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The cytological and genetic characterisation of doubled haploid lines derived from triticale × wheat hybrids

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Summary. Anther culture, when applied to hexaploid triticale × wheat hybrids, offers the opportunity to re-assort wheat D genome and rye R genome chromosomes into homozygous doubled haploid lines in a single generation. The characterisation of such lines is the first step in their utilisation in wheat improvement. Two lines, M24 and M25 from the cross of 'Beagle' × 'Kedong 58', and one line, M27, from the cross 'Beagle' × 'Jinghua No. 1' have been characterised using different methods including conventional cytology and chromosome banding, and by using marker systems for storage protein composition (glutenins and gliadins), isozymes (α-amylase, aminopeptidase, glutamate oxaloacetic transaminase (GOT)) and RFLP markers. The results from all approaches were consistent in proving that M24 is a whole chromosome 6R/6D substitution line, while M25 and M27 are whole chromosome 1R/1D substitution lines. The relative advantages and disadvantages of each method of identification are also discussed.

Key words: Wheat – Doubled haploids – Isozymes – RFLPs

Introduction

A number of techniques are now available for introducing genes from the relatives of bread wheat into wheat (*Triticum aestivum*) itself (Feldman 1988). Among these methods, anther culture has been proved to be an effective approach because of two important advantages: firstly, various types of recombinant gametes can be fully expressed at a homozygous plant level; secondly, gametoclonal variation can give rise to new recombinants and variants that are usually difficult to obtain by conventional methods (Hu and Huang 1987). Using the F_1 hybrid of 6x triticale × bread wheat as the parental plants for anther culture, pollen plant populations containing diverse chromosome constitutions have been obtained and investigated cytologically by Wang and Hu (1985) and Tao and Hu (1989). A number of rye-wheat substitution lines, addition lines and multi-addition lines were subsequently developed (Hu et al. 1988). These unique lines contain varied combinations of wheat and rye chromosomes and possess agronomically important characters such as powdery mildew resistance, early maturity and short straw. Characterisation of these lines in terms of their genotypic constitution is essential for both further genetic study and use in breeding.

Many methods can be applied in alien-wheat chromosome identification. Among the cytological techniques telocentric analysis is the most commonly used method (Sears and Sears 1978). However, the advent of differential staining techniques, such as C-banding, N-banding and in situ hybridization, can now provide precise identification of the individual somatic chromosome constitution, and these techniques have been used extensively in many programmes (Gill et al. 1988).

The application of biochemical and molecular markers in plant breeding is an area of interest that is expanding rapidly. Improved electrophoretic techniques allow many different proteins to be recognised and their genetic control determined. This is particularly the case for isozymes, where more than one hundred different genes have been recognised and their location assigned to chromosome arms. Both storage protein and isozyme gene systems are good markers for alien-wheat chromosome identification (Hart 1983a; Gale and Sharp 1988). In addition, molecular markers – restriction fragment

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length polymorphisms (RFLPs) – are now available and offer the advantages of effectively unlimited number, co-dominant inheritance, and the requirement for using just a single technique.

The purpose of the present work was to identify the chromosome constitutions of doubled haploid lines derived from anther culture by applying these different marker systems. Initially, cytological techniques were applied to recognise the rye chromosome contribution. Subsequently, marker systems for genes located on homoeologous chromosome groups 1 and 6 were applied to elucidate the exact chromosome constitution of each line for these groups.

Materials and methods

Plant material

Three doubled haploid lines derived by culturing anthers of two triticale × wheat hybrids were the basis of the present study. M24 and M25 were derived from the cross of the triticale var 'Beagle' with the wheat var 'Kedong 58' (K58), and M27 was derived from the cross of 'Beagle' with the wheat var 'Jinghua No. 1' (JH No. 1) (Tao and Hu 1989). Seeds for the present studies were obtained from self-pollination of plants derived after four generations of selfing from the original doubled haploid plants. As controls for the analysis of biochemical and molecular markers and for cytological studies, 'Chinese Spring' (CS), its aneuploid lines and rye additions and substitution lines of chromosomes of rye var 'Imperial' and 'King II' into 'Chinese Spring' and 'Holdfast' were also included in these studies.

Cytological analysis

The cytological constitution of each line was evaluated by examining mitosis in root-tip cells using Feulgen and C-banding preparations according to the method described by Tao and Hu (1989). Identification of individual C-banded rye chromosomes was based on the standard pattern described by Sybenga (1983). Subsequently, plants of each line were crossed to their respective wheat parents and to 'Chinese Spring' double ditelocentric lines to evaluate pairing behaviour at meiosis. M24 was also crossed to the substitution line of rye chromosome 6R for wheat chromosome 6D in the var 'Holdfast'.

Analysis of biochemical and molecular markers

Four biochemical marker systems were used to characterise each of the doubled haploid lines:

1. Endosperm protein sub-unit composition. The composition of storage proteins (glutenins and gliadins) was analysed by polyacrylamide gel electrophoresis (PAGE) with and without sodium dodecyl sulfate (SDS), according to Payne and Corfield (1979) and Lafiandra and Kasarda (1985), respectively.

2. α-Amylase. Isozyme variation for malt α-amylase was evaluated using isoelectric focusing (IEF) following the method described by Gale et al. (1983) with a modification of Liu (personal communication). A pericarp from a 1-week-old germinating seed was crushed in 70 µl distilled water and centrifuged. The supernatant was incubated at 70 °C for 10 min to remove β-amylase activity prior to recentrifuging. Isoelectric focussing was carried out on 0.25-mm thick, 12-cm wide polyacrylamide gels containing 2% (w/v) ampholyte (Isolyte 6–8, Pharmalyte 5–7 in the ratio 2:1). The zymograms were visualised by immersion of the gels in 1.5% starch solution for 10 min, followed by staining in a solution of 1.5×10^{-3} *M* iodine, 3.5×10^{-3} *M* potassium iodide and 3% acetic acid.

3. Aminopeptidase. Isozyme variation for aminopeptidase phenotype was analysed as described by Koebner and Martin (1989).

4. Glutamate oxaloacetic transaminase (GOT). PAGE was used for GOT analysis. The electrophoresis procedure was the same as the one for glutenin sub-unit separation except that the gel was prepared without SDS. The sample preparation and staining method were as described by Hart (1983b).

RFLPs were examined using the techniques for DNA extraction, restriction enzyme digestion, agarose gel electrophoresis, alkaline "Southern" blotting to nylon membranes, probe preparation, hybridisation and fragment size determination as described by Sharp et al. (1988). A modification was that gene screen plus (NEN) membranes were used. Three probes were used to examine RFLPs between the lines and their parents. The probe, pTa71 is a full-length nuclear rDNA repeat unit and encodes the 26S, 18S and 5.8S rRNA structural genes, as well as spacers (Gerlach and Bedbrook 1979). The probe pTag 1290 has been shown to be a glutenin high molecular weight (HMW) subunit gene (Bartels and Thompson 1983). The probe PSR154 is an anonymous cDNA probe developed from leaf tissue and is located on the long arms of the homoeologous group 6 chromosomes (Sharp et al. 1989).

Results

1. *Cytological results*

An examination of mitotic cells in Feulgen-stained preparations revealed that all three lines have a chromosome number of 2n = 42. Chromosome banding of mitotic cells (Fig. 1) indicated that one pair of rye chromosomes is present in all three lines, 1R in M25 and M27, and 6R in M24. Meiotic analysis of each of the lines indicated normal diploid pairing (21 bivalents), while in the hybrids of M24, M25 and M27, together with their respective wheat parents there was either 20 bivalents plus 2 univalents, or 18 bivalents, 2 univalents and a quadrivalent. This indicates that one reciprocal translocation exists in each line relative to their original wheat parents. This was either donated by the triticale parent, and therefore must be between chromosomes of the A and B genomes (either A/B, A/A or B/B), or alternatively, this translocation may have arisen by gametoclonal variation during anther culture. These alternative hypotheses remain to be resolved. Twenty-one bivalents were generally found at meiosis in the hybrid of M24 with the 'Holdfast' 6R (6D) substitution line, although a quadrivalent was present in some pollen mother cells (PMCs) of this hybrid (not shown).

In the test crosses of M27 and M25 with the CS double ditelosomics for chromosomes of homoeologous group 1, a telosomic trivalent was found in lines from

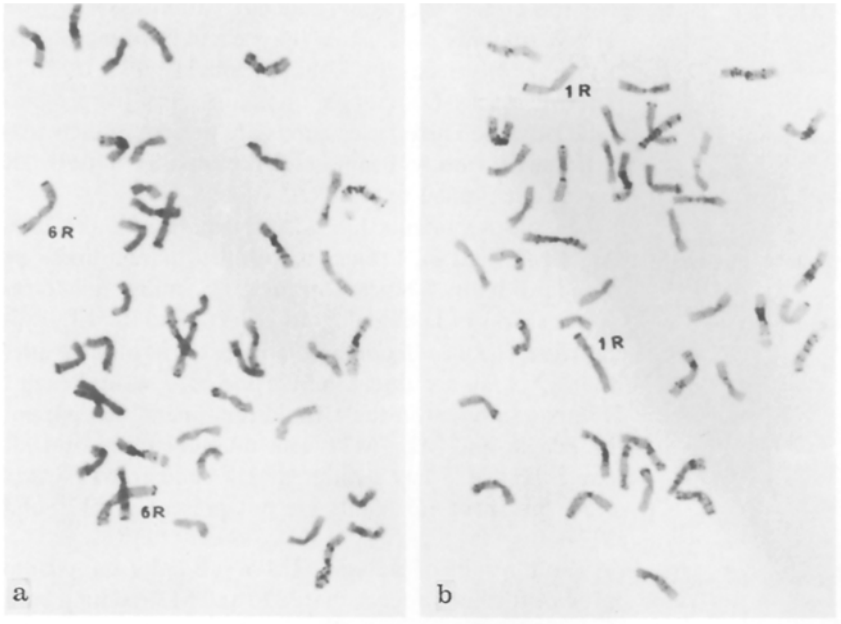

Fig. 1 a, b. C-banded mitotic metaphase somatic chromosomes of M24 (**a**) and M27 (**b**) showing a pair of rye chromosome 6R in M24 and 1R in M27

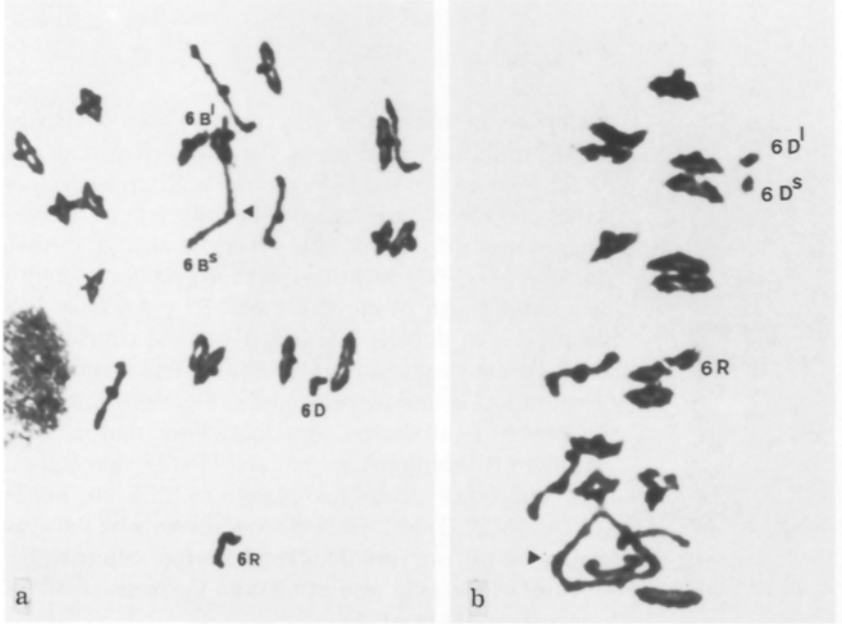

Fig. 2 a, b. Meiotic metaphase I of (**a**) CS DDT 6B × M24 with 19 bivalents +1 ditelotrivalent +2 univalents and (**b**) CS DDT 6D × M24 with 18 bivalents +1 quadrivalent +1 univalent +2 telo-univalents. The quadrivalent, trivalent, univalents and telocentrics are marked

crosses with 1A and 1B double ditelosomics, but not in the 1D test cross. In the test crosses involving M24 with the double ditelosomic lines of homoeologous group 6 chromosomes, pairing of telosomics was found in crosses of double ditelosomics of 6A and 6B, but not of 6D (Fig. 2). These results indicate that in M25 and M27 wheat chromosome 1D is substituted by rye chromosome 1R, and chromosome 6D in M24 is substituted by rye chromosome 6R.

2. *Storage protein composition*

The genes encoding HMW sub-units of glutenin, *Glu-1*, occur on the long arm of chromosomes 1A, 1B and 1D (Payne 1987). Lawrence and Shepherd (1981) also located the gene encoding HMW subunits of rye glutenin to the long arm of 1R. Structural genes encoding gliadin proteins are located on the short arms of chromosomes of group 1, and group 6 and genes encoding γ-gliadin and

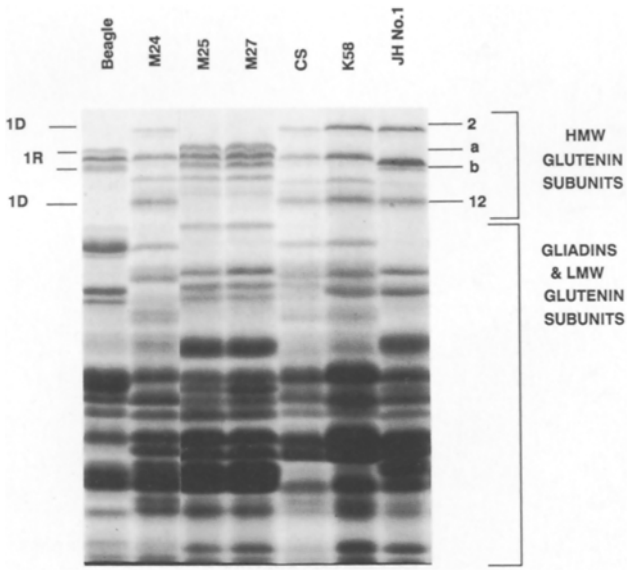

Fig. 3. SDS-PAGE patterns of endosperm protein (reduced glutenins) of DH lines and parents

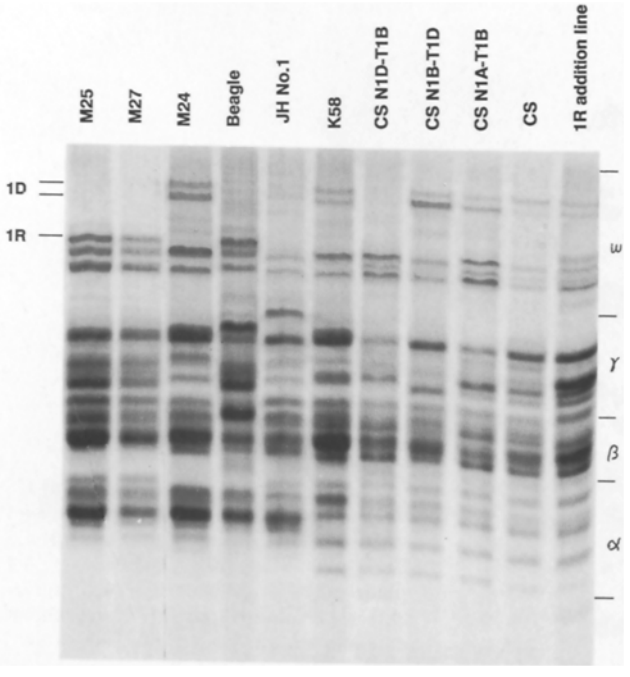

Fig. 4. PAGE patterns of endosperm proteins (prolamins) of DH the lines, parents and controls

Ω-gliadin (*Gli-1*) are located on the short arms of chromosomes of group 1 (Payne 1987). The rye storage protein, secalin is also controlled by a gene located on the short arm of 1R (Shewry et al. 1985).

Figure 3 shows the SDS-PAGE patterns of reduced glutenins of the lines M24, M25, M27 and their original triticale and wheat parent varieties together with the standard line 'Chinese Spring'. Wheat parent, 'Kedong 58' has nearly the same glutenin pattern as CS with HMW subunits 2+12, 7+8 (Payne and Lawrence 1983), while JH No. 1 has 2+12 and, probably, 17+18. M25 and M27 lack 1D subunits 2+12 and carry the proteins labelled a, b. These latter bands are present in their triticale parent variety 'Beagle' and, presumably, must be 1R bands determined by *Glu-R1*.

Figure 4 presents the gliadin patterns of the lines M25, M27 and M24 together with their parent lines, the standard lines 'Chinese Spring', CS nullisomic-tetrasomic stocks of homoeologous group 1 and the 1R addition line. It is clear from the gliadin patterns of these lines that 1D bands are absent in M25 and M27, as in 'Beagle'. The band present in the 1R addition line is also present in 'Beagle' and M25, M27. This must therefore be a 1R band. JH No. 1 has a different 1D band from CS and K58, but these 1D bands are not present in M25 and M27.

Both results of glutenin HMW sub-units and gliadin provide further evidence that M25 and M27 contain both long and short arms of 1R, and therefore, appear to be complete 1R-1D substitution lines.

3. *Isozyme variation*

α-Amylase. In wheat, α-amylase production is controlled by two triplicate sets of genes, the *α-Amy-1* set with loci on the long arm of the homoeologous group 6 chromosomes and the *α-Amy-2* set on the long arm of homoeologous group 7 chromosomes (Gale et al. 1983). Ainsworth et al. (1987) identified homoeoloci for *α-Amy-1* on the long arm of chromosome 6R. α-Amylase IEF phenotypes of the DH lines, their original triticale and wheat parent varieties, the 6R addition line and the 6R-6D substitution line are presented in Fig. 5. One isozyme was present in all the rye containing lines, that is, 'Beagle', the 6R addition line and the 6R-6D substitution lines. This isozyme was also present in M24, but not in M25 and M27. The 6D isozyme was absent in all the lines having the 6R isozyme. This result further confirms the presence of the long arm of 6R and the absence of the long arm of 6D in M24.

Aminopeptidase. The aminopeptidase enzyme was first reported to be controlled by a triplicate set of genes by Hart (1973) and its locus, designated *Amp-1*, is located on the short arms of chromosomes 6B and 6D (Koebner and Martin 1989). Aminopeptidase IEF phenotypes of DH lines, their original parent varieties, 'Chinese Spring' and CS nulli-tetrasomic stocks of homoeologous group 6 chromosomes showed that there was no 6D isozyme in M24, just as in its triticale parent 'Beagle', but there was no evidence of an alternative 6R isozyme (data not shown).

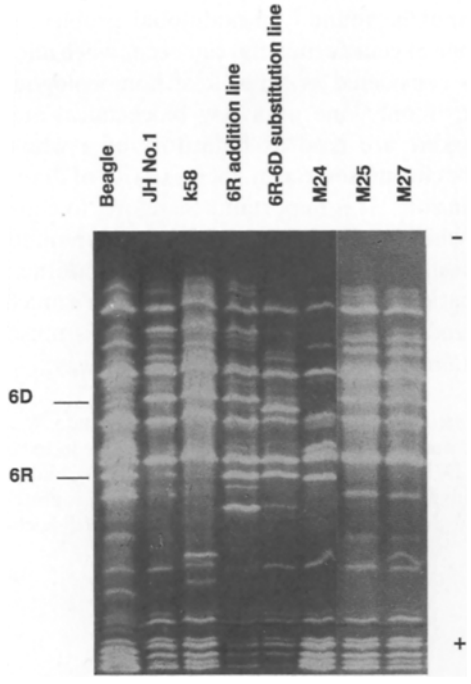

Fig. 5. α-Amylase IEF phenotypes of DH lines, parents and controls

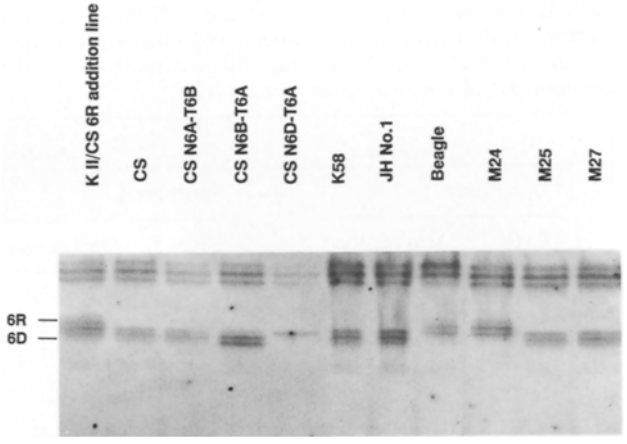

Fig. 6. GOT PAGE patterns of DH lines, parents and controls

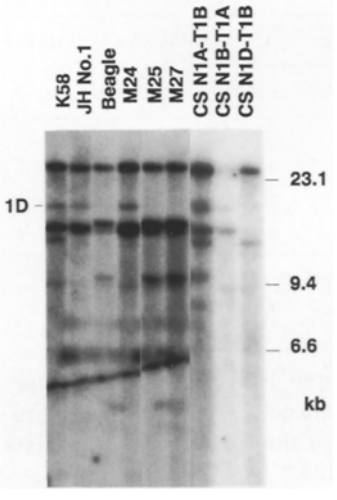

Fig. 7. Hybridization of probe pTa71 to DraI-restricted DNA of DH lines and parents

Glutamate Oxaloacetic Transaminase (GOT). Isozymes of GOT of wheat and rye were first studied by Hart (1975). Genetic and biochemical analysis of the activity of this enzyme using 'Chinese Spring' indicated that there are three genetically independent GOT systems: *Got-1, Got-2* and *Got-3* (Hart 1975). *Got-2* is encoded by a triplicate set of structural genes located on the long arms of homoeologous group 6 chromosomes, while *Got-3* is controlled by genes on the group 3 chromosomes. *Got-R2* and *Got-R3* are found on chromosome 6R and 3R in rye and triticale, respectively (Tang and Hart 1975). Only two sets of GOT isozymes, *Got-2* and *Got-3*, were found in this study. The GOT PAGE patterns of the DH lines, their original parent varieties, CS and CS nulli-tetrasomic stocks of homoeologous group 6 and the 6R addition line are shown in Fig. 6. In this case, both extra or missing isozymes can be found. M24 has two 6R isozymes, but lacks the 6D isozyme, as does 'Beagle'.

All the above results are consistent with the conclusion that M24 contains a 6R locus instead of a 6D locus for all of these isozyme systems and that both 6R arms are present.

4. *RFLP analysis*

Figure 7 shows hybridization patterns of probe pTa 71 to DraI-restricted DNA. Two new fragments were apparent in the pattern of M25, M27 and 'Beagle', and these must be from chromosome 1R. In addition, the hybridization of probe pTag 1290 to DraI-restricted DNA of the three DH lines, their triticale and wheat parents and stocks of compensating nulli-tetrasomics of 'Chinese Spring' for chromosomes of homoeologous group 1 showed that the 1D fragment was missing in M25 and M27, and from their triticale parent, 'Beagle' (not shown). The hybridization of probe PSR154 to EcoRV- and SstI-restricted DNA of these lines was also carried out, and it was clearly shown that the 6D fragment was absent in 'Beagle' and M24, and that a 6R fragment was present instead in both lines (not shown).

Discussion

The results from all methods of genotypic assessment converge to provide convincing evidence that M25 and

Table 1. The characterisation of M25 and M27 using different markers for the chromosomes of homoeologous group 1 (I), and the characterisation of M24 using different markers for the chromosome of homoeologous group 6 (II)

I

	Long Arm			Short Arm		
	Cyto-logical analysis	Marker loci		Cyto-logical analysis	Marker loci	
		Glu-1	*XGlu-1*		*Nor*	*Gli-1*
1A	+	+	+	+		+
1B	+	+	+	+	+	+
1D	−	−	−	−		−
1R	+	+	+	+	+	+

II

	Long Arm				Short Arm	
	Cyto-logical analysis	Marker loci			Cyto-logical analysis	Marker loci
		Got-2	*Xpsr154*	*α-Amy-1*		*Amp-1*
6A	+	+	+	+	+	
6B	+	+	+	+	+	+
6D	−	−	−	−	−	−
6R	+	+	+	+	+	

+ indicates presence of arm/marker, − indicates absence

M27 are whole chromosome 1R-1D substitution lines and that M24 is a whole chromosome 6R-6D substitution line. Table 1 presents a summary of these analyses for M25 and M27, and M24.

It is clear from a comparison of the methods applied in this study that each had its own particular advantages which could not be substituted for completely by any of the other approaches; there were disadvantages as well. Thus, conventional cytological analysis, as when using morphological markers, requires considerable experience and is also very time consuming. For example, it was quite difficult to distinguish specific somatic chromosomes, particularly the wheat chromosomes. Nevertheless, at the present time, cytological techniques still, generally, provide the most accurate method of chromosome identification (Gill et al. 1988). This study gives strong support to this argument.

Biochemical and molecular markers, on the other hand, provide a quick and precise method for chromosome segment identification. In the present study this was helped by the fact that wheat and rye show clear polymorphisms for the systems examined. However, not all isozymes or DNA probes can be used as markers, since among more than the 100 isozymes in wheat, only a few display polymorphisms. So in many cases, particularly in intraspecific studies, differences between any two desired genotypes cannot be found. An additional problem is that an exchange of genetic material can occur when alien germ plasm is considered as a result of homoeologous pairing. Thus, if only one or a few biochemical and molecular markers are used as indicators of a whole chromosome or chromosome arm, there is a risk of drawing false conclusions. It is important, therefore, to combine methods which provide different types and amounts of information and which can complement each other. Such a combination of different methods can give a much more precise and reliable analysis, although this must, ideally, be obtained in a quick and convenient way.

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