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Visualization of *Secale cereale* DNA in wheat germ plasm by fluorescent in situ hybridization

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Abstract Homozygous wheat/rye (1BL/1RS or 1AS/1RL) translocation lines have significantly contributed to wheat production, and several other wheat/rye translocation lines show a potential promise against biotic and abiotic stresses. Detecting the presence of rye at the chromosome level is feasible by C-banding and isozyme protocols, but the diagnostic strength of genomic in situ hybridization for eventually analyzing smaller DNA introgressions has greater significance. As a first step we have applied the genomic in situ hybridization technique to detect rye chromosomes in a wheat background using germ plasm of agricultural significance. By this method rye contributions to the translocations 1BL/1RS, 1AL/1RS, 5AS/5RL and 6BS/6RL could be identified. Differential labelling has further enabled the detection of rye and *Thinopyrum bessarabicum* chromosomes in a trigeneric hybrid of *Triticum aestivum/Th. bessarabicum//Secale cereale*.

Key words Alien chromosome additions
Fluorescent in situ hybridization · Genomic DNA
Intergeneric hybrids · Wheat/rye translocations

Introduction

Alien genetic transfer programs in wheat have been associated with exploiting the genetic variability of distant Triticeae relatives (intergeneric hybridization) as well as the variation prevalent amongst the closer wheat relatives (interspecific hybridization) (Mujeeb-Kazi 1993). These hybridization methodologies allow for the incorporation of novel genetic variation into wheat for biotic/abiotic stresses as a consequence of alien DNA exchange mechanisms; complex for intergeneric and relatively simplistic for interspecific hybridization. For intergeneric introgression the choice of an alien species has tremendous significance and the use of alien diploid species is a priority, of which *Secale cereale* L. is one option.

The crop × Triticosecale Wittmack in its hexaploid or octoploid forms represents rye (*S. cereale*) utilization in agriculture. Individual wheat/rye chromosomal translocations (1BL/1RS and 1AL/1RS) have significantly contributed to global wheat production with over five million hectares cultivated to these wheat cultivars (Peña et al. 1990). Several other wheat/rye translocations holding potential agricultural promise for biotic/abiotic stresses are 5AS/5RL (copper efficiency) and 6BS/6RL (cereal cyst nematode). Though appropriate wheat/rye translocations have not yet been developed, disomic 4R and 6R chromosome additions to wheat (K. S. Gill personal communication) also hold promise for Karnal bunt (*Tilletia indica*) resistance.

Though the detection of rye chromosomes and translocated arms can be achieved readily by utilizing plant morphology or cytology, biochemical parameters are also an effective means of diagnosing alien DNA presence (Asiedu et al. 1989; Schwarzacher et al. 1992). The latter comprise the applications of isozymes, restriction fragment length polymorphisms (RFLP), in situ hybridization with labelled cloned DNA probes (species-specific or repetitive clones), or the use of total genomic DNA. Though the limits of definition need elaboration, genomic in situ hybridization including fluorescence in situ hybridization has been proven to have wide usage in identifying alien DNA introgression (Rayburn and Gill 1985; Schwarzacher et al. 1989).

In the study reported here, we apply the fluorescent in situ hybridization technique to several rye segments being utilized in bread wheat (*Triticum aestivum* L.) and spring durum (*T. turgidum* L.) breeding. Nonradioactive in situ hybridization using labelled total genomic DNA of the alien species was successfully utilized with the unlabelled blocking DNA of *T. aestivum* or *T. turgidum* (2n=6x=42, AABBDD or 2n=4x=28, AABB). These results are presented together with the details of the procedures that give high resolution of alien genetic materials.

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Materials and methods

Plant material

Germ plasm possessing the total rye (*S. cereale* L.) chromosomes (× Triticosecale Wittmack), disomic rye chromosome additions (1R and 6R), wheat/rye translocations (1BL/1RS; 1AL/1RS; 5AS/5RL; 6BS/6RL), or the rye genome in a trigeneric hybrid (*T. aestivum/Thinopyrum bessarabicum//S. cereale*) was utilized for fluorescence in situ hybridization (Table 1).

Chromosome preparation

Root tips collected from germinating seeds in petri dishes were pretreated with 8-OH-quinoline + colchicine + dimethylsulfoxide (DMSO) for 3–3.5 h (Mujeeb-Kazi and Miranda 1985) and fixed in 3 ethanol:1 acetic acid. Root meristems were next incubated at 37°C in an enzyme solution (5% cellulase R-10+1% pectolyase Y-23 in 0.01 *M* citric acid/sodium citrate buffer, pH 4.5) for 30 min. After incubation the meristems were first rinsed with buffer, then water, and an individual meristem was transferred onto a ethanol-washed glasslide. The preparation was made by the ordinary squash method.

Plant DNA preparation and probe labelling

DNA of *T. aestivum, T. turgidum, S. cereale*, and *Th. bessarabicum* was extracted following standard protocols (Hoisington 1992). Prior to use, the DNA was mechanically sheared to 10–20 kb by passing the DNA about 200 times through a 26-gauge, 100-µl syringe. DNA of the species to be used for blocking was autoclaved to degrade it into 300- to 1000-bp fragments. Both sheared and autoclaved DNA was stored at 4°C. Total genomic DNA was labelled by nick-translation using either biotin-14-dATP (Gibco, BRL) or digoxigenin-11-dUTP (Boehringer Mannheim) as the in situ probe. Unincorporated nucleotides were removed by a Sephadex G50 column. The label incorporation was evaluated by means of dot blots using the streptavidin-alkaline phosphatase-detection system (Gibco, BRL).

In situ hybridization, detection, and visualization

The hybridization procedure was essentially similar to that of Rayburn and Gill (1985). A slight modification, however, was that freshly prepared slides were treated with RNase-A (25 µg/ml) in 2 × SSC for 45–60 min at 37°C. Chromosomal DNA was denatured by immersing the slides in 70% deionized formamide (Fisher, Molecular grade), 2 × SSC for 3 min at 70°C. The slides were then dehydrated for 4–5 min each in a graded series of 70%, 90% and absolute ethanol at –20°C. The hybridization mixture generally comprised 50% deionized formamide, 2 × SSC, 10% dextran sulphate, and 40–80 ng probe DNA (differentially labelled probes also mixed together). The blocking DNA was 15–20 times the amount of the probe DNA (details in Table 2).

The hybridization mixture was denatured for 10 min at 80°C and then immediately quenched in ice for 8–10 min at which time 25–30 µl was applied to each preparation. A good quality coverslip (22 × 30 mm) was placed on the preparation and its margins sealed with rubber solution. The slides were initially incubated for 8 min at 80°–82°C and then overnight at 37°C in a humidity chamber.

Table 1 Germ plasm used for fluorescent in situ hybridization analyses with cytological and origin details

Germ plasm	Cytological details	Origin
× TriticoSecale	2n=6x=42, AABBRR	CIMMYT
T. aestivum cv CS+1R1R	2n=6x=42+2=AABBDD+1R1R	University of Missouri, USA
T. aestivum cv CS+6R6R	2n=6x=42+2=AABBDD+6R6R	University of Missouri, USA
T. aestivum cv TAM 200	2n=6x=42;1AL/1RS,1AL/1RS	Texas A & M University, USA
T. aestivum cv Seri 82	2n=6x=42;1BL/1RS,1BL/1RS	CIMMYT
T. aestivum	2n=6x=42;1BL/1RS, 1BL/1RS and 5AS/5RL, 5AS/5RL	CIMMYT & Cambridge Laboratory, IPSR
T. aestivum	2n=6x=42;6BS/6RL, 6BS/6RL	University of Illinois, USA
T. turgidum cv Altar 84	2n=4x=28;1BL/1RS, 1BL/1RS	CIMMYT
T. aestivum/Th. bessarabicum// S. cereale	2n=5x=35;ABDJR	CIMMYT

[a] CIMMYT, International Maize and Wheat Improvement Center, Mexico; IPSR, Institute of Plant Science Research, Norwich, UK

Table 2 Specific details of the hybridization mixture used for different germ plasm that consisted of *Triticum aestivum* (ABD), *T. turgidum* (AB), *Secale cereale* (R), and *Thinopyrum bessarabicum* (J)

Germ plasm	Deionized formamide (%)	Dextran sulphate (%)	SSC ×	Labelled probe		Block DNA[a]	Volume used (µl/slide)
				Biotin	Digoxigenin		
× TriticoSecale	44.01	8.80	3.52	60 ng R		15 × AB	28.0
T. aestivum cv CS+1R1R	46.99	9.40	3.76	40 ng R		20 × ABD	27.0
T. aestivum cv CS+6R/6R	47.17	9.43	3.77	40 ng R		20 × ABD	26.0
T. aestivum cv TAM 200	41.00	8.25	1.47	40 ng R		20 × ABD	27.0
T. aestivum cv Seri 82	41.00	8.25	1.47	40 ng R		20 × ABD	27.0
T. aestivum	41.00	8.60	1.72	40 ng R		20 × ABD	29.0
T. aestivum	41.00	8.25	1.47	40 ng R		20 × ABD	27.0
T. turgidum cv Altar 84	48.45	9.69	3.88	35 ng R		20 × AB	25.0
T. aestivum/Th. bessarabicum// S. cereale	38.09	7.94	3.17	40 ng J	40 ng R	20 × ABD	30.0

[a] 15 or 20 times the amount of probe DNA as an example of values in the column

After hybridization, the rubber solution was pealed off carefully, and the coverslips were removed by dipping the slides into 2 × SSC at 40°C. The slides were next washed in 2 × SSC twice (5 min each), 50% formamide in 2 × SSC for 10 min, and in 2 × SSC twice (5 min each) at 40°C in a shaking waterbath to remove the unhybridized and weakly hybridized probes. The slides were then washed in 2 × SSC for 5 min at room temperature before detection of the hybridization sites by avidin-conjugated fluorochromes (F-avidin DCS, Vector Laboratories). The fluorochrome signal was amplified with biotinylated anti-avidin D (Vector Laboratories) followed by another layer of avidin-conjugated fluorochromes, as described by Schwarzacher et al. (1989) and Leitch et al. (1990).

The slides were washed in detection buffer (4 × SSC/0.2% Tween-20) for 5 min and incubated with 200 µl 5% BSA (Bovine Serum Albumin-Fraction V, Sigma A2153) in detection buffer for 5 min at room temperature. The blocking solution was drained off, and the slides were placed in the humidity chamber taking care that the preparations did not dry out during detection. The preparations were covered with 75 µl of fluoresceinated avidin-DCS (10 µg/ml) in the detection buffer that contained 5% BSA. A plastic coverslip was placed, and slides incubated for 1 h at 37°C. After incubation the slides were washed three times (6 min each) in the detection buffer at 37°C in a shaking waterbath.

The preparations were then blocked with 200 µl 5% (v/v) normal goat serum (Vector Laboratories) in the detection buffer for 5 min at room temperature. The slides were next drained, again placed in the humidity chamber, and incubated with 75 µl biotinylated anti-avidin D (20 µg/ml) in detection buffer containing 5% (v/v) normal goat serum for 45–60 min at 37°C. After incubation the slides were washed three times (6 min each) in detection buffer at 37°C in a shaking waterbath.

The preparations were blocked again with 200 µl 5% BSA as above for further amplification of the biotinylated probe hybridization signal with another layer of 75 µl fluoresceinated avidin DCS (10 µg/ml) in detection buffer containing 5% BSA. After incubation, the slides were washed three times in the detection buffer as before. The slides were next counter-stained with 200 µl DAPI (4′, 6-diaminodino-2-2-phenylindole) (3 µg/ml) in McIlvaine's citrate buffer (0.01 *M* citric acid, 0.08 *M* sodium hydrogen phosphate, pH 7.0) for 20–25 min at room temperature. They were then washed briefly in detection buffer, drained and again counter-stained with 200 µl propidium iodide (20 µg/ml) in 2 × SSC for 10–15 min at room temperature followed by washing in 2 × SSC. After the slides had been drained of the 2 × SSC, they were mounted in Vectashield (Vector Laboratories) to reduce fading of the fluorescence. The slide preparations were kept at 4°C for 1–2 days to stabilize the fluorochromes before they were examined with a Zeiss Axiophot epifluorescence research microscope with filter sets 02, 09, and 23. Representative cells were photographed on a Fujicolor Super HG400 or G400 color print film.

Differential labelling

The detection of differentially labelled probe hybridization sites for the trigeneric hybrid (Table 2) was similar to that observed by Leitch et al. (1991) and is briefly described here. The detection of the biotinylated probe with 'Texas Red'-labelled avidin DCS (Vector Laboratories) and the digoxigenin probe with sheep anti-digoxigenin fluorescein (Boehringer-Mannheim) was performed simultaneously. Procedures for washing and blocking the preparations were similar to those described earlier. The preparations were covered with 75 µl of 'Texas Red'-labelled avidin DCS (10 µg/ml) and anti-digoxigenin-fluorescein (20 µg/ml) in detection buffer containing 5% BSA. A plastic coverslip was placed, and the preparations incubated for 1 h at 37°C. After the incubation and washing, the signals for biotin- and digoxigenin-labelled probe hybridization sites were amplified with biotinylated anti-avidin D (Vector Laboratories) and FITC conjugated rabbit anti-sheep (Dakapotts, USA), respectively. The preparation incubation was done with 75 µl biotinylated anti-avidin D (20 µg/ml) and rabbit anti-sheep (1:4) in detection buffer containing 5% normal goat serum for 45–60 min at 37°C after a 5-min block with 5% normal goat serum. After washing and blocking the preparations with 5% BSA, the biotinylated probe hybridization sites were further amplified with another layer of 75 µl 'Texas Red'-labelled avidin DCS (10 µg/ml) in detection buffer containing 5% BSA. After incubation, the slides were washed three times in detection buffer, counter-stained with DAPI, and mounted in the antifade solution.

Results and discussion

Alien chromosome detection

Total genomic DNA of *S. cereale* as a probe enabled identification of complete rye chromosomes (Fig. 1a), disomic rye chromosome additions (Fig. 1b, c), and wheat/rye chromosome translocations in metaphase cells (Figs. 1d, e; 2a–e) or at interphase (Fig. 1f). Distortion of chromosome morphology was not prevalent. The translocation breakpoints were exceptionally clear and appeared as centric break-fusion products. The in situ hybridization signals with fluorescein-avidin and propidium iodide were distinctly identified by their yellow and orange-to-red fluorescein, respectively, under Zeiss filter 09 (Figs. 1b, c inserts, e; 2d, e) and filter 23 (Figs. 1a, inserts in c and d, f (left); 2a). The DNA-specific dye DAPI used earlier gave uniform blue fluorescence (Schwarzacher et al. 1992) with filter 02, and the alien chromosomes could not be distinguished from those of wheat. We observed hybridization signals with DAPI using Zeiss filter 02 and obtained exceptional clarity in contrast (Figs. 1b–d, f (right); 2b, c). Differently labelled probes also enabled the detection of *Th. bessarabicum* and *S. cereale* chromosomes (Fig. 2 f).

Previous workers have successfully applied fluorescence in situ hybridization, including the multi-color type, for wheat and its relatives and alien transfer stocks (Schwarzacher et al. 1989, 1992; Leitch et al. 1990, 1991; Mukai et al. 1993a, b; Lagudah et al. 1993; Nkongolo et al. 1993).

In all our samples analyzed the quality of in situ hybridization was highly satisfactory for the diagnosis of alien materials. The technique is sensitive and accurate for identifying the presence of alien chromosomes or their segments and holds tremendous potential for resolving cryptic wheat/alien exchanges both at metaphase and interphase. Schwarzacher et al. (1992) identified an array of alien chromosomes in wheat backgrounds for which they did not consider complete metaphases to be essential. Schwarzacher et al. (1992), Cremer et al. (1988), Maluszynska and Heslop-Harrison (1991) and Tkachuk et al. (1991) have indicated that interphase cytogenetic analysis will be of increasing importance since high quality metaphase preparations require skilled methodology. We face minimal constraints in obtaining superior metaphase spreads with a high mitotic index, which leads to quality in situ resolution of alien chromatin presence (Figs. 1 and 2). The interphase results are also of exceptional clarity

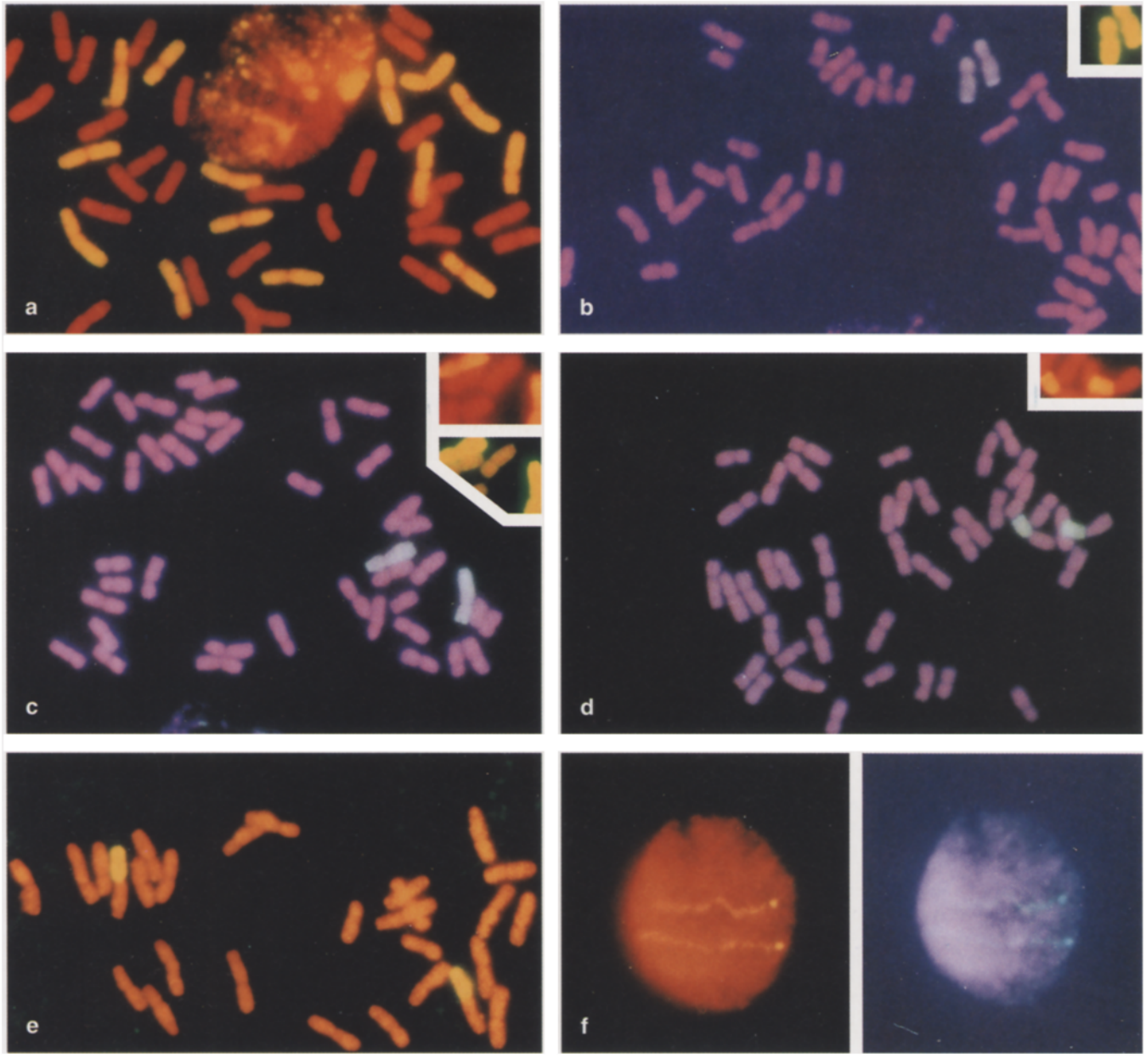

Fig. 1a–f **a** A fluorescence micrograph of × *Triticosecale* (2n=6x=42, AABBRR) under filter 23. The unlabelled wheat chromosomes are *orange-red* while the 14 rye chromosomes are labelled *yellow*. **b** Rye chromosome 1R disomic addition line to *Triticum aestivum* L. cv 'Chinese Spring' (CS); 2n=6x=42+1R1R. Filter 02 allowed detection of the 2 rye chromosomes (DAPI stain). *Insert:* 1R resolution under filter 09. **c** Rye chromosome 6R disomic addition line to *T. aestivum* L. cv 'CS'; 2n=42+6R6R; detected under filter 02 (DAPI). *Insert:* Detection using filter 23 (*top*) and 09 (*bottom*) of the 6R chromosomes. **d** A translocated 1BL/1RS wheat cultivar with 42 chromosomes in which the two rye segments (1RS) are visible. The unlabelled wheat chromosomes are *bluish-purple* whereas with filter 02 (DAPI stain) the translocated rye arm was detectable. The *insert* shows the rye arm under filter 23. **e** A 1BL/1RS homozygous translocation in a durum wheat (*T. turgidum* L., 2n=4x=28) showing the *bright yellow* rye arm (1RS) when rye DNA was used as a probe and unlabelled durum wheat DNA was used for blocking with filter 09. **f** An interphase mitotic cell of a homozygous 1AL/1RS *T. aestivum* cultivar. The labelled detection resolves the rye (1RS) arm using filter 23 (*left*) and 02 (*right*)

(Fig. 1f). The identification of primary constrictions giving a clear arm ratio is another positive aspect of our in situ end-products. Some recent in situ preparations (Friebe et al. 1993; Mukai et al. 1993a, b) also demonstrate the detection of alien DNA inserts in complete metaphase spreads. The adopting of the mitotic technique of Mujeeb-Kazi and Miranda (1985) with the recent modifications (Mujeeb-Kazi et al. 1994) to in situ somatic applications currently leaves us with a minor constraint where the cytoplasm does not totally clear in all metaphase cell spreads. This constraint has yet to be resolved. Interphase details are automatically captured in all preparations, but if a greater concentration is required for interphase analyses a reduced root-tip pre-treatment time of 2.5 h may be adopted.

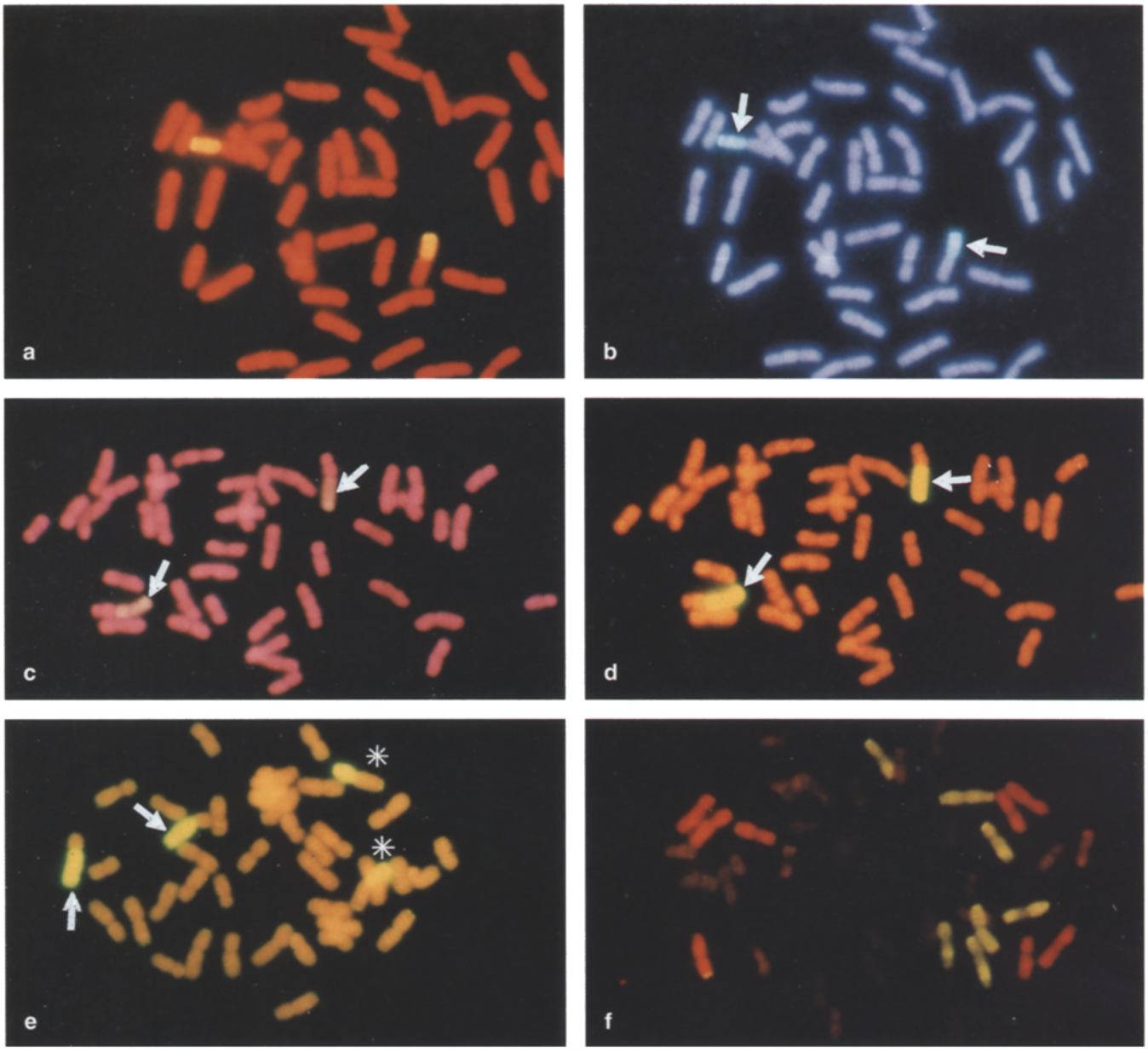

Fig. 2a–f **a** A 1AL/1RS metaphase cell with 42 chromosomes. The labelled rye DNA discriminated the 1RS arm under filter 23. **b** The same cell as in **a** under filter 02 (DAPI). The 1RS arm is *arrowed*. **c** A homozygous 6BS/6RL translocation bread wheat with 42 chromosomes showing the *arrowed* 6RL arms as detected under filter 02 with rye DNA as a probe. **d** The same cell as in **c** under filter 09. The 6RL arms are *arrowed*. **e** A homozygous double translocation in *T. aestivum* cv 'Alondra'/'Pavon' backcross background showing the 2 5AS/5RL and 1BL/1RS chromosomes (*arrowed* and asterisk, respectively) as detected with filter 09 and rye DNA as a probe. **f** Double labelling on an aneuploid trigeneric hybrid of *T. aestivum/Th. bessarabicum//S. cereale* with 40 chromosomes. Wheat DNA was unlabelled, *Th. bessarabicum* was labelled with biotin, and *S. cereale* with digoxigenin. Eight *Th. bessarabicum* chromosomes are *reddish* and 7 *S. cereale* chromosomes are *yellowish* under filter 23

Practical applications

Our applied intergeneric crosses program has relied very heavily on conventional cytogenetical techniques in which isozyme analyses and chromosome banding have made major contributions. The outputs from distant hybridization programs are anticipated as being futuristic. These may be reduced by using radical approaches (Mujeeb-Kazi 1993) or by the use of diploid alien sources like *S. cereale* and the *Thinopyrum* species. We have elaborated here an extremely accurate diagnostic procedure for screening *S. cereale* chromosomal sources toward practical agricultural goals. Understandably, for all these rye stocks a similar inference can be readily made by the conventional banding or isozyme methodologies. It is when the exchanges become cryptic that conventional diagnostics are limiting

and the current fluorescent in situ hybridization procedure is strengthened. The merits of fluorescence in situ hybridization are further elaborated by Nkongolo et al. (1993) and Wang et al. (1993). These resolution limits have to be tested on genetic stocks that are in various developmental phases for which we are utilizing manipulation procedures different than those reported by Friebe et al. (1993) and Mukai et al. (1993a, b).

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