FLUORESCENT *In Situ* HYBRIDIZATION AND ALIEN GENETIC TRANSFER DIAGNOSTICS

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ABSTRACT

Fluorescent *in situ* hybridization was used as a diagnostic tool to detect the presence of alien introgression in *Triticum* species. Detection of entire alien chromosomes or translocated arms has been routinely positive for *Secale cereale* cultivars associated with wheat and possessing an agricultural significance. Extension of the technique to various intergeneric Triticeae hybrids has elucidated several cytogenetic mechanisms associated with genome relationships, genome elimination and categorization. Double labelling allowed differentiation of genomes in a trigeneric in a hybrid. The detection of smaller introgressed alien segments; more complex but needed; has been successfully accomplished for leaf rust genes *Lr9, Lr19, Lr24, Lr25, Lr29.* These are transfers from *Aegilops unmbellulata, Thinopyrum elongatum* and *S. cereale.* The complexity of detection of the *Ae. ventricosa Lr37* transfer is also presented.

INTRODUCTION

The crop x *Triticosecale* WITTMACK in its hexaploid or octoploid forms represents rye (*Secale cereale* L.) 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 wheats. Several other wheat/rye translocations holding potential agricultural promise for biotic/abiotic stresses are 6BS/6RL (Cereal cyst nematode) and 5AS/5RL (Copper efficiency). Individual complete rye chromosome additions to wheat also hold promise for Karnal bunt (*Tilletia indica*) resistance but require genetic manipulations.

Though detection of rye chromosomes and translocated arms can be achieved readily by C-banding or isozyme analyses, application of *in situ* hybridization methodology has additional advantages for diagnosing cryptic introgressions not marked by the former techniques.

We report in this study the application of the fluorescent *in situ* hybridization technique to several rye segments being utilized in spring durum and bread wheat breeding. Nonradioactive *in situ* hybridization using labelled total genomic DNA of the alien species with the unlabelled blocking DNA of *Triticum aestivum* (2n=6x=42; AABBDD) or *T. turgidum* (2n=4x=28; AABB) was successfully utilized. Extension of the above technique has been made to various cytogenetic aspects of some intergeneric Triticeae hybrids. Further, alien introgressions from alien species categorized for leaf rust genes has been possible with fluorescent *in situ* hybridization. These results are presented.

MATERIALS AND METHODS

Plant Material. *Triticum aestivum* and *T. turgidum* germplasm possessing a rye chromosomes as addition lines or as wheat/rye chromosome translocations or present in trigeneric hybrids were utilized fluorescence *in situ* hybridization. For the leaf rust alien gene inserts isogenic lines of these *Lr* genes in *T. aestivum* cv. Thatcher background were utilized (Table 1).

Chromosome Preparation. Root-tips collected from germinating seeds in a petri dish were pre-treated with 8-OH-quinoline+colchicine+DMSO for 3 to 3½h (Mujeeb-Kazi and Miranda, 1985) and fixed in 3 ethanol: 1 acetic acid. Root meristems were incubated at 37°C in an enzyme solution (5% Cellulase R-10+1% Pectolyase Y-23 in 0.01M sodium citrate buffer, pH 4.5) for 30 minutes. After incubation the meristems were rinsed first with buffer then water, and an individual meristem transferred onto a ethanol washed glass slide. A drop of 3:1 fixative was added, the tissue macerated, a 45% acetic acid drop added, cover-glass applied coupled with gentle pressure on the cover-glass to spread the chromosomes. The procedure followed was similar to that described by Islam-Faridi *et al.*, (1993).

DNA Preparation. DNA of *Triticum aestivum* (2n=6x=42), *T. turgidum* (2n=4x=28), *Secale cereale* (2n=2x=14), *Aegilops umbellulata* (2n=2x=14), *Ae. squarrosa* (2n=2x=14), *Thimopyrum bessarabicum* (2n=2x=14) and *Th. elongatum* (2n=2x=14) was extracted following standard protocols. Prior to use, the DNA was mechanically sheared to 10-20 Kb. Total genomic DNA was labelled by nick-translation using either biotin-14-dATP (GIBCO, BRL) or digoxigenin-11-dUTP (Boehringer Mannheim) for use as the *in situ* probe.

Hybridization Procedure. *In situ* hybridization procedures were essentially similar to those of Rayburn and Gill (1985) and the detection diagnostic utilized fluorescence (Schawarzacher et al. 1989, Islam-Faridi and Jewell, 1993). Double labelling detection followed the procedure of Islam-Faridi et al. (1993) using biotin with fluorescein labelled avidin (Vector Laboratory) and digoxigenin with fluorescein sheep anti-digoxigenin (Boehringer Mannheim). The hybridization procedure for *Lr37* utilized biotin with Texas red labelled avidin (Vector Laboratories) and digoxigenin with fluorescein sheep anti-digoxigenin (Boehringer Mannheim).

RESULTS AND DISCUSSION

Alien chromosome detection. Total genomic DNA of *S. cereale* as a probe enabled identification of complete rye chromosomes, rye chromosome additions and wheat/rye translocations in metaphase cells or in interphase (data not shown). Distortion of chromosome morphology was not prevalent. The translocation breakpoints were exceptionally clear and appear 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 9 and 23 (data not shown). The DNA-specific dye DAPI used earlier gave uniform blue fluorescence (Schwarzacher et al. 1992) with filter 2 (Zeiss) and the alien chromosomes could not be distinguished from those of wheat.

Table 1. Germplasm used for fluorescent *in situ* hybridization analyses with cytological and origin details

Germplasm	Cytological Details	Seed Origin
X TriticoSecale	2n=6x=42, AABBRR	CIMMYT
T. aestivum cv. CS+1R1R	2n=6x=42+2=AABBDD+1R1R	late E.R. Sears
T. aestivum cv. CS+6R6R	2n=6x=42+2=AABBDD+6R6R	late E.R. Sears
T. aestivum cv. TAM 200	2n=6x=42;1AL/1RS,1AL/1RS	Texas A & M
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 Lab., IPSR
T. aestivum	2n=6x=42;6BS/6RL, 6BS/6RL	Univ. of Illinois
T. turgidum cv. Altar 84	2n=4x=28;1BL/1RS, 1BL/1RS	CIMMYT
T. aestivum/Th. bessarabicum// S. cereale	2n=7x=35;ABDJR	CIMMYT
T. aestivum cv. Thatcher	2n=6x=42; Isogenic *Lr9*	R. Singh, CIMMYT
T. aestivum cv. Thatcher	2n=6x=42; Isogenic *Lr19*	R. Singh, CIMMYT
T. aestivum cv. Thatcher	2n=6x=42; Isogenic *Lr24*	R. Singh, CIMMYT
T. aestivum cv. Thatcher	2n=6x=42; Isogenic *Lr25*	R. Singh, CIMMYT
T. aestivum cv. Thatcher	2n=6x=42; Isogenic *Lr29*	R. Singh, CIMMYT
T. aestivum cv. Thatcher	2n=6x=42; Isogenic *Lr37*	R. Singh, CIMMYT

CIMMYT= International Maize and Wheat Improvement Center, Mexico
IPSR = Institute of Plant Science Research, Norwich, U.K.

Table 2. Specific details of the hybridization mixture used for different germplasm comprising of *Triticum aestivum* (CS), *T. turgidum* (A), *Secale cereale* (R), *Thinopyrum bessarabicum* (J), *Th. elongatum* (E), *Aegilops umbellulata* (U) and *Ae. squarrosa* (D)

Germplasm	Deionized Formamide (%)	Dextran Sulphate (%)	SSC X	Labelled Probe		Block DNA*	Volume used µl/slide
				Biotin	Digoxigenin		
X TriticoSecale	44.01	8.80	3.52	60ng R		18 x A	28.40
T. aestivum cv. CS+1R1R	46.99	9.40	3.76	40ng R		20 x CS	26.60
T. aestivum cv. CS+6R/6R	47.17	9.43	3.77	40ng R		20 x CS	26.50
T. aestivum cv. TAM 200	41.00	8.25	1.47	40ng R		20 x CS	27.25
T. aestivum cv. Seri 82	41.00	8.25	1.47	40ng R		20 x CS	27.25
T. aestivum	41.00	8.60	1.72	40ng R		20 x CS	29.00
T. aestivum	41.00	8.25	1.47	40ng R		20 x CS	27.25
T. turgidum cv. Altar 84	48.45	9.69	3.88	35ng R		25 x A	25.80
T. aestivum/Th. bessarabicum// S. cereale	38.09	7.94	3.17	40ng J	40ng R	20 x CS	31.50
T. aestivum Lr9	48.08	9.62	3.85	50ng U		20 x CS	26.00
T. aestivum Lr19	48.54	9.71	3.88	40ng E		20 x CS	25.75
	36.23	7.25	2.90	40ng E	50ng D	1000ng Al**	34.50
T. aestivum Lr24	58.54	9.71	3.88	40ng E		20 x CS	25.75
T. aestivum Lr25	44.91	8.98	3.59	50ng R		20 x CS	27.80
T. aestivum Lr29	48.54	9.71	3.88	40ng E		20 x CS	25.75
T. aestivum Lr37	44.56	8.91	3.57	70ng D		18 x Al**	28.00
T. aestivum cv. Thatcher	44.56	8.91	3.57	70ng D		18 x Al**	28.00

* 20x = 20 times amount of probe DNA as an example of values in the column

Al** = *T. turgidum* cv. Altar 84

We have been able to observe the hybridization signals with DAPI using Zeiss filter 2 repeatedly and obtained exceptional clarity in contrast. Double labelling also enabled the detection of *Th. bessarabicum* and *S. cereale* chromosomes, thus opening avenues of using alien species amphiploids as bridge parents in crosses with wheat. Probe DNA of *Ae. umbellulata, Th. elongatum, S. cereale* and *Ae. squarrosa* with *T. aestivum* blocking DNA enabled detection of the introgressions for *Lr9; Lr19, Lr24, Lr29; Lr25; r37* respectively. The *Lr* genes are located on chromosomes 6B (*Lr9*); 7DL (*Lr19*), 3DL (*Lr24*), 7DS (*Lr29*); 4A**ß** (*Lr2*) and 2AS (*Lr37*).

Practical Applications. The rye translocations used in this study have been attributed with characteristics that contribute to resistances/tolerances to biotic/abiotic stresses. These specifically are:

. **1BL/1RS**: genes for leaf, stem, stripe rust and muldew resistance
. **1AL/1RS**: green-bug resistance
. **5AS/5RL**: copper efficiency
. **6BS/6RL**: cereal cyst nematode resistance

Other rye chromosomes not yet involved in translocations have, as disomic additions to wheat, been associated with resistance to Karnal bunt. *Th. bessarabicum* contributes to salinity tolerance.

Though virulence exists for most of the *Lr* genes physically in this study, we envision that the current detection methodology shall facilitate their pyramiding with other durable leaf rust genes or complexes conventionally present in wheat cultivars like *Lr13* and *Lr34*. Multiple inserts could then be identified quite simplistically. Should the size of the alien introgressions becomes an adverse factor, one may envisage genetic manipulation strategies targeted towards reducing the DNA insert size from what is currently prevalent for the various *Lr* alien genes.

CONCLUSIONS

It has been fortunate that whole arm translocations (1BL/1RS or 1AL/1RS) have yielded superior cultivars that are grown on over 5 million hectares globally. Utilizing wild relatives in plant improvement however, requires that alien transfer/s be cryptic. We anticipate that when cryptic transfers are engineered through genetic manipulation strategies the diagnostic strength of genomic *in situ* hybridization shall be fully realized. The technique is rapid, sensitive and gives superior resolution provided that somatic preparations possess quality chromosome separation with minimum cytoplasmic debris and have a desirable mitotic index.

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