Theor Appl Genet (1995) 90: 952–956

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Biochemical and molecular diagnostics of *Thinopyrum bessarabicum* chromosomes in *Triticum aestivum* germ plasm

Received: 25 April 1994 / Accepted: 18 August 1994

Abstract *Thinopyrum bessarabicum* (2n=2x=14, JJ) is a self-fertile salt-tolerant grass species, and its hybridization with *Triticum aestivum* to achieve the transfer of this attributes has been promoted. For the detection of alien introgression, development of diagnostic markers of *Th. bessarabicum* chromosomes in the wheat background has emerged as an important aspect in our intergeneric hybridization program. Six proteins/isozymes–high-molecular-weight glutenins, superoxide dismutase, grain esterase, β-amylase, glutamate oxaloacetate transaminase and α-amylase – were identified as positive markers for detecting the presence of *Th. bessarabicum* chromosomes in the advanced backcross derivatives of *T. aestivum/Th. bessarabicum//*n* T. aestivum*. Fluorescent *in situ* hybridization further enabled the detection of complete and translocated arms of *Th. bessarabicum* chromosomes in the *T. aestivum* background. These diagnostic markers served for tentatively characterizing a distinct set of *Th. bessarabicum* disomic additions to wheat (2n=44) and have facilitated establishing the homoeology of these added chromosomes.

Key words *Triticum aestivum* · *Thinopyrum bessarabicum* · Protein/isozyme markers · *In situ* hybridization · Alien disomic additions

Introduction

Thinopyrum bessarabicum (2n=2x=14, JJ); syn *Agropyron junceum*; is a self-fertile maritime grass (Dewey 1984) possessing salinity tolerance (Gorham et al. 1985; Mujeeb-Kazi et al. 1993). This important character makes *Th. bessarabicum* a potential candidate for transferring this attribute into cultivated wheat (*Triticum aestivum* L.; 2n=6x=42, AABBDD) since salinity tolerance limits wheat cultivation in many locations. Colchicine treatment of the F_1 hybrid between *T. aestivum* cv 'Chinese Spring' and *Th. bessarabicum* led to the production of the *T. aestivum/Th. bessarabicum* amphiploid (2n=8x=56, AABBDDJJ; Forster et al. 1987). A set of disomic addition lines (2n=44) was produced by carrying out backcrosses of wheat onto the F_1 hybrid (at CIMMYT) or onto the amphiploid (Forster et al. 1987, 1988). Six of the seven disomic addition lines have been tentatively identified with distinct protein/isozyme markers, and the remaining addition line possesses a morphological marker. The biochemical markers utilized in this study for characterizing the disomic addition line set have been previously reported as being diagnostic for *Th. bessarabicum* and being expressed in the *T. aestivum/Th. bessarabicum* amphiploid (William and Mujeeb-Kazi 1993). These biochemical markers were high-molecular-weight glutenins (HMW glutenins), superoxide dismutase (SOD, E.C. 1.15.1.1), grain esterase (EST-5, E.C. 3.1.1.1), β-amylase (α-1,4-glucan malto hydrolase, E.C. 3.2.1.2.), glutamate oxaloacetate transaminase (GOT, E.C. 2.6.1.1) and α-amylase (α-AMY, α-1,4-glucan-4-glucan hydrolase, E.C. 3.2.1.1).

Fluorescent *in situ* hybridization (FISH) using a biotin-labeled genomic DNA probe has been widely used for identifying alien chromosomes and chromatin *in situ* wheat/alien hybrid derivatives (Rayburn and Gill 1985; Schwarzacher et al. 1992; Mukai et al. 1993; Islam-Faridi and Mujeeb-Kazi 1995). FISH is a powerful diagnostic tool for detecting small introgressed alien segments in a wheat background, but it was used here for ascertaining the presence of complete chromosomes after the homoeology of the added alien chromosomes to wheat was established. Although some *in situ* hybridization work with *Th. bessarabicum* has been reported (Schwarzacher et al. 1992; Rayburn et al. 1993) our approach here is an integrated one that extends the utility of *Th. bessarabicum* germ plasm into the practical agricultural domain.

Communicated by K. Tsunewaki

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Table 1 Methods used in electrophoretical analysis of six protein/enzymes

Protein or enzyme	Separation method	Tissue used	Cathode buffer	Anode buffer
HMW glutenins	SDS-PAGE	Mature kernel	–	–
SOD	IEF (pH 4.0–6.5)	Mature kernel	0.1 *M* β-alanine	0.5 *M* H_3PO_4
EST-5	IEF (pH 3.5–9.5)	Mature kernel	1 *M* NaOH	1 *M* H_3PO_4
β-AMY	Native-PAGE	Mature kernel	–	–
GOT	Native-PAGE	Young leaf	–	–
α-AMY	IEF (pH 3.5–9.5)	Germinating seed	1 *M* NaOH	1 *M* H_3PO_4

Table 2 *Triticum aestivum* and *Thinopyrum bessarabicum* derivatives utilized for fluorescence *in situ* hybridization (CIMMYT International Maize and Wheat Improvement Center, Mexico, IPSR Institute of Plant Science Research, Norwich, UK

Line	Cytological constitution	Origin
T. aestivum cv CS	2n=6x=42; AABBDD	Late E.R. Sears
1J disomic addition	2n=44; AABBDD+1J1J	CIMMYT
2J disomic addition	2n=44; AABBDD+2J2J	CIMMYT/IPSR
3J disomic addition	2n=44; AABBDD+3J3J	CIMMYT
4J disomic addition	2n=44; AABBDD+4J4J	CIMMYT
5J disomic addition	2n=44; AABBDD+5J5J	CIMMYT/IPSR
6J disomic addition	2n=44; AABBDD+6J6J	CIMMYT
7J disomic addition	2n=44; AABBDD+7J7J	CIMMYT
Amphiploid	2n=8x=56; AABBDDJJ	IPSR

Materials and methods

A series of meiotically stable disomic addition lines (2n=44) were analyzed electrophoretically for biochemical markers using protocols described by William and Mujeeb-Kazi (1993). These addition lines were developed by CIMMYT's Wheat Wide Crosses program. *Th. bessarabicum* seeds were obtained from the late Dr. D.R. Dewey of the USDA/ARS Laboratory in Logan, Utah and the *T. aestivum/Th. bessarabicum* amphiploid seed from Dr. B. Forster (then at PBI, Cambridge). For all of the electrophoretic analyses *T. aestivum* cvs 'Chinese Spring'and 'Genaro-81', *Th. bessarabicum* and the *T. aestivum/Th. bessarabicum* amphiploid served as controls. Enzyme/protein extraction and electrophoretic details were similar to those of William and Mujeeb-Kazi (1993). A summary of the electrophoretic techniques used is presented in Table 1. α-AMY, SOD and EST-5 isozymes were separated on Ampholine PAG plates (Pharmacia) with isoelectric focusing (IEF). The flat-bed LKB Multiphor II electrophoresis unit was used for IEF with water cooling. GOT and β-AMY isozymes were separated by native polyacrylamide gel, electrophoresis using 6% and 8.5% acrylamide gels, respectively. HMW glutenin subunits were separated on 10% polyacrylamide gels with 3% stacking gels in the presence of sodium dodecyl sulfate. Staining for EST-5, β-AMY, and α-AMY was according to William and Mujeeb-Kazi (1992), SOD according to Newman and Hart (1986) and GOT according to Vallejos (1983). HMW glutenin proteins were stained with 0.1% coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid.

Fluorescent *in situ* hybridization

The plant materials used for *in situ* hybridization are given in Table 2. Root-tip collection and slide preparation was essentially according to Mujeeb-Kazi and Miranda (1985). Root-tip meristematic tissue was digested with 5% cellulase R-10 + 1% pectolyase Y-23 in 0.01 M citrate buffer and distilled water. The tissue was macerated on a clean slide with forceps in a drop of 3:1 ethanol:acetic acid. A drop of acetic acid was further added to spread the cell contents. The fluorescent *in situ* hybridization procedure was similar to the protocols of Schwarzacher et al. (1992) and Islam-Faridi and Mujeeb-Kazi (1994). Genomic *T. aestivum* cv 'Chinese Spring' DNA sheared by autoclaving at 10 psi for 5 min was used 15 times in excess of biotin-14-dATP-labeled *Th. bessarabicum* genomic DNA. *Th. bessarabicum* DNA was biotin-labeled by nick translation following the instructions accompanying the GIBCO-BRL bionicktranslation kit. Stringency washes were done twice at 37 °C with 30% formamide in 0.5×SSC, and twice again with 4×SSC+tween-20 (0.2%) at room temperature. Biotin-labeled *Th. bessarabicum* DNA detection utilized fluorescein-avidin coupled with biotinylated anti-avidin for signal amplification following Vector Labs Inc. protocols.

Results and discussion

Addition line with group 1 chromosome

Separation of HMW glutenins for *T. aestivum* (cvs 'Chinese Spring', 'Genaro-81'), *Th. bessarabicum* and the amphiploid are shown in Fig. 1. The 'Chinese Spring' and 'Genaro-81' patterns differed from each other due to their allelic nature (Payne and Lawrence 1983). *Th. bessarabicum* had a single band in the HMW glutenin region of slightly faster mobility as compared to the 'Chinese Spring' and 'Genaro-81' patterns. This was the marker band used in screening several disomic addition lines and provided positive identification. The diagnostic band was present in *Th. bessarabicum* and in its amphiploid with wheat (Fig. 1). Since the HMW glutenin genes are located in the long arms of homoeologous group 1 chromosomes in wheat (Beitz et al. 1975; Lawrence and Shepherd 1981) this protein marker enabled characterization of the *Th. bessarabicum* disomic addition lines with a group 1 chromosome of wheat.

Addition line with group 2 chromosome

Th. bessarabicum had a single SOD activity band, as compared to the two more basic activity bands of bread wheat (Fig. 2a). *Th. bessarabicum* did not express any polymorphism for SOD isozymes among its different accessions

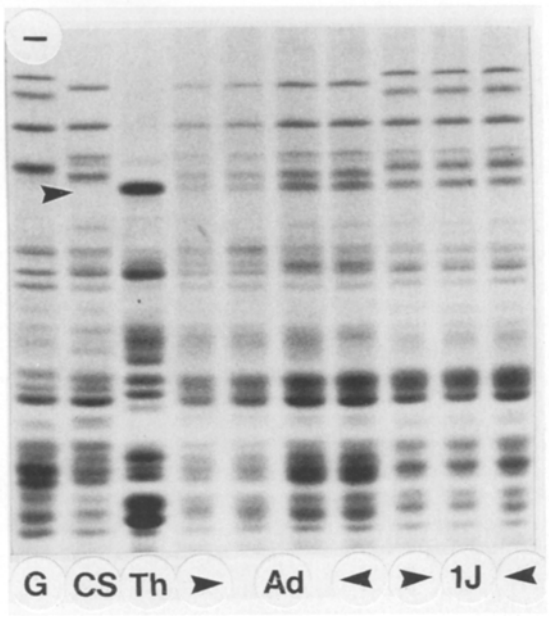

Fig. 1 SDS-PAGE of total proteins of *T. aestivum* cvs 'Genaro 81' (*G*) and 'Chinese Spring' (*CS*), *Th. bessarabicum* (*Th*), CS/*Th. bessarabicum* amphiploid (*Ad*) and disomic addition lines of group 1 chromosome (*1J*). Marker *arrowed*

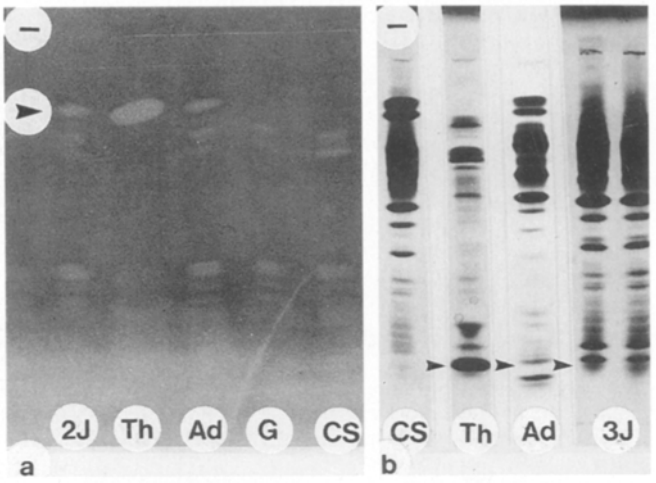

Fig. 2a, b Electrophoretic profiles of grain SOD and EST-5. *CS*, *G*, *Ad*, *Th*, *2J* and *3J* are *T. aestivum* cvs 'Chinese Spring', and 'Genaro 81', CS/*Th. bessarabicum* amphiploid, *Th. bessarabicum* and the disomic additions of the 2J and 3J chromosome, respectively. Markers *arrowed*. **a** Grain SOD profiles on IEF (pH 4.0–6.5) polyacrylamide gels, **b** grain EST profiles on IEF (pH 3.5–9.5) polyacrylamide gels

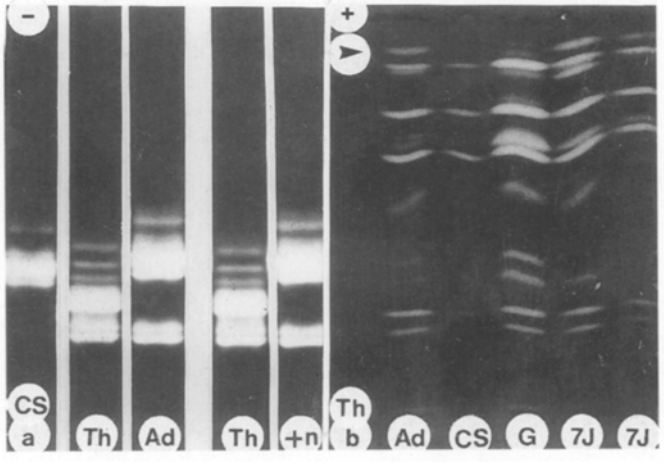

Fig. 3a, b Electrophoretic profiles of β-amylase and α-AMY-2 profiles. *CS*, *G*, *Th*, *Ad*, *J+* and *7J* are *T. aestivum* cvs 'Chinese Spring' and' 'Genaro 81'. *Th. bessarabicum*, CS/*Th. bessarabicum* amphiploid and the disomic addition lines of the 4J or 5J and 7J chromosome, respectively. Markers *arrowed*.
a Native PAGE of β-amylase, **b** α-AMY-2 profiles

(William and Mujeeb-Kazi 1993). This band was also expressed in its amphiploid with wheat. SOD isozyme genes are located in homoeologous group 2 wheat chromosomes (Newman and Hart 1986), hence they serve as a marker band for characterizing the *Th. bessarabicum* disomic additions with a group 2 chromosome (Fig. 2a).

Addition line with group 3 chromosome

Although individual *Th. bessarabicum* seeds exhibit some polymorphism for EST-5 patterns on IEF, marker bands were present when comparisons were made with the IEF patterns of bread wheat (William and Mujeeb-Kazi 1993). Figure 2b shows the EST-5 banding patterns of 'Chinese Spring', *Th. bessarabicum*, the *T. aestivum/Th. bessarabicum* amphiploid and a disomic addition line. Since EST-5 genes have been associated with homoeologous group 3 chromosomes in wheat (Ainsworth et al. 1984), the diagnostic band observed (Fig. 2b) characterized the *Th. bessarabicum* disomic addition line with the group 3 chromosome.

Addition line with group 4 or 5 chromosomes

β-AMY isozyme separation on native polyacrylamide gels exhibited polymorphism among different *Th. bessarabicum* accessions (William and Mujeeb-Kazi 1993). The β-AMY patterns of bread wheat and *Th. bessarabicum* are shown in Fig. 3a. The *Th. bessarabicum* polymorphisms for β-AMY do not limit the use of β-AMY as a diagnostic marker since there is no overlap with the pattern of the bread wheat controls. The amphiploid and the disomic addition line showed the marker band (Fig. 3a). β-AMY genes are located on chromosome arms 4AL (β-*Amy*-A1), 5AL (β-*Amy*-B1), and 4DL (β-*Amy*-D1) (Ainsworth et al. 1983; McIntosh 1988; Sharp et al. 1988). Some alien species follow a similar trend where β-AMY genes are located on group 4 or 5 chromosomes (Ainsworth et al. 1987). We did not observe our addition line group to possess blue aleurone, a morphological marker related to group 4. Also, the phosphoglucomutase (PGM) group 4 marker did not prove to be diagnostic for these additions. This line had already

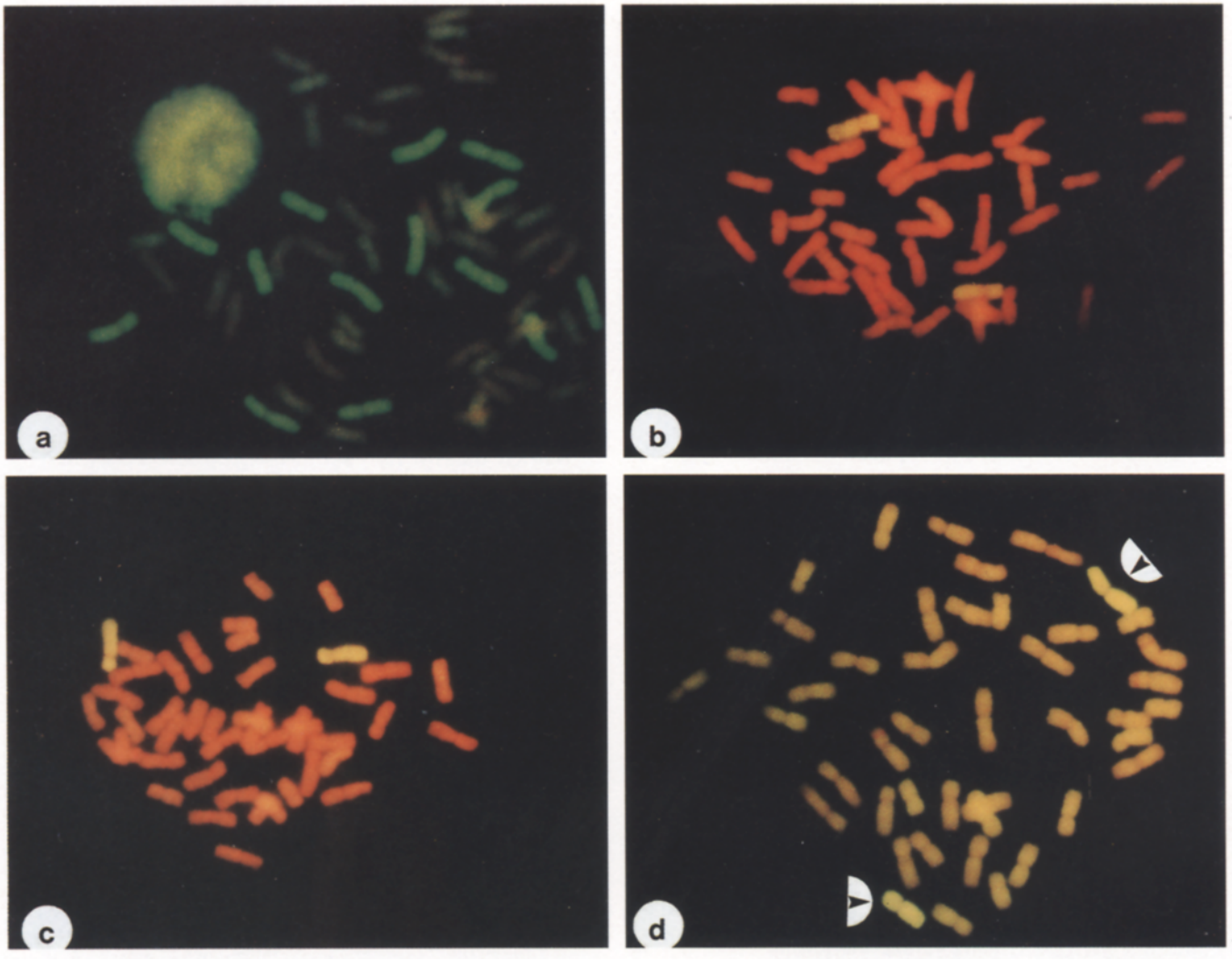

Fig. 4a–d Fluorescence micrographs of an amphiploid, *T. aestivum* cv 'Chinese Spring'/*Th. bessarabicum* (2n=8x=56, AABBDDJJ) and three disomic additions of *Th. bessarabicum* chromosomes to 'Chinese Spring'. Wheat chromosomes were blocked with sheared unlabeled *T. aestivum* DNA. Biotin-labeled *Th. bessarabicum* DNA used as a probe enabled detection of *Th. bessarabicum* chromosomes with yellow green or bright yellow fluorescence using a 09 Zeiss filter. **a** Amphiploid; **b, c, d** Disomic additions of 4J, 6J and 7J, respectively

been categorized as a 5J disomic addition (Forster et al. 1988), before it was obtained by us. We have now backcrossed it to cv Genaro 81 in order to improve its plant type. Nevertheless, we consider the β-AMY identified disomic addition line derived after this backcross as being homoeologous to group 4 or 5. It would be analogous to the 5J group according to the published report of Forster et al. (1988).

Addition line with group 6 chromosome

Genetic analysis of GOT-2 isozymes has established that the controlling genes are associated with homoeologous group 6 wheat chromosomes (Hart 1975). *Th. bessarabicum* expressed a single band in the GOT-2 zone of activity with identical mobility to the GOT-2 band 1 of 'Chinese Spring'. The amphiploid and the disomic addition line expressed three bands with increased staining intensity for GOT-2 band 1. This intensely stained band allows for characterization of the *Th. bessarabicum* disomic addition lines of group 6 chromosomes.

Addition line with group 7 chromosome

α-AMY genes are located on homoeologous group 6 (α-*Amy* 1) and 7 chromosomes (α-*Amy* 2) of wheat (Nishikawa and Nobuhara 1971; Gale et al. 1983). In the α-AMY 2 isozyme region (Fig. 3b) *Th. bessarabicum* had a band that was not present in bread wheat (William and Mujeeb-Kazi 1993). This α-AMY 2 marker band expressed in the amphiploid and in the disomic addition line enabled characterization of the *Th. bessarabicum* disomic addition lines of group 7 chromosomes.

In situ hybridization

Fluorescent *in situ* hybridization (FISH) analyses of root-tip chromosome preparations in the amphiploid of 'Chinese Spring'/*Th. bessarabicum*, and some disomic addition lines enabled identification of the three *Th. bessarabicum* chromosomes, 4J, 6J and 7J, added to 'Chinese Spring' (Fig. 4). We consider the FISH procedure to be advantageous over other molecular techniques in that DNA acquisition for probing occurs readily and subtle alien introgressions can also be ascertained. In the germ plasm evaluated in this study, the chromosome morphology was not distorted and chromosome constrictions were generally clear. The ratio of the blocking:labeled DNA was 15:1. When this excessive amount of blocking DNA was not used the differentiation of *Th. bessarabicum* chromosomes became unclear, suggestive of the genomic homoeology between bread wheat (ABD) and *Th. bessarabicum* (J).

The quality of alien chromosome detection (Fig. 4) by FISH shall facilitate detection of subtle introgressions from addition lines linked to useful traits, as has been proven for *Secale cereale* chromosome transfer (Mukai et al. 1993; Islam-Faridi and Mujeeb-Kazi 1994). Use of a *ph* mutant or *Ph* suppressor in wide hybridization shall allow subtle alien DNA introgressions to occur, but their size might prove to be a constraint for FISH based detections if introgressions are very subtle and beyond the current detection limit.

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