Biochemical and Molecular Markers for the Detection of *Thinopyrum Bessarabicum* Chromosomes in Its Hybrid Derivatives with *Triticum Aestivum*

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ABSTRACT

Thinopyrum bessarabicum (2n=2x=14, JJ) possesses a high level of salt tolerance and is a potential source of transferring this important and complex trait into *Triticum aestivum* L. through intergeneric hybridization. This makes it necessary to establish several diagnostic markers to enable detection of *Th. bessarabicum* chromosomes in a wheat background, As an aid to cytological markers, seven proteins/isozymes (MDH, HMW-Glutenins, SOD, EST, GOT, β-Amy and α-Amy) were identified as positive markers. These markers have been used to tentatively characterize disomic chromosome additions of *Th. bessarabicum* to different wheat homoeologous groups. Additionally, RAPD markers have also been established for some of the tentative disomic additions to strengthen diagnosis. *In-situ* genomic blocking has further enabled differential characterization of *Th. bessarabicum* chromosomes in the amphiploid of Chinese Spring/*Th. bessarabicum* (2n=8x=56, AABBDDJJ) as well as alien disomic chromosome additions. These multiple diagnostic markers should hopefully enable identification of *Th. bessarabicum* chromosome introgressions.

INTRODUCTION

Thinopyrum bessarabicum (*Agropyron junceum*; 2n=2x=14, JJ) is a self-fertilizing maritime grass, rhizomatous and noted for its high tolerance to salinity. It has been successfully hybridized to *Triticum aestivum* L. with the hybrids providing: (i) information about the phytogenetic relationships, and (ii) germplasm to be exploited for transferring the salt tolerance attribute of *Thinopyrum bessarabicum* to *Triticum aestivum*. Amphiploids were induced by treating the F_1 hybrid plants with colchicine[1,2]. Using the two parental species together with the amphiploid has thus formed the basis necessary to study biochemical characteristics and establish markers to facilitate detection of alien chromosomal introgression in *T. aestivum*. Disomic addition lines of different *Th. bessarabicum* chromosomes in a wheat background were additionally utilized.

The diagnostic biochemical/protein markers used bave been complemented by establishing PCR based RAPD[3,4] and genomic *in situ* hybridization markers[5,6] also, for detecting *Th. bessarabicum* chromosomes in a wheat background.

EXPERIMENTAL

Biochemical/protein markers

Four different accessions of *Thinopyrum bessarabicum*, PI 531710, PI 571711, 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, PGM, β-AMY, α-AMY, SOD and HMW-glutenins. Leaves from yound seedlings of individual accessions as well as a composite were analyzed for GOT. *Triticum aestivum* cv. 'Chinese Spring' was utilized together with the amphiploid of 'Chinese Spring'/*Th. bessarabicum* for the comparison of banding patterns. Disomic addition lines (2n=6x=44) of *Th. bessarabicum* chromosomes were also tested.

Details of enzyme extractions, protein separation on Isoelectric focusing (IEF), native polyacrylamide gel electrophoresis (native-PAGE), SDS-PAGE and staining for different enzyme systems have been earlier described[7].

RAPD analysis

Genomic DNA isolation was done according to the recently published procedures[3]. Arbitrary primers of 10 nucleotides were obtained from Operon Inc. (Alameda, CA). Primers (a total of 80 from kits A, L, O and N) were initially screened on *T. aestivum* cv. Chinese Spring, *Th. bessarabicum* and the amphiploid. When the polymorphisms were detected these primers were used on disomic addition lines together with the wheat parents, *Th. bessarabicum* and the amphiploid as controls.

The PCR reactions were carried out in a 25 μl reaction mixture consisting of 20 ng genomic DNA, 0.1mM each dATP, dCTP, dGTP and dTTP (Perkin-Elmer Co., CT), 0.001% Gelatin (Sigma chemicals Co.), 2.0mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl (pH 9.0), 0.1% triton x -100, 1.0 units of Taq polymerise (Promega chemicals Co. Madison, WI; USA) and 15ng of respective primers. An Ericomp thermocycler was used with the following cycles. One cycle of 2 min at 93°C, 45 cycles of 92° for 1 min, 36°C for 1 min and 72°C for 1 min. Samples were maintained at 72°C for 5 min prior to termination of the reaction. Approximately 10 μl the reactions were separated on 3.0% agarose (Nusieve 3:1) gels at 50 m Amp for 5.0 and viewed under U.V.

***In-situ* hybridization**

For chromosome preparations, seeds of the amphiploid were germinated and treated as previously described[9]. Total genomic DNA was isolated[8] from both Chinese Spring and *Th. bessarabicum*. The Chinese Spring DNA which is to be used as blocking DNA was sheared to 400-800 bp by passing it several times through a 1 ml tuberculin syringe with a 24 G needle attached. The *Th. bessarabicum* DNA was nick translated with biotinylated -11- dUTP; kit from Enzo Diagnostics, Inc. New York, NY, USA.

The in situ hybridization procedures were essentially similar to those earlier established[9,10]. The detection of the biotinylated probe was performed with the detek 1-hrp kit also from Enzo Diagnostics. The substrate for the horse radish peroxidase enzyme was changed to diaminobenzidine tetrahydrachloride (DAB) (Sigma). The detection conditions have been described previously[10].

RESULTS AND DISCUSSION

Biochemical markers

Seven isozymes/proteins were identified as positive biochemical markers. The results of PGM; initially considered a positive marker; have not been consistently reproducible and therefore not used in characterization of addition lines. The blue aleurone marker however, is a diagnostic index. The other biochemical markers tentatively identified[11] for characterization of the *Th. bessarabicum* disomic chromosome additions are listed in Table 1.

Table 1. Biochemical markers and their homoeologous group location for disomic chromosome additions (2n=6x=44) of *Thinopyrum bessarabicum* in a wheat (*Triticum aestivum*) background.

Biochemical Marker	Homoeologous Group location
MDH	Group 1
HMW-Glutenin	Group 1
SOD	Group 2
Seed EST	Group 3
PGM	Group 4
β-AMY	Group 4/5
GOT-2	Group 6
α-AMY-2	Group 7

RAPD markers

Genomic DNA can be amplified and polymorphisms detected by single primers of arbitrary nucleotide sequences using PCR[12,13]. Polymorphisms among bread wheat cultivars were observed[14] using pairwise arbitrary defamers. We have been able to observe adequate polymorphisms between bread wheat and *Th. bessarabicum* using arbitrary single defamers. This is not unexpected since the chances of detecting polymorphisms among the amplified products is greater when alien species are involved. The possible utility of RAPD markers in the production of alien chromosome addition and substitution lines as well as detecting introgressions in wheat has been suggested[15]. Polymorphisms in wheat using PCR with primer sequences derived from the sequence of a gamma gliadin gene were observed[16]. Primers that target α-amylase genes and intron/exon splice junctions to detect polymorphisms in barley and identify chromosomal locations for the amplified fragments were used in analyses of wheat/barley addition lines[17].

Our results also indicate that genomic DNA in a complex genome such as that of wheat, can be amplified using arbitrary primers of 10 nucleotides in length. A total of 80 primers were evaluated in the study using the *T. aestivum* cv. 'Chinese Spring', *Th.bessarabicum*, and the amphiploid between the two species. When polymorphisms were detected between two parents and the amphiploid showed at least some, these primers were screened on the disomic addition lines.

Since PCR is a novel technique compared to the other conventional markers (morphological and protein/isozyme) in identifying alien genetic material, it may not be possible as yet to locate marker band/s to specific chromosomes or arms unless the material used has previously been characterized for homoeologous relationships by conventional techniques. However, if a given addition or substitution line is identified using conventional techniques, RAPD markers can further strengthen addition line diagnostics. Although at this stage the nature of the amplified sequences is inconclusive, it is more likely that repetitive sequences are amplified when defamers are used as primers[15].

The disomic addition lines analyzed are in agronomically superior plant type, possessing high fertility and cytologically stable with a high frequency of 22 bivalents at meiosis[11]. The amplification patterns of the disomic addition lines together with the parents and the amphiploid with primers 0-5, 0-6 and N-17 indicate that these primers give rise to amplification products that are specific to some addition lines. Primer N-17 had an amplification product (760bp) which was also present in the alien disomic addition line with group 1 homoeology to wheat. Similarly, primer 0-6 had two amplification products that were present in the disomic addition line with group 2 (590bp) and group 6 (500bp) homoeology. Primer 0-5 had an amplification product of 470 bp that was present in the group 5 disomic addition line.

***In situ* hybridization**

Upon genomic *in situ* hybridization of the Chinese Spring/*Th. bessarabicum* amphiploid, fourteen of the fifty-six chromosomes were observed to be brown in colour. The remaining forty two chromosomes were all light blue in appearance. The unlabelled Chinese Spring effectively kept the labelled *Th. bessarabicum* DNA from hybridizing to the forty two wheat chromosomes. When Chinese Spring DNA was not used as a blocking DNA, all fifty-six of the amphiploid chromosomes appeared brown.

The results presented above indicate that enough homoeology exists between *Th. bessarabicum* and wheat that unless wheat DNA is used as a blocking DNA, *Th. bessarabicum* and wheat DNA duplexes are formed during DNA-DNA hybridization conditions. Another indication of this residual homoeology is the stringency of the post hybridization washing. When the 37°C temperature earlier used[10] was adopted in the present study, the forty two wheat chromosomes did not appear blue. Their appearance was slightly brown. Differentiation between the light brown wheat and dark brown *Th. bessarabicum* chromosomes was still possible but not optimal. Upon raising the washing temperature to 45°C, the forty-two wheat chromosomes appeared blue with very little if any brown colour. These results indicated that there was enough homoeology between wheat and *Th. bessarabicum* and that more stringent washing conditions were necessary. The conditions used in this study were more stringent (42°C in 50%

formamide) than those used earlier[18]. We also used twenty to thirty times less blocking DNA than used previously[18]. This difference in blocking DNA concentration could be a factor in obtaining cross hybridization between alien and wheat DNA. Obtaining optimum differentiation between wheat and alien chromosomes with genomic *in situ* hybridization may be a function of a proper balance between blocking DNA amount and stringency of post hybridization washing conditions.

The biochemical/protein markers identified can positively assist in the detection of *Th. bessarabicum* chromosomes homoeologous to wheat. Once the homoeology of the added chromosome is established, additional markers such as RAPDs will be useful in providing more diagnostic markers. Although the nature of the amplified fragment in RAPDs is not conclusive at this stage, RAPD markers may be useful in detecting introgressions involving alien chromosomes. Genomic *in-situ* hybridization is a powerful and rapid technique for the detection of *Th. bessarabicum* chromosomes in wheat background. Subtle alien introgressions could be detected in wheat background using genomic *in-situ* hybridization.

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