1. Hybridization of the selected homoeologous group 5 probes to HindIII- and BamHI-digested DNA of the CS group 5 nullisomic-tetrasomic and ditelosomic lines, and of three group 5 alien addition lines. The probes and plant lines used are indicated

Table 3. The 14 clones chosen and the approximate length of the largest EcoRI + HindIII cDNA insert fragment used as a probe

Clone	Insert (kb)
101	0.6
118	0.7
123	0.5
128	0.5
129	1.2
135	1.0
144	1.3
152	0.8
154	0.4
156	0.9
161	0.7
162	2.0
163	0.5
167	0.7

DNA from the goup 5 NT and DT lines, Betzes, the group 5 barley addition, the CS/Imperial rye and CS/*Ae. umbellulata* amphiploids, and the group 5 additions derived from each of them. Probe 118 clearly detects a set of loci on the short arms of the wheat group 5 chromosomes, as it detects DNA fragments absent from DT5AL, DT5BL and DT5DL, while 128 detects loci on the long arms of these chromosomes, as the hybridizing DNA fragments, absent from N5A-T5B, N5B-T5D and N5D-T5A, are present in the long arm DT lines (Fig. 1).

Each of the probes usually detects one or two fragments on the appropriate arm following digestion with each of the two restriction enzymes used, but in three cases, the fragments from two or three wheat homoeologues are of similar size and hence co-migrate upon electrophoresis. For example, probe 163, which reveals

Table 4. Sizes (kb) of HindIII fragments detected by the 14 probes in the genomes analysed. The chromosomal location in the alien species is given by the position in the table, except where indicated in the footnotes

Probe	Arm location[a]	Genome					
		A	B	D	H	R	U
161	1S	4.2	2.8	6.0	13.5*	?	4.7
162	1L	3.5	2.8	3.3	11.0*	6.0	4.0
135	2S	11.8	13.5	9.4	7.4	?	8.8
101	2L	13.0	9.0	7.0	6.5	5.4	8.0+2.4
123	3S	2.1	5.2	1.6	3.5	5.0	4.5*
156	3L	4.1	7.4	3.9	10.2+7.6	4.4	4.5*
144	4S[+]	4.4	6.0	10.0	5.0	?	8.0
163	4L[+]	5.0	1.7	3.0	4.6	2.7[b]	3.5[c]
118	5S	9.0	5.6	6.2	4.0+3.6	?	3.5
128	5L	5.4	11.0	9.0	7.0	10.5	5.9
167	6S		(5.8+2.6)[e]		13.0	2.4[d]	12.0+7.5[f]
154	6L	2.3	5.6+3.0	6.6+2.5	4.7	7.2+1.6	?
152	7S	4.5	6.4	11.5	9.0+2.8	6.0[d]	9.2
129	7L	4.0	7.2	10.5	4.8	12.5	11.5

[a] In the hexaploid wheat genomes
[b] Located on 7R
[c] Present in the CS × *Ae. umbellulata* amphiploid, but not in the presumed group 4 addition, nor in the five other available addition lines
[d] Located on 4R
[e] Neither band is absent in stocks lacking 6A, 6B or 6D, so each represents at least two co-migrating fragments
[f] The 12.0-kb fragment is located on 7U and the 7.5-kb fragment on 2U
[?] No extra fragments can be identified in the amphiploids, or in any of the addition lines analysed
* These fragments are present in either the species or the amphiploid, but not in any of the six available addition lines, so they are presumed to be located on the chromosome not represented in each addition series (group 1 in barley, group 3 in *Ae. umbellulata*), in agreement with the location in the other two series of additions
[+] 4S signifies arms 4AL, 4BS and 4DS; 4L signifies arms 4AS, 4BL and 4DL (evidence for homoeologous relationships listed in McIntosh 1988)

Table 5. Sizes (kb) of BamHI fragments detected by the 14 probes in the genomes analysed. The chromosomal location in the alien species is given by the position in the table, except where indicated in the footnotes

Probe	Arm location[a]	Genome					
		A	B	D	H	R	U
161	1S	13.5+4.0	7.8+2.3	4.0	4.2*	6.2	?
162	1L	4.8	5.2	5.4	5.8*	5.7	?
135	2S	2.6	5.2+2.0	10.4+4.4	4.5+2.3	3.4	14.0
101	2L	21.0	19.0+13.0	3.4	8.0	2.0	9.5
123	3S	8.8	11.4	8.5+7.2	14.0	?	8.0*
156	3L	18.0+10.4	7.0	9.0	14.0+10.0	21.0	20.0*
144	4S[+]	9.5	3.6	14.0	11.0	17.0[b]	13.0
163	4L[+]	13.5[d]	13.5[d]	16.5	11.0	15.0[b]	13.3[c]
118	5S	13.0	15.0	7.8	18.0+14.0	11.0	6.8+4.8
128	5L	3.4	3.8+2.5	19.0+18.0	2.0	8.8+7.4	11.0
167	6S	9.0	5.6	7.6	9.8	?	10.5+6.4[e]
154	6L	9.8	14.0	10.6	7.2+5.4	?	5.8
152	7S	6.4	4.3	6.6	2.6	?	?
129	7L	13.0[f]	15.5	13.0[f]	5.5	8.0+6.0	?

[a], [b], [c], [?], [*], [+] as in Table 4
[d] Neither band is absent in stocks lacking 4A or 4B, so each represents at least two comigrating fragments
[e] The 10.5-kb fragment is on 2U, and the 6.4-kb fragment is on 7U
[f] The 13.0-kb band is present in stocks lacking 7A or 7D, so represents two co-migrating fragments

loci on 4AS, 4BL and 4DL in HindIII digests (Table 4), detects a band of hybridization 13.5 kb in BamHI digests, which does not disappear when either 4AS or 4BL are absent, although there is a single BamHI fragment on 4DL (Table 5). The use of two separate digests with HindIII and BamHI, however, enables the probes to detect loci on each of the chromosome arms in each homoeologous group (Tables 4 and 5). In addition, the locations of DNA fragments hybridizing to the probes on the arms within each group are in agreement with previously determined homoeologies (Gale and Miller 1987; Sharp and Soltes-Rak 1988; McIntosh 1988), and the detected loci are not involved in any of the translocations now known to have occurred between wheat chromosomes during the evolution of hexaploid wheat (Naranjo et al. 1987).

Figure 1 also shows that probe 128 identifies and HindIII fragment in Betzes present in the addition, and the Imperial and *Ae. umbellulata* amphiploids each have an additional fragment which is present in the respective group 5 additions, in addition to the three in CS. The same is evident with probe 118 with the Betzes and *Ae. umbellulata* addition lines, but in this case no discrete Imperial HindIII fragment is visible in either the amphiploid or the group 5 addition. However, the BamHI digests reveal that there are unique fragments on chromosome 5R, and it is likely that in HindIII digests this probe detects an Imperial fragment of similar size to that from the CS 5B chromosome. The 5B band is of equal or greater intensity to the 5D fragment in both the amphiploid and 5R addition, whereas the ratios of intensities of these bands is reversed in the other tracks (Fig. 1).

Overall using two restriction enzymes, the 14 probes were found to hybridize to DNA fragments from the 3 alien species, and the majority identify the same fragments in particular addition lines (Tables 4 and 5). Figures 1 and 2 and Tables 4 and 5 also indicate a difference between HindIII and BamHI, in that HindIII tends more often to give rise to one fragment per genome (and thus a simpler pattern), and that these fragments are more often within the more easily analysed size range (2–10 kb) than BamHI fragments. HindIII, therefore, seems to be the enzyme of first choice for these analyses.

Homoeology of chromosomes in the addition lines studied

In the Betzes addition series, all of the detected fragments are located to the addition lines expected by the homoeologies outlined in Table 1. Tables 4 and 5 also show that in the complete Imperial series, probes for groups 1, 2, 3 and 5 identify fragments in the single appropriate addition line, while the group 4, 6 and 7 probes reveal translocations in *S. cereale* relative to the wheat chromosomes. Probes 163 (wheat 4AS, 4BL, 4DL) and 152 (wheat group 7, S arm), which detect loci on chromosomes 7R and 4R respectively, therefore corroborate the known group 4/group 7 translocation present in *Secale* (Koller and Zeller 1976; Miller 1987). In addition, probe 144 (4AL, 4BS, 4DS) also detects a locus on 4R, indicating the non-centric breakpoint of the 4/7 *Secale* translocation, as suggested by Koller and Zeller (1976). A further complication is that probe 167 (group 6, S arm) detects a locus on 4R, indicating that this chromosome also contains some group 6 genetic material (Tables 1 and 2). Although Koller and Zeller (1976) suggested the involvement of 6R in a translocation with 7R, they did not find that 4R has any homoeology with 6R. However, Rao and Rao (1980) have found loci controlling an identical 6-PGD isozyme on 4R and 6R by analysis of the Imperial addition lines, which may suggest some relationship between these chromosomes.

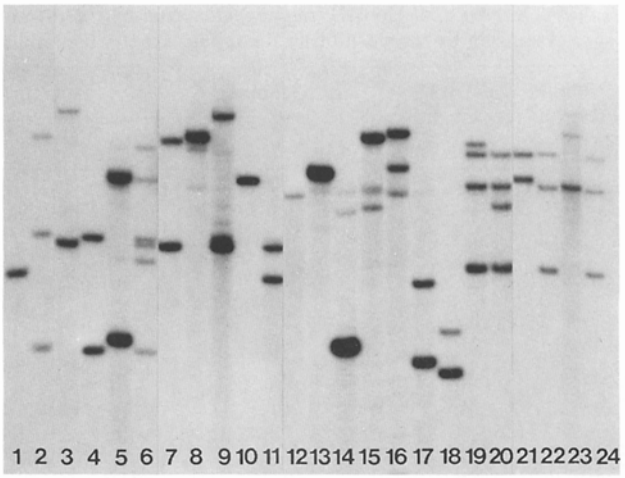

Fig. 2. Hybridization of probe 128 to HindIII restricted DNA from a range of Triticeae species. *Track* 1 – *Aegilops sharonensis* (2x), 2 – *Ae. vavilovii* (6x), 3 – *Ae. biuncialis* (4x), 4 – *Ae. crassa* (4x), 5 – *Ae. cylindrica* (4x), 6 – *Ae. juvenalis* (6x), 7 – *Ae. ovata* (4x), 8 – *Ae. uniaristata* (2x), 9 – *Ae. triaristata* (6x), 10 – *Ae. squarrosa* (2x), 11 – *Ae. variabilis* (4x), 12 – *Agropyron junceum* (2x), 13 – *Haynaldia villosa* (2x), 14 – *Ae. caudata* (2x), 15 – *Ae. speltoides* (2x), 16 – *Ae. mutica* (2x), 17 – *Ae. longissima* (2x), 18 – *Hordeum bulbosum* (2x), 19 – CS/5H^{ch} (group 5 *H. chilense* addition into CS), 20 – CS/*Ag. elongatum* amphiploid (8x), 21 – *Triticum durum* (4x), 22 – *T. spelta* (6x), 23 – *Ae. ventricosa* (4), and 24 – *T. aestivum* cv CS (6x)

In the *Ae. umbellulata* series, the group 1, 2, 5 and 7 probes identify fragments in the appropriate addition line only, but the other probes give a complicated pattern (Tables 4 and 5). The group 3 probes hybridize to alien fragments in the amphiploid which are not present in the six available additions and, therefore, the missing addition is certainly of homoeologous group 3.

Probes 163 and 167 indicate that the chromosomes in the *Ae. umbellulata* addition series have a number of translocations relative to wheat. Probe 163 (wheat group 4) does not detect DNA fragments in the addition shown by probe 144 to be of group 4, while the group 6 short

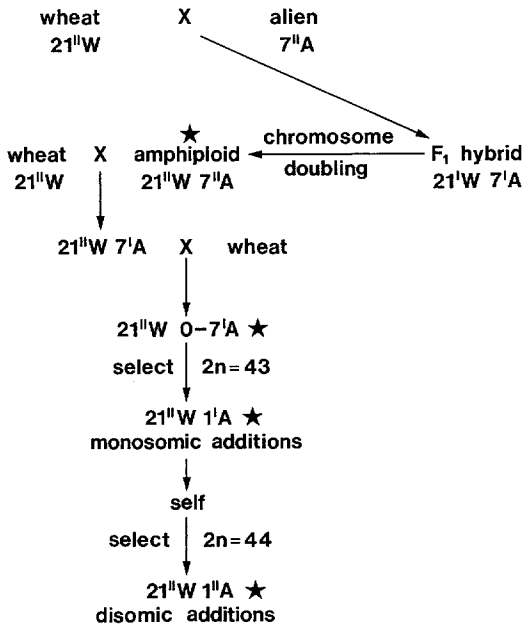

Fig. 3. Simplified scheme for the development of alien addition lines incorporating RFLP analysis to reduce the amount of test crossing and chromosome analysis needed. The *stars* indicate where RFLP analysis may be beneficial (see text). The meiotic chromosome pairing expected in the genotypes is indicated: 21"W – 21 pairs of hexaploid wheat chromosomes, 7'A – 7 unpaired alien chromosomes

arm probe, 167, detects two DNA fragments, one on each of the additions shown by the group 2 and 7 probes to be of groups 2 and 7 (Tables 4 and 5). It is not possible to deduce from these locations if the translocations are present in the *Ae. umbellulata* parent or if they arose during the development of the addition lines.

Tables 4 and 5 also indicate a closer relationship between the R and U genomes of *S. cereale* and *Ae. umbellulata,* respectively, and the three wheat genomes, than between the H genome of *H. vulgare* and the hexaploid wheat genomes, since all of the H genome fragments differ from the A, B and D genome fragments, whereas eight R genome and five U genome fragments are of the same size as wheat fragments. This is in agreement with current ideas of the evolutionary relationships within the Triticeae (Baum 1978).

Evolutionary conservation of cDNA sequences in Triticeae species

The widespread use of these probes in facilitating alien gene transfers to wheat will require that they hybridize to fragments in all the relatives of wheat which have or may be used in such work, i.e. species of the tribe Triticeae. Tables 4 and 5 show that they hybridize to fragments in *H. vulgare, S. cereale* and *Ae. umbellulata.* Figure 2 shows the hybridization of one of the probes (128) to HindIII-digested DNA of a number of other *Triticum, Hordeum, Aegilops, Agropyron* and *Haynaldia* species. In all of the species examined, the probe hybridizes to specific fragments, which are usually of different lengths from those present in CS. It is probable that all of the 14 selected probes will behave in this way, since they were all selected by their hybridization to fragments in *H. vulgare, S. cereale Ae. umbellulata* DNA (Tables 4 and 5), and many of the probes have been found to hybridize to the species shown in Fig. 2 (P. J. Sharp, S. Chao and S. Desai, unpublished results). This wide hybridization presumably reflects the origin of these probes as cDNA clones, i.e. copies of active genes. The amount of evolutionary DNA sequence divergence in coding regions between species of the Triticeae may not have been extensive enough to result in a lack of significant hybridization of wheat cDNA probes to DNA from the various species studied under the hybridization conditions used here.

It is also clear from Fig. 2 that the number of fragments detected by the probe in the alien species is related to the ploidy level of the species; hexaploid *Triticum* species have three bands, the tetraploid *Triticum* species has two bands and some of the diploid *Aegilops* species have a single hybridizing fragment. However, others, notably the outbreeding species, may have more fragments than genomes, suggesting either heterozygosity for RFLP differences, or that the restriction enzyme used cuts within the region of DNA detected by the probe (Fig. 2).

Use of probes in the manipulation and analysis of wheat and alien chromosomes within wheat

Figure 3 shows a very simplified programme of hybridization and cytological screening necessary to produce a collection of wheat-alien addition lines, each containing a single alien chromosome in disomic dose. The lines produced by the scheme would be uncharacterized with respect to homoeologous group.

RFLP screening using a set of probes such as those described here could be applied at various levels in the process in order to make it more directed and efficient. Firstly, the amphiploid produced by chromosome doubling of the F_1 hybrid could be screened with the 14 probes to identify alien DNA fragments, which could be followed subsequently in the procedure. The screening would also identify variant wheat fragments from the wheat parent if it was not CS. Subsequently, the $2n=43$ plants selected by root-tip chromosome counts could be screened to assign the alien chromosomes present in different plants to homoeologous group and eliminate any wheat trisomics. If a particular homoeologous group was not present in any monosomic addition plants, then plants with $2n>43$ would be screened to identify those with the lowest chromosome number containing the alien chromosome of interest. These could then be taken through further rounds of self-fertilization. In addition,

screening 2n = 44 plants at this stage will identify the rare disomic additions directly. After a further round of selfing of the monosomic addition plants, root-tip chromosome counts would identify disomic addition lines. The same process, combined with RFLP analysis, would identify monosomic or disomic additions from the 2n > 43 plants, if necessary. RFLP analyses of the disomic addition lines produced would also indicate the absence of any other alien chromosomes in the background. These disomic additions would not need to be intercrossed and meiosis examined in the hybrids in order to identify duplicates, as the RFLP analyses would have identified such duplicates earlier in the process. However, if the RFLP analyses indicate chromosomal translocations between separate additions, then these could be backcrossed to the amphiploid and meiosis in the hybrids examined, to identify disomic additions with the same alien chromosomal type as the amphiploid and those which had become translocated during isolation.

The ease of detection of translocations with each of the probes will depend on their intrachromosomal location. More terminally located probes will be able to detect more of the non-centric translocations which may occur. RFLP analysis, therefore, has the potential both to increase the likelihood of obtaining a complete addition series where biologically possible [some addition lines are weak or sterile (Islam et al. 1981)], and to greatly reduce the test crossing and meiotic screening of the additions needed to assign the additions to homoeologous group and to detect any translocations which may have arisen (Gale and Miller 1987). Similarly, RFLP analysis is a particularly efficient tool for the subsequent isolation of alien chromosome substitution lines, since the probes not only allow the presence of the alien chromosome to be assayed, but also enable the direct and positive identification of the absent wheat chromosome.

The probes can also be used to analyse the chromosomal constitution of wheat itself. Figures 1 and 2 show that the tetrasomic chromosome in the NT lines can be identified by the increased dose of the appropriate fragment, since this is revealed as a more intense band on the autoradiogram. In addition, it is possible to identify the chromosome present in a single dose in 2n = 41 plants by the reduced dose of DNA restriction fragments located on particular chromosomes (Gale et al. 1988).

These probes therefore represent a new resource for use in the analysis of alien transfers and wheat chromosome manipulation. They provide an extra set of markers which can be used to facilitate the directed construction of genetic stocks for breeding novel characteristics into the wheat crop.

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