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Thinopyrum bessarabicum: biochemical and cytological markers for the detection of genetic introgression in its hybrid derivatives with *Triticum aestivum* L.

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Summary. *Thinopyrum bessarabicum* (2n = 2x = 14, JJ) with its unique property of salt tolerance provides a potential means for the transfer of this important and complex trait into cultivated wheat through intergeneric hybridization. To accomplish this, diagnostic markers for detecting the presence of *Th. bessarabicum* chromosomes in a wheat background have to be established. The C-banded karyotype of *Th. bessarabicum* distinctly identifies individual *Th. bessarabicum* chromosomes and separates them from those of *Triticum aestivum*. Also, seven protein/isozymes, i.e., malate dehydrogenase, high-molecular-weight glutenin, superoxide dismutase, grain esterase, glutamate oxaloacetate transaminase, β-amylase and α-amylase, were identified as being positive markers specific to *Th. bessarabicum*; these were also expressed in the *T. aestivum*/*Th. bessarabicum* amphiploid. These diagnostic biochemical markers could be useful in detecting and establishing homoeology of *Th. bessarabicum* chromosomes in *T. aestivum*/*Th. bessarabicum* intergeneric hybrid derivatives.

Key words: *Triticum aestivum* – *Thinopyrum bessarabicum* – Protein-isozyme markers – Chromosome banding – Intergeneric hybridization

Introduction

Thinopyrum bessarabicum (*Agropyron junceum*; 2n = 2x = 14, JJ) is a self-fertilizing maritime grass, rhizomatous (Dewey 1984) and noted for its high tolerance to salinity (Forster et al. 1987, 1988; Gorham et al. 1985). It has been successfully hybridized to *Triticum aestivum* L., and the hybrids have provided: (1) information about phylogenetic relationships (Alonso and Kimber 1980) and (2) germ plasm to be exploited for transferring the salt tolerance attribute of *Th. bessarabicum* to *T. aestivum* (Mujeeb-Kazi et al. 1983; Sharma and Gill 1983a, b). Amphiploids have been induced by treating the F_1 hybrid plants with colchicine (Alonso and Kimber 1980; Forster et al. 1987). Using the two parental species together with the amphiploid has thus formed the basis necessary to study biochemical characteristics and establish markers to facilitate the detection of alien chromosomal introgression in *T. aestivum*. Such biochemical markers are reported in the study described here as they are present in *Th. bessarabicum* and can be diagnosed in its amphiploid (2n = 8x = 56, AABBDDJJ) with *T. aestivum*. The biochemical markers used were malate dehydrogenase (MDH, E.C. 1.1.1.37), grain esterase (EST-5, E.C. 3.1.1.1), β-amylase (β-AMY α-1,4-glucan maltohydrolase E.C. 3.2.1.2.), α-amylase (α-AMY, α-1,4-glucan-4-glucan hydrolase E.C. 3.2.1.1.), superoxide dismutase (SOD, E.C. 1.15.1.1), high-molecular-weight glutenin (HMW-glutenins) and glutamate oxaloacetate transaminase (GOT, E.C. 2.6.1.1).

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Materials and methods

Four different accessions of *Th. bessarabicum*, PI 531710 (Jaaska), PI 571711 (Jaaska), D-3583 and D-3584, from geographically diverse regions were utilized in this study. Seed material for *Th. bessarabicum* was obtained from Dr. D. R. Dewey of the USDA/ARS Laboratory in Logan, Utah, USA. Four to eight individual seeds and a composite of 10 seeds from each accession were evaluated for MDH, EST, β-AMY, α-AMY, SOD and HMW-glutenins. Leaves from young seedlings of individual accessions as well as a composite were analyzed for GOT. *T. aestivum* cv "Chinese Spring" was utilized together with the amphiploid of "Chinese Spring"/*Th. bessarabicum* for the comparison of banding patterns.

Table 1. List of enzymes analyzed, separation methods used, tissue analyzed and cathode and anode buffers used for IEF

Enzyme/protein	Separation method	Plant tissue	Cathode buffer	Anode buffer
MDH	IEF (pH 3.5–9.5)	Mature kernel	1 *M* NaOH	1 *M* H_3PO_4
EST	IEF (pH 3.5–9.5)	Mature kernel	1 *M* NaOH	1 *M* H_3PO_4
SOD	IEF (pH 4.0–6.5)	Mature kernel	0.1 *M* β-alanine	0.5 *M* H_3PO_4
α-AMY	IEF (pH 4.0–6.5)	Germinating seed	0.1 *M* β-alanine	0.5 *M* H_3PO_4
β-AMY	Native-PAGE	Mature kernel	–	–
GOT	Native-PAGE	Young leaf	–	–
HMW-glutenins	SDS-PAGE	Mature kernel	–	–

Enzyme extraction

For all of the enzyme systems except GOT and α-AMY mature seeds of *Th. bessarabicum*, "Chinese Spring" (CS) and the CS x *Th. bessarabicum* amphiploid were used. Individual seeds of *Th. bessarabicum* were dehulled and, ground, and the flour was extracted with 50 μl extraction buffer. A composite of 10 seeds of *Th. bessarabicum* as well as individual seeds of "Chinese Spring" were extracted with 300 μl of buffer solution. The extraction buffer for MDH and SOD was 0.05 *M* TRIS-HCl (pH 7.5). The buffer for β-AMY was 0.05 *M* TRIS-HCl (pH 7.5) with 0.2 *M* 2-mercaptoethanol containing 10% sucrose. For EST, 0.05 *M* Na_2HPO_4 (pH 7.5) was used. MDH, EST and SOD extractions were done at 4 °C for 1 h with occasional shaking, whereas for β-AMY overnight extractions were made at 4 °C. For α-AMY 5-day-old germinating seeds were homogenized and then extracted overnight at 4 °C with 0.05 *M* TRIS-HCl (pH 7.0) containing 0.002 *M* $CaCl_2$. Leaves from young fast-growing seedlings were macerated (1:2, w/v) with 0.05 *M* TRIS-HCl (pH 7.5) with 10% sucrose at 4 °C and immediately used for GOT analysis. Samples were vortex mixed, centrifuged for 5 min, and the supernatants were used for enzyme analysis. Extracts for α-AMY were heated at 70 °C for 10 min to inactivate the β-amylase.

Isoelectric focusing (IEF)

Ampholine PAG plates (Pharmacia) were used for IEF, which was performed on a flat-bed LKB Multiphor 11 electrophoresis unit. Gels were prefocused for 30 min at a constant power of 12 W. Paper wicks (Whatman 3 mm) were laid over the gel 1 cm from the cathode, and 20 μl of sample extracts were applied. Isoelectric focusing was continued for a total of 6 h at a constant power of 12 W with the maximum voltage set at 1300 V. Paper wicks containing samples were removed after 45 min into the run. The pH gradients of the gels used for different enzyme systems, the plant tissue used and the cathode and anode buffers are shown in Table 1.

Native polyacrylamide gel electrophoresis (Native-PAGE)

β-AMY and GOT isozymes were separated on native-PAGE. Running gels of 8.5% and 6.0% acrylamide were used for β-AMY and GOT, respectively, and 5% acrylamide stacking gel was used for both systems. The gel compositions and the running buffers were similar to those described in Davis (1964). The extracts were mixed with 0.005% bromophenol-blue, and 30-μl samples was used for electrophoresis in a Hoeffer verticle electrophoresis unit. The gel dimensions were (180 x 160 x 1.5 mm), and electrophoresis was conducted at 40 mA constant current at 4 °C until the dye front reached the bottom of the gel.

SDS-PAGE

High-molecular-weight glutenin subunits were separated by SDS-PAGE on a Hoeffer vertical electrophoresis unit. A 10% running gel and a 3% stacking gel were used in combination with sample extraction and electrophoretic conditions adopted from Payne et al. (1980). The gel dimensions were the same as those for native-PAGE with electrophoresis conducted at 5 mA per gel for 2 h followed by 10 mA per gel unit the dye front reached the bottom of the gel. Gels were rinsed in a solution of 30% ethanol to remove SDS and stained in a solution of 0.1% coomassie brilliant blue R-250, 50% methanol and 10% acetic acid. Destaining was in a solution of 40% methanol and 10% acetic acid.

Enzyme staining

Staining for MDH, EST and β-AMY was according to William and Mujeeb-Kazi (1992), SOD was stained according to Newman and Hart (1986) and GOT according to Vallejos (1983). Staining for α-AMY was similar to that for β-AMY.

Cytology

Seeds of *Th. bessarabicum* accession PI-531710 (JAASKA-11) were germinated and transplanted into peat pots. Root tips were periodically sampled and processed according to the procedure of Mujeeb-Kazi and Miranda (1985). After pretreatment, the root tips were fixed in 0.1% aceto-carmine for 48 h, squashed in 45% acetic acid, following which the cover glass was removed by the dry-ice method. The C-banding procedure was essentially similar to that of Bennett et al. (1977) and Jahan et al. (1990).

Photography

Representative C-banded cells were photographed on a Kodak Technical Pan Film 2415 (Estar-AH base) at 18 din, 12 V with a Collins special green filter and a yellow 54 barrier filter combination. A composite was made and photographed to produce the glossy plate used here.

Results and discussion

High-molecular-weight glutenins

In the region of high-molecular-weight glutenins there was no polymorphism within different seeds of a given accession of *Th. bessarabicum* or between accessions. The banding patterns common to *Th. bessarabicum*, the amphiploid, and "Chinese Spring" are shown in Fig. 1. According to the numbering system developed for the high-molecular-weight subunits (Payne and Lawrence 1983) "Chinese Spring" is assigned the allelic combinations 2+12 (*Glu-D1a*) and 7+8 (*Glu-B1b*). *Th. bessarabicum* has a single unique band in the high-molecular-weight subunit region with a faster mobility that is absent in the

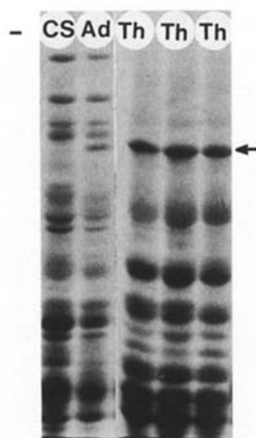

Fig. 1. SDS-PAGE of total proteins of *T. aestivum* cv "Chinese Spring" (*CS*), amphiploid of CS/*Th. bessarabicum* (*Ad*) and *Th. bessarabicum* (*Th*). The marker band in the amphiploid is indicated by an *arrowhead*

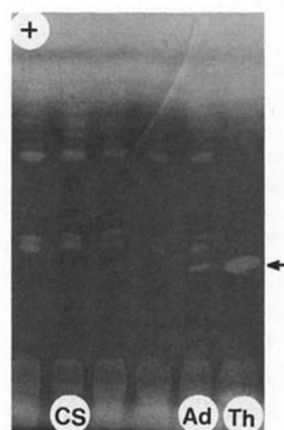

Fig. 3. Grain SOD banding profiles on IEF (pH 4.0–6.5) polyacrylamide gels; banding profiles of *T. aestivum* cv "Chinese Spring" (*CS*), the CS/*Th. bessarabicum* amphiploid (*Ad*) and *Th. bessarabicum* (*Th*). The marker band is indicated by an *arrowhead*

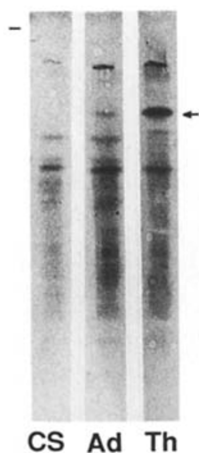

Fig. 2. Grain MDH banding profiles on IEF polyacrylamide gels (pH 3.5–9.5) showing banding characteristics of *T. aestivum* cv "Chinese Spring" (*CS*), the amphiploid of CS/*Th. bessarabicum* (*Ad*) and *Th. bessarabicum* (*Th*). The marker band is indicated by an *arrowhead*

banding profile of "Chinese Spring". This marker band is also present in the amphiploid (Fig. 1). The high-molecular-weight glutenin genes are located in the long arms of homoeologous group-1 chromosomes in wheat (Beitz et al. 1975; Lawrence and Shepherd 1981). With this marker band as a biochemical marker, it would be possible to identify the *Th. bessarabicum* chromosome homoeologous to the group-1 chromosomes of wheat.

Malate dehydrogenase

The MDH banding patterns of 8 individual seeds of each of the four *Th. bessarabicum* accessions did not show polymorphism. "Chinese Spring" had a banding pattern with 12–13 bands. The resolution of the banding patterns was better on the basic side of the gel than on the acidic side. All individual seeds of the *Th. bessarabicum* accessions analyzed had 1 band on the basic side that was not present in "Chinese Spring"; this marker band was also expressed in the amphiploid (Fig. 2). Since MDH gene locations have been assigned to the long arms of homoeologous group-1 chromosomes (Benito and Salinas 1983) and to the short arms of homoeologous group-5 chromosomes (Liu and Gale 1989), it would be possible to identify the *Th. bessarabicum* chromosome homoeologous to these chromosomes in wheat by using this marker band.

Superoxide dismutase

There was no polymorphism within individual seeds of a given accession or among different accessions of *Th. bessarabicum* for SOD. *Th. bessarabicum* had a marker band that was not present in "Chinese Spring" (Fig. 3). This marker band was expressed in the amphiploid (Fig. 3) and therefore could serve as a potential marker to identify the *Th. bessarabicum* chromosome homoeologous to group 2 of wheat.

Grain esterase

Numerous bands were observed for cv "Chinese Spring" with slight polymorphisms prevalent in the banding patterns within individual seeds of all four *Th. bessarabicum* accessions (data not shown), but their composites did not exhibit polymorphism. All of the individual seeds analyzed in the four accessions of *Th. bessarabicum* had 1 band in the acidic region of the gel that did not show much polymorphism. This band was not present in the bread wheat cultivars, but was clearly expressed in the

amphiploid (Fig. 4). Genes for grain esterase in wheat (EST-5) are located in the long arms of homoeologous group-3 chromosomes (Ainsworth et al. 1984). It would be possible to use this distinctive band to detect a *Th. bessarabicum* chromosome homoeologous to the group-3 chromosomes of wheat.

β-Amylase

"Chinese Spring" had a banding pattern with 3–4 bands when β-amylase was separated on 8.5% acrylamide native-PAGE. Individual seeds of *Th. bessarabicum* had a banding profile with 2–4 bands with faster mobilities than in "Chinese Spring". The banding profiles of 4 individual seeds of *Th. bessarabicum* accessions D-3584 and PI 531711, their composites together with patterns of "Chinese Spring" are shown in Fig. 5a. The individual seeds had banding patterns ranging from 2–4 bands. Seed 1 and 2 of accession D-3584 had 4 bands, whereas seed 3 had only the 2 slower moving bands. Seed 4 also had 2 bands, but in this case only 2 faster moving bands were present. In the case of accession 531711 all 4 seeds had 2 bands each; 2 seeds lacked the 2 faster moving bands and the other 2 lacked the 2 slower moving bands. In both cases the composites of the two accessions showed all 4 bands (Fig. 5a).

The variability expressed by individual seeds of *Th. bessarabicum* does not limit our use of β-amylase as a marker. The banding patterns of individual seeds of *Th. bessarabicum*, whether they had 2 or 4 bands had distinctly faster mobilities than the banding patterns of the wheat cultivars. Figure 5b shows the banding patterns of the amphiploid together with that of "Chinese Spring" and a composite of *Th. bessarabicum*. In addition to having the bands present in the wheat cultivar, the amphiploid possessed 2 faster moving bands, which is a characteristic of the banding pattern shown by some individual *Th. bessarabicum* seeds. It is possible that the *Th. bessarabicum* accession used in the production of the F_1 hybrid and subsequently its amphiploid had only 2 faster moving bands, which is characteristic of the banding pattern of some individual seeds of *Th. bessarabicum* (Fig. 5a). Since genes controlling β-AMY are located on 4Aβ, 4DL (*β-Amy1* locus), and 5AL (*β-Amy2* locus) (Ainsworth et al. 1983), it would be possible to identify the *Th. bessarabicum* chromosome homoeologous to these chromosomes in wheat by using this marker band.

Glutamate oxaloacetate transaminase

There are three zones of enzymes activity when GOT isozymes are separated on native-PAGE (Hart 1975). In the region of GOT-2 3 bands have been observed (Hart 1975). Genetic analysis of GOT-2 isozymes has established that the genes responsible for the production of band 1 are located on chromsomes 6DL, band 2 on 6AL, 6BL, and 6DL with band 3 on 6AL and 6BL (Hart 1975).

Fig. 4. Grain EST banding profiles on IEF (pH 3.5–9.5) polyacrylamide gels of *T. aestivum* cv "Chinese Spring" (*CS*), the amphiploid of CS/*Th. bessarabicum* (*Ad*) and *Th. bessarabicum* (*Th*). The marker band is indicated by an *arrowhead*

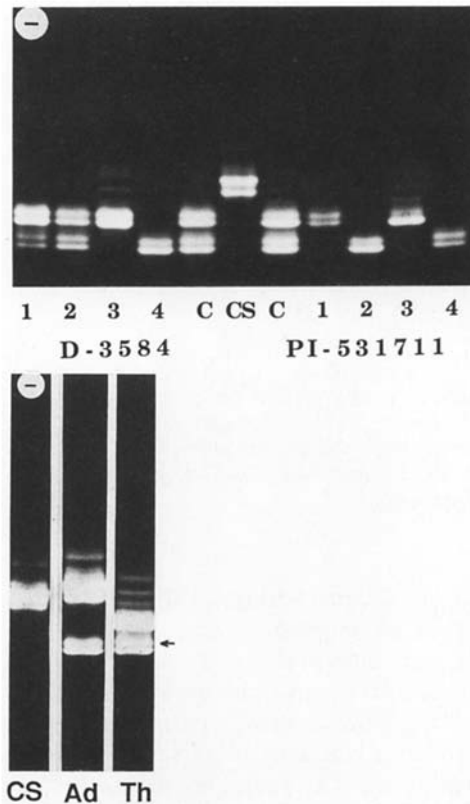

Fig. 5A, B. Native-PAGE of β-amylase. **A** β-Amylase banding pattern of individual seeds (*lanes 1–4*) within accessions D-3584 and PI 531711 together with the composites (*C*) of these *Th. bessarabicum* accessions and *T. aestivum* cv "Chinese Spring (*CS*). **B** Banding pattern of the "Chinese Spring" (*CS*), amphiploid of CS/*Th. bessarabicum* (*Ad*) and *Th. bessarabicum* (*Th*). The marker band is indicated by an *arrowhead*

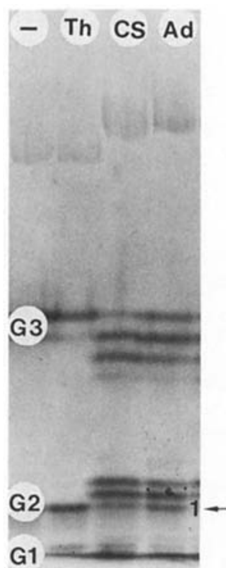

Fig. 6. Native-PAGE of leaf GOT isozymes of *Th. bessarabicum* (*Th*), *T. aestivum* cv "Chinese Spring" (*CS*) and the amphiploid of CS/*Th. bessarabicum* (*Ad*). GOT-1 to -3 zones are *numbered*. Of the 3 bands in the GOT-2 zone note the variation in intensity of band 1 (marked by an *arrowhead*) in the amphiploid and CS. *Th. bessarabicum* GOT-2 zone has band 1 only

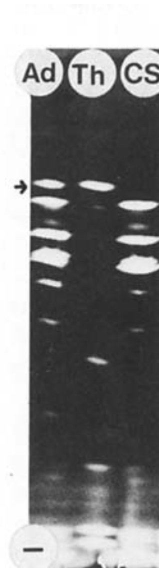

Fig. 7. IEF (pH 4.0–6.5) on polyacrylamide gels of α-amylase (α-AMY2) isozymes; banding patterns of the amphiploid of *T. aestivum* cv "Chinese Spring" CS/*Th. bessarabicum* (*Ad*), *Th. bessarabicum* (*Th*) and *T. aestivum* cv "Chinese Spring" (*CS*). The marker band is indicated by an *arrowhead*

An analysis of leaf extracts from individual seedlings of each of the four *Th. bessarabicum* accessions showed no polymorphism for GOT-2 banding patterns (data not shown). *Th. bessarabicum* expressed a single band in the GOT-2 zone that coincided with that of band 1 in the GOT-2 region in "Chinese Spring" (Fig. 6). The amphiploid showed an increase in staining intensity of band 1 in the GOT-2 zone (arrowhead marker, Fig. 6). The other two bands in the GOT-2 zone had similar intensities in "Chinese Spring" and the amphiploid. Band 1 in the GOT-2 zone with increased activity can therefore be used as a marker for the detection of the *Th. bessarabicum* chromosome homoeologous to group-6 chromosomes of wheat.

α-Amylase

α-Amylase isozymes have two activity zones when separated on IEF-polyacrylamide gels, the genes of which are located in the long arms of homoeologous group-6 (*α-Amy1*) and group-7 (*α-Amy2*) chromosomes in wheat (Nishikawa and Nobuhara 1971; Gale et al. 1983). α-AMY2 isozymes which have more acidic isoelectric points (pIs) are controlled by genes located on group-7 chromosomes in wheat (Gale et al. 1983). Individual seeds in different accessions of *Th. bessarabicum* indicated the presence of 1 strong band and 2 more of lesser activity in the α-AMY2 region (Fig. 7). There was no polymorphism for the single strong band among or within accessions (data not shown). The most acidic band of

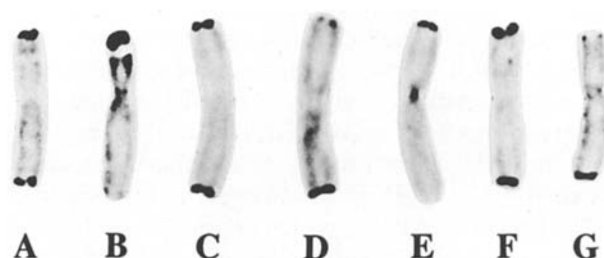

Fig. 8. A C-banded karyotype of *Th. bessarabicum* PI 531710 ($2n = 2x = 14$, JJ) with band-positive sites for each of the seven chromosomes

Th. bessarabicum was not present in "Chinese Spring" (Fig. 7). The amphiploid, in addition to having the banding profile of "Chinese Spring", clearly expressed the marker band specific to *Th. bessarabicum*. Therefore the α-AMY2 band specific to *Th. bessarabicum* can serve as a marker to detect the presence of the *Th. bessarabicum* chromosome homoeologous to group-7 chromosomes of wheat.

Cytology

The C-banded karyotypes of *Th. bessarabicum* (Fig. 8) show diagnostic band-positive sites for each of the seven chromosomes that are quite distinct from those of *T. aestivum*. The banding details obtained in our investigation (Fig. 8) conform to those published earlier (Endo and Gill 1984) with maintenance of similar alphabetical

designations. Most of the bands present were terminal and of a medium or large size. Chromosomes A (short arm satellited), C and F had terminal bands on both arms; chromosomes B (short arm satellited) and E possessed terminal banding sites on the short arms; chromosomes D and G had terminal sites on the long arms with very faint and variable terminal sites on the short arms also. There were no significant polymorphic variations observed in the seedlings of the analyzed accession (PI-531710). This banding distinctiveness is anticipated to readily and accurately assist in identifying *Th. bessarabicum* chromosomes in a wheat background.

Conclusion

The biochemical markers identified can positively assist in the detection of six of the seven *Th. bessarabicum* chromosomes homoeologous to wheat. Unique C-banding sites of some *Th. bessarabicum* chromosomes further serve as diagnostic markers. When disomic addition lines of an alien species are being made, intercrossing these addition lines characterizes the disomic addition line set distinctly. Establishing the homoeology of the added chromosome(s) is a rather cumbersome process that involves substituting alien chromosomes and demonstrating compensation for the loss of a particular wheat chromosome. Since the chromosomal locations of biochemical markers in wheat are well established, the presence of a particular biochemical marker for a given disomic addition line readily indicates homoeology of this added alien chromosome. This helps in our program aimed at inducing alien introgressions from *Th. bessarabicum*, with the objective of transferring a complex and important trait (salt tolerance) into cultivated wheat, *T. aestivum* L. (Mujeeb-Kazi and Asiedu 1990).

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