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Characterization of *Thinopyrum bessarabicum* chromosome segments in wheat using random amplified polymorphic DNAs (RAPDs) and genomic in situ hybridization

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Abstract. Ten random amplified polymorphic DNA (RAPD) markers specific to chromosome $5E^b$ of *Thinopyrum bessarabicum* were detected. Genomic in situ hybridization and standard cytological observations revealed that six of the markers are located on the $5E^b$ short arm and four are located on the $5E^b$ long arm. These RAPD markers have been used to confirm the identity of putative $5E^b$ (5A) and $5E^b$ (5D) substitution individuals. The potential of RAPDs for the detection of wheat/alien recombinants is discussed.

Key words: Random amplified polymorphic DNA – Genomic in situ hybridization – *Wheat* – *Thinopyrum bessarabicum* – Isozymes

Introduction

The introgression of genetic variation into wheat (*Triticum aestivum*) from non-homologous genomes often entails the production first of wheat/alien addition or substitution lines. Such lines are generally unsuitable for agronomic purposes, as many unwanted genes are introduced along with those giving rise to the trait of interest. Homoeologous recombination techniques can be used to reduce the size of the alien segment including the target gene(s) incorporated into the wheat chromosome. The major problem is the selection of such recombinants, which occur only at low frequency. Selection would be greatly enhanced by the availability of genetic markers spaced along the alien chromosome. It has recently been proposed that random amplified polymorphic DNAs (RAPDs) can be exploited as a source of genetic markers (Williams et al. 1990). RAPDs, which are attractive since no prior knowledge of target DNA sequence is required and commercially produced random primers can be used, may be a valuable source of markers for alien chromatin and hence have an application in wheat/alien introduction (Devos and Gale 1992). Genetic markers can be complemented by genomic in situ hybridization (GISH), which allows the direct visualization of alien chromatin (Schwarzacher et al. 1992).

The work described in this paper evaluates the use of RAPDs, complemented by genomic in situ hybridization and isozymes, as a source of markers for characterizing lines of wheat carrying all or part of chromosome $5E^b$ from *Thinopyrum bessarabicum*. This chromosome is thought to carry much of the genetic information that allows. *Th. bessarabicum* to tolerate high levels of soil salinity (Forster et al. 1988) and is therefore of interest in an ongoing programme to improve the salt tolerance of wheat.

Materials and methods

Materials

The following genotypes were used: the hexaploid wheat variety 'Chinese Spring' (CS), CS aneuploids monosomic for chromosome 5A (CS5A), 5B (CS5B) or 5D (CS5D) (Sears 1954), CS double ditelosomic for either chromosomes 5A (CSDDT5A) or 5D (CSDDT5D) (Sears and Sears 1978), lines of CS carrying an added pair of $2E^b$, $5E^b$ (Forster et al. 1988) and $5E^bS$ ($5E^b$ short arm telosome) chromosomes and a CS line disomic for a $5AS/5E^bL$ translocation. The $5E^bS$ telosome and the $5AS/5E^bL$ (terminology of Koebner and Miller 1986) – translocated chro-

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mosome were isolated in the progeny of a plant doubly monosomic for chromosome 5A and $5E^b$, and their identity was verified by cytological analysis of relevant test-crosses.

Attempts to substitute chromosome $5E^b$ for each of its wheat group 5 homoeologues were made by pollinating CS5A, CS5B and CS5D with the $5E^b$ addition line. Plants with 42 chromosomes were selected from the resulting F_1 progeny. Monosomic (2n = 41) and disomic substitution (2n = 42) were selected in the F_2 and F_3 generations, respectively, with the aid of biochemical, molecular and morphological markers, as described below.

Isozyme analysis

Isoelectric focussing (IEF) separations of β-amylases and iodine-binding factors were carried out following published procedures (Sharp et al. 1988a; Liu and Gale 1989, respectively) with the exception that the gels used contained a 1:1:1 mix of the ampholytes 3–5 (Isolabs), 4.2–4.9 and 4.5–5.4 (Pharmacia).

RAPD analysis

The polymerase chain reaction (PCR) was performed as follows: genomic DNA was extracted from either leaves, as described by Sharp et al. (1988b), or from endosperm. In the latter case the endosperm half (brush end) of a single seed was weighed and crushed, and the resulting material incubated in 500 µl CTAB buffer in a 1.5-ml eppendorf tube with occasional vortexing for 2 h. Chloroform (200 µl) was added, and the tube was vortexed and then spun at 13 k rpm for 5 min to separate the phases. The top (aqueous) phase was decanted to a fresh tube, 500 µl of isopropanol was added and the DNA was left to precipitate at −20 °C for at least 1 h, after which the tube was briefly spun to pellet the precipitate. The liquid was aspirated from the pellet, which was washed in 70% ethanol and dissolved in 1 × TE (2 µl/mg endosperm) containing 20 ng/µl RNase. PCR amplification reactions were performed in volumes of 50 µl containing 25 ng of template DNA. The reaction buffer consisted of 100 µ*M* each of dATP, dCTP, dGTP and dTTP, 200 n*M* oligonucleotide primer (Operon Technologies) and 0.8 units of *Taq* polymerase (Boehringer Mannheim), in 10 m*M* Tris HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 100 µg/ml gelatine, 0.05% v/v Tween 20 and 0.05% v/v Nonidet P-40. The reaction mixture was overlaid with mineral oil prior to amplification in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 45 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C using the fastest possible transitions between temperatures, with a final extension of 72 °C for 5 min followed by cooling to 4 °C until recovery of the samples. Aliquots (15 µl) of the amplification products were electrophoresed through 1.5% agarose gels and detected by staining with ethidium bromide. A total of 120 primers from Operon kits A, C, D, W, X, Y, Z were tested.

Primers which gave two distinguishable RAPD profiles in a comparison between CS and $5E^b$ leaf DNA were then used to amplify template from $5E^bS$, $5AS/5E^bL$ and $2E^b$.

Genomic in situ hybridization

In situ hybridization using total *Th. bessarabicum* DNA, or the probe pTa71, (9-kb *Eco*RI fragment of wheat ribosomal DNA, Gerlach and Bedbrook 1979) was performed as described by Schwarzacher et al. (1992) with the exception that root tips were harvested when they were approximately 1 cm in length and then placed in water at 4 °C for 22 h prior to fixation. Slides were analysed by epifluorescence microscopy using a Nikon Microphot-SA microscope.

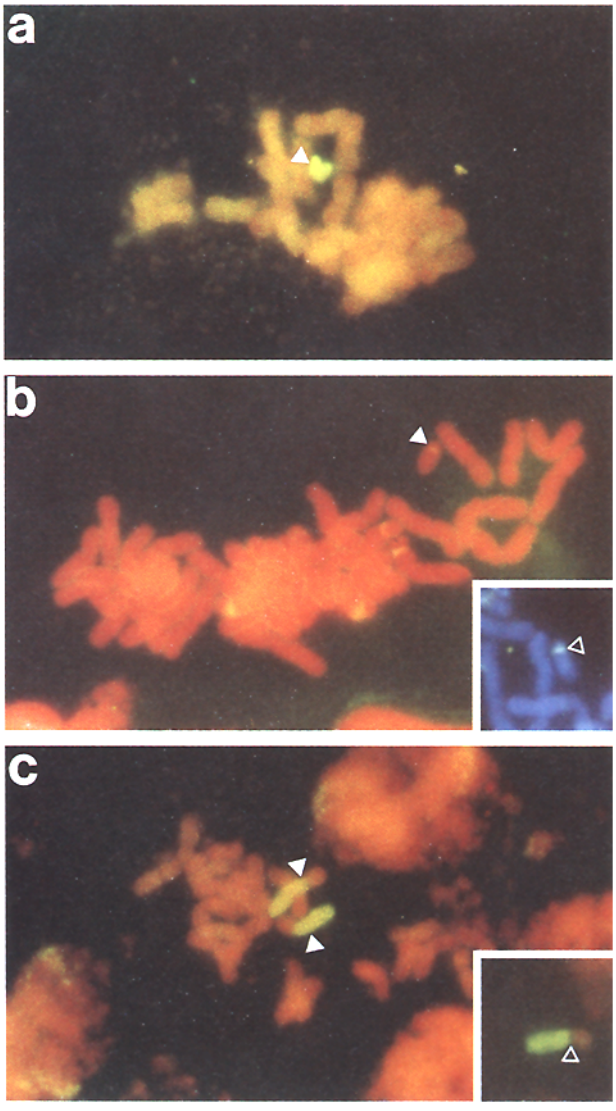

Fig. 1. **a** In situ hybridization, using total genomic *Th. bessarabicum* DNA, labelled with Digoxigenin, as probe (counter stained with propidium iodide), showing the $5E^bS$ telosome (*arrow*). **b** In situ hybridization, using pTA71, labelled with Digoxigenin, as probe (counter stained with propidium iodide), showing a hybridization site on $5E^bS$ (*arrow*). The hybridization site is more clearly visible in the *insert* (*bottom right-hand corner*), a double exposure firstly for FITC and then for DAPI, in the absence of counter stain. **c** In situ hybridization, using total genomic *Th. bessarabicum* DNA, labelled with Digoxigenin, as probe (counter stained with propidium iodide), showing a pair of $5AS.5E^bL$-translocated chromosomes (*arrows*). From the chromosome in the *inset* it can be seen that the translocation is Robertsonian

Results

Characterization of lines carrying $5E^bS$ and $5AS/5E^bL$

GISH, which has previously been used to detect the complete $5E^b$ chromosome in a wheat background

(Schwarzacher et al. 1992), allowed the direct visualization of the $5E^bS$ telosome (Fig. 1a). Further in situ hybridizations revealed that the telosome carries a pTa71 hybridization site (Fig. 1b). GISH of lines disomic for $5AS/5E^bL$ revealed that the translocation was the result of a centric fusion (Fig. 1c), and the stock can thus be referred to as $5AS.5E^bL$, following the terminology of Koebner and Miller (1986). Plants disomic for the $5AS.5E^bL$ translocation are morphologically distinct from CS since the ears of the former are speltoid as a result of the absence of the *Q* gene, which is located on the long arm of chromosome 5A (Mac Key 1954).

Lines carrying the complete $5E^b$ chromosome were found to express a novel β-amylase isozyme encoded by the gene designated here as *β-Amy-E^b1* (Fig. 2). The observation that *β-Amy-E^b1* was absent in individuals carrying $5E^bS$ but present in those carrying the $5AS.5E^bL$ translocation demonstrates that the gene encoding this isozyme is located on $5E^bL$.

RAPD analysis

Most of the 120 10-mer primers tested produced amplification profiles consisting of between two and ten products. Some, however, completely failed to prime, while others produced in excess of ten products. Ten primers (A05, C02, C09, D12, W03, W20, X11, Y11, Z11 and Z13) amplified one distinct product (the W20 product co-migrated with a faint CS product) (Fig. 3), while another (Y10) amplified two distinct products from the $5E^b$ template as compared to the CS template. Of these primers 6 (A05, D12, W03, W20, Y11 and Z11) (Fig. 3) amplified the non-wheat product from the $5E^bS$ but not from the $5AS.5E^bL$ template, while 4 of them (C09, X11, Y10 and Z13) only amplified the non-wheat product from the $5AS.5E^bL$ template (Fig. 3). The extra product amplified by primer C02 was present in the profiles of both $CS5E^bS$ and $5AS.5E^bL$ (Fig. 3). Of these 11 primers 10 gave identical profiles with CS and $2E^b$ templates. Primer C02, however, displayed the same non-wheat product from the $2E^b$ template as

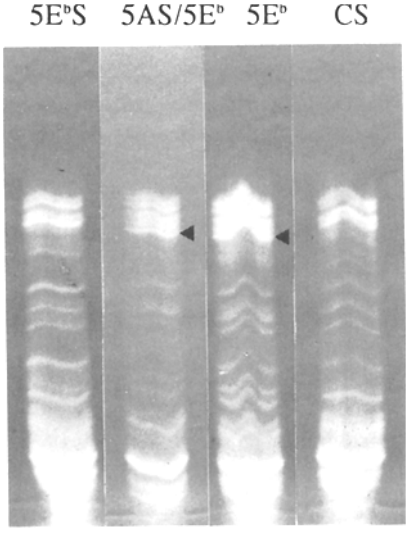

Fig. 2. β-Amylase phenotypes of CS, $5E^b$, $5AS.5E^bL$ and $5E^bS$. The extra band present in $5E^b$ and $5AS.5E^bL$ is *arrowed*

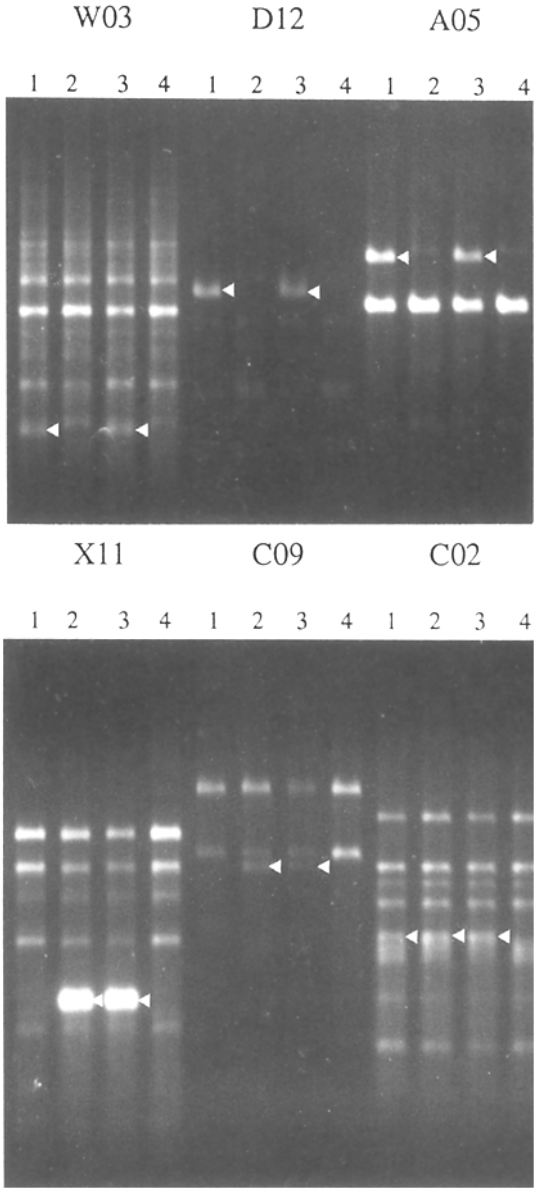

Fig. 3. PCR profiles produced by three primers (*W03*, *D12* and *A05*) that amplify products from lines carrying $5E^bS$, two primers (*X11* and *C09*) that amplify products from lines carrying $5E^bL$ and one primer (*C02*) that amplifies a product from lines carrying both $5E^bS$ and $5E^bL$ (*1* $5E^bS$, *2* $5AS.5E^bL$, *3* $5E^b$, *4* CS). The specific bands are *arrowed*

observed from the $5E^b$, $5E^bS$, and $5AS.5E^bL$ templates (Fig. 4).

PCR profiles obtained from template DNA from leaf and seed were compared for 1 primer (X11) for the four lines, CS, $5E^b$, $5E^bS$, and $5AS.E^bL$. Typically, an extract from approximately 10 mg of endosperm yielded 5 µg of high-molecular-weight DNA, sufficient for about 200 PCR reactions. The patterns obtained from leaf- and seed-derived template for this primer were indistinguishable for any given genotype (data not shown).

Production of substitution lines

Thirty-eight CS5A × $5E^b$, 30 CS5B × $5E^b$ and 27 CS5D × $5E^b$ 41-chromosome F_2 plants were isolated. Putative monosomic substitutions were selected from these plants on the basis of the presence of *β-Amy-E*^{b}1, located on $5E^bL$, and the absence of either *Ibf-A1*, *Ibf-D1*, or *Ibf-D1*, located on the long arms of chromosome 5A, 5B or 5D, respectively (Liu and Gale 1989). One such CS5D × $5E^b$ F_2 plant and 9 such CS5A × $5E^b$ F_2 plants were selected, but all of the 2n = 41 CS5B × $5E^b$ F_2 progeny possessed *Ibf-B1* and were therefore not studied further. The 10 selections were allowed to self-fertilize and 42-chromosome F_3 plants were selected. DNA from one 42-chromosome plant from each of the 9 putative $5E^b$(5A) F_3 progenies and the single $5E^b$(5D) F_3 progeny were screened using primers D12 and X11, which amplify sequences on $5E^bS$ and $5E^bL$, respectively. Each of the ten 42-chromosome individuals were found to carry both RAPD markers (Fig. 5a, b). The 9 putative disomic $5E^b$(5A) plants and the putative disomic $5E^b$(5D) plant were test-crossed with CSDDT5A and CSDDT5D, respectively. In each case the hybrids formed 20 bivalents, two telocentric univalents and one bibrachial univalent at metaphase I of meiosis, confirming that these plants were disomic substitutions.

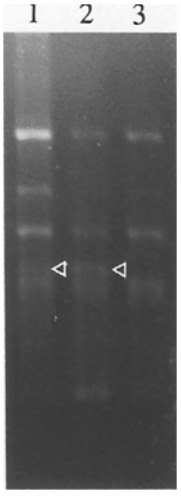

Fig. 4. PCR profiles produced by primer C02 of: *1* $2E^b$, *2* $5E^b$, *3* CS. The extra band produced from $2E^b$ and $5E^b$ is *arrowed*

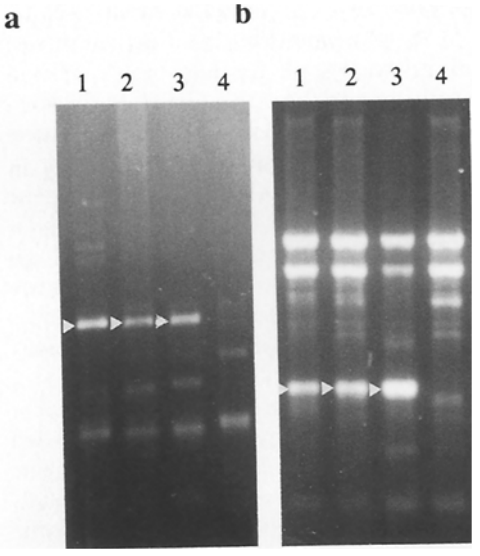

Fig. 5. PCR profiles of CS, $5E^b$, and two lines, one a putative $5E^b$(5D) substitution and the other a putative $5E^b$(5A) substitution, analysed with **a** $5E^bS$ marker, primer D12 (*1* $5E^b$(5A), *2* $5E^b$(5D), *3* $5E^b$, *4* CS) and **b** $5E^bL$ marker, primer X11 (*1* $5E^b$(5A), *2* $5E^b$(5D), *3* $5E^b$, *4* CS). The $5E^b$-specific bands are *arrowed*

Discussion

The marker systems presently available for identifying wheat/alien recombinants (e.g. RFLPs and isozymes) all have a number of drawbacks, such as speed of assessment or tissue specificity of the marker. There is, therefore, considerable interest in new marker systems such as RAPDs, which rely on PCR procedures. RAPDs have recently been exploited as a source of markers for the identification of alien chromosomes in *Brassica napus* (Struss et al. 1992) and as tags for *Pseudomonas* resistance in tomato (Martin et al. 1991). RAPD markers can be generated rapidly and easily using commercially produced random oligonucleotide primers, and the assay can be performed quickly. In contrast, RFLP technology is a time-consuming procedure requiring the isolation of tens of micrograms of DNA, the production of DNA probes, Southern blotting and hybridization. The main advantage of RFLPs is that there is prior knowledge of their location relative to each other from genetic maps. Thus the chance of identifying recombinants can be increased by screening for recombinants using dispersed markers, providing that there is gene synteny between the alien

chromosome and its wheat homoeologues. In contrast the location of RAPD markers is not known. Thus, considerably more RAPDs relative to RFLPs will have to be used during screening to increase the chance of using dispersed markers.

The main disadvantages of RAPDs lie firstly in their sensitivity to reaction conditions, which are not always easy to control, and secondly that, although RAPDs can be used to identify the presence or absence of alien chromatin, they give no information regarding the presence or absence of homoeologous wheat chromatin. In the investigation reported here RAPDs were used to confirm the presence of both arms of chromosome $5E^b$ in the putative substitution lines. However, cytological analyses were required to confirm the absence of either chromosome 5A or 5D. Similarly, although RAPDs provide a quick method of screening for recombinants, characterization of these will best be achieved by using other marker systems, such as RFLPs and cytological analyses combined with GISH.

The results described in this paper demonstrate that RAPD markers are a powerful tool for the identification of wheat/alien additions, substitutions and recombinants, particularly given the possibility of a rapid extraction of template DNA from dormant seed, since selections can be made without the need for plants to be grown. In under 2 weeks ten markers specific to either $5E^bS$ or $5E^bL$ were obtained. With the exception of C02 none of the primers amplified sequences on both arms of $5E^b$ nor did they amplify sequences on $2E^b$, demonstrating that they are not present on both chromosomes.

Several of the RAPD markers specific to chromosome $5E^b$ identified in this work are relatively weakly amplified by the 10-mer primers. Therefore, a further development of the RAPD assay will be to clone the alien amplification products and end-sequence them to derive 20- to 35-mer primers that would be expected to improve the robustness of the assay. Such markers could be used in conjuction with 20- to 35-mer primers derived from RFLP probes (Tragoorung et al. 1992). Ultimately, electrophoretic separations may be unnecessary as successful amplification of the locus being assayed could be detected spectrophotometrically.

In this work GISH made it possible to confirm in advance, firstly, the identity of the $5E^b$ short arm telosome, and, secondly that $5AS.5E^bL$ was the result of a Robertsonian translocation. However, GISH is a time-consuming procedure and will, therefore, be of limited use for the identification of wheat/alien recombinants that occur at low frequency. In practise it will be more practical to first isolate recombinants using genetic markers such as RAPDs, and then to use GISH to determine the physical size of the alien segments introduced. Furthermore, if a range of recombinants containing different amounts of alien chromatin are isolated it should be possible, by relating the presence or absence of genetic and morphological markers to the size of the alien segment, to create a physical gene map of the wheat and alien chromosomes in question.

Chromosome $5E^b$ is of particular interest as it carries a gene(s) responsible for conferring salt tolerance to wheat (Forster et al. 1988). At present, attempts are being made to induce homoeologous recombination between chromosome $5E^b$ and its wheat homoeologues in an attempt to transfer the gene(s) for salt tolerance to wheat. It is envisaged that the identification and characterization of recombinants will be facilitated using the RAPD markers, *β-Amy-E^b1*, the pTa71 hybridization site identified in this work, and GISH. The reduction in the size of the alien chromatin in recombinants which contain the gene responsible for salt tolerance may, however, still be insufficient to exclude all of the agronomically deleterious genes. If this is the case the size of the alien segment will have to be reduced further. This could be achieved by intercrossing individuals homozygous for an exchange proximal and an exchange distal to the gene for salt tolerance such that recombination between homologous segments of alien chromatin will give rise to a recombinant chromosome containing a reduced alien segment (Sears 1981). RAPDs provide one way in which chromosomes containing overlapping alien segments and individuals carrying a very small segment of alien chromatin could be identified quickly and easily.

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