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ORIGINAL PAPER

Trigenomic chromosomes by recombination of *Thinopyrum intermedium* and *Th. ponticum* translocations in wheat

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Abstract Rusts and barley yellow dwarf virus (BYDV) are among the main diseases affecting wheat production world wide for which wild relatives have been the source of a number of translocations carrying resistance genes. Nevertheless, along with desirable traits, alien translocations often carry deleterious genes. We have generated recombinants in a bread wheat background between two alien translocations: TC5, ex-*Thinopyrum* (*Th*) *intermedium*, carrying BYDV resistance gene *Bdv2*; and T4m, ex-*Th. ponticum*, carrying rust resistance genes *Lr19* and *Sr25*. Because both these translocations are on the wheat chromosome arm 7DL, homoeologous recombination was attempted in the double hemizygote (TC5/T4m) in a background homozygous for the *ph1b* mutation. The identification of recombinants was facilitated by the use of newly developed molecular markers for each of the alien genomes represented in the two translocations and by studying derived F_2, F_3 and doubled haploid populations. The occurrence of recombination was confirmed with molecular markers and bioassays on families of testcrosses between putative recombinants and bread wheat, and in F_2 populations derived from the testcrosses. As a consequence it has been possible to derive a genetic map of markers and resistance genes on these previously fixed alien linkage blocks. We have obtained fertile progeny carrying new tri-genomic recombinant chromosomes. Furthermore we have demonstrated that some of the recombinants carried resistance genes *Lr19* and *Bdv2* yet lacked the self-elimination trait associated with shortened T4 segments. We have also shown that the recombinant translocations are fixed and stable once removed from the influence of the *ph1b*. The molecular markers developed in this study will facilitate selection of individuals carrying recombinant *Th. intermedium–Th. ponticum* translocations (Pontin series) in breeding programs.

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Introduction

Translocations involving chromosomes of wild relatives of wheat have provided diverse sources of resistance to rusts and barley yellow dwarf virus (BYDV) diseases of wheat. Unfortunately alien translocations have been reported to carry several undesirable traits. A translocation from *Thinopyrum* (*Th*) *ponticum* (Podp.) Barkworth and Dewey ($2n = 10x = 70$) [syn *Agropyron elongatum* (Host) Beauvois and syn. *Lophopyrum ponticum* (Podp.) Love] onto the wheat chromosome arm 7DL carries rust resistance genes *Lr19* and *Sr25* (Sharma and Knott 1966; McIntosh et al. 1995). A translocation from *Th. intermedium* (Host) Barkworth and Dewey ($2n = 6x = 42$) [syn. *Agropyron intermedium* (Host) Beauvois and syn. *Agropyron glaucum*

Roem.et Schult.] also onto 7DL carries the BYDV resistance gene *Bdv2* (Banks et al. 1995). In addition, both translocations are reported to carry putative yield enhancement genes (Singh et al. 1998; Monneveux et al. 2003). On the negative side the *Lr19* and, to a lesser extent, *Bdv2* translocations carry undesirable genes for yellow flour color, an important quality defect. The *Th. ponticum* derived translocation carries gene(s) causing segregation distortion (Zhang and Dvorak 1990; Prins et al. 1997; Sibikeeva et al. 2004). Such deleterious genes could affect the use of the alien translocations in breeding programs.

Rust diseases are a very dynamic group of plant pathogens whose genetic flexibility has caused breeders to respond with a constant search for new resistance sources. *Lr19* is effective against most of the *Puccinia triticina* (leaf rust) pathotypes present in the South Pacific. Although virulence for *Lr19* has been reported in Mexico, India and Ukraine (Huerta-Espino and Singh 1994; Bhardwaj et al. 2005; Elyasi-Gomari and Panteleev 2006), it still provides effective protection against leaf rust in many parts of the world (McCallum and Seto-Goh 2006). The translocation carrying *Lr19* has been the subject of a number of studies. A physical map was initially constructed in 'Indis' using deletion mutants produced by gamma radiation (Marais 1991; Prins et al. 1996). Numerous efforts have been made to shorten the size of the fragment (Prins et al. 1997; Groenewald et al. 2005) and lines with smaller fragments and less yellow pigment have been identified (Knott 1980). Some mutant genotypes containing less yellow pigment lack the stem rust resistance gene *Sr25* (Knott 1980; Marais 1992). In other modifications the translocation was moved from 7DL to 7BL; this modification is also shorter but has not been deployed because of a strong tendency for self-elimination (Prins et al. 1997; Prins and Marais 1998). In attempts to further reduce the size of the translocation and make it more stable, homoeologous recombination was induced in the absence of the homoeologous pairing suppressor gene, *Ph1*. As a result, secondary smaller translocations were obtained; two of them had overlapping and non-overlapping regions. These two lines were subjected to homologous recombination and the progeny screened with the available mapped markers. A tertiary smaller recombinant translocation was identified (Groenewald et al. 2005), but it has not been used in wheat breeding because it still retains the tendency to self-eliminate during segregation in heterozygotes (Groenewald et al. 2005). The disruption of the integrity of the original translocation, that comprised almost the whole chromosome 7DL arm, seems to have caused imbalance that was not complemented by the other wheat genomes (Prins et al. 1997; Prins and Marais 1998; Marais et al. 2001; Groenewald et al. 2005).

BYDV is an endemic disease distributed worldwide, caused by a complex of viruses in the Luteovirus family, including those causing barley yellow dwarf and cereal yellow dwarf diseases. These viruses are transmitted by aphids and thrive wherever the vector is present. The importance of the disease varies depending on the strain of the virus, suitable conditions for the survival and spread of the vector, and on the germplasm being grown. BYDV is a disease for which there is no resistance known in the wheat gene pool. Previously a gene conferring some degree of tolerance, based on field observations, was described and named *Bdv1* (Singh et al. 1993). Alien introgressions in wheat are the sole sources of resistance to the virus (Banks et al. 1995; Sharma et al. 1995). *Bdv2* was the first gene for BYDV resistance available for wheat breeding and a number of translocations carrying *Bdv2* from *Th. intermedium* onto the wheat chromosome arm 7DL have been developed (Banks et al. 1995; Xin et al. 2001). Of these translocations, the most widely used in wheat breeding are TC5, TC6 and the one carrying the smallest fragment, TC14. Several molecular markers to detect these translocations are available and have been used for marker-assisted selection (Ayala et al. 2001; Stoutjesdijk et al. 2001). Named cultivars released with *Bdv2* include the winter wheat Mackellar (with TC14) and the spring wheat cultivar Glover (with TC6). Despite the successful deployment of *Bdv2* in commercial cultivars, it remains desirable to reduce the size of the translocation to delete undesirable genes and diminish the amount of alien chromatin displacing wheat genes. A second translocation, P98134, carrying BYDV resistance was also derived from *Th. intermedium* on the wheat chromosome 7DL (Crasta et al. 2000; Ohm et al. 2005). This translocation was derived from a different source than the TC lines, but no commercial cultivars have been released so far.

It has been demonstrated that it is possible to reduce the size of alien translocations by inducing recombination between the translocation and the corresponding unaltered wheat chromosome (e.g., Marais 1992; Lukaszewski 2000). This paper demonstrates that it is possible to induce recombination between two different translocations and thereby eliminate unwanted genes and accumulate beneficial genes into new recombinant translocations. The re-engineered chromosomes involve three genomes from three species, and may be referred to as trigenomic chromosomes. To achieve this we have brought together the *ponticum* and *intermedium* translocations, both on 7DL, in a homozygous *ph1b* mutant background. The *ph1b* mutation enhances the likelihood of pairing and cross-over between homoeologous chromosomes. We have employed existing and newly developed molecular markers together with bioassays for the *Lr19* and *Bdv2* mediated resistances to confirm the identity of recombinant trigenomic chromosomes. The information generated during this study enabled mapping of genes and markers on the two progenitor translocations.

Materials and methods

Plant materials

For the development of molecular markers the following lines were used: (1) Wheat lines ($2n = 42$; genome AABBDD): 'Chinese Spring', 'Hartog', 'Spear' and 'Cook'; (2) three translocation lines carrying different sized segments of a group seven chromosome from *Th. intermedium* ($2n = 42$; genome JJJ^{s}J^{s}SS) onto the chromosome arm 7DL: TC5 and TC14 in 'Hartog' background (Banks et al. 1995), and YW642 (Xin et al. 2001), all derived from the same source and containing the BYDV resistance gene *Bdv2*; and (3) a wheat line Cook*3/*Lr19* containing a translocation (T4) from *Th. ponticum* ($2n = 70$; genome JJJJJJJ^{s}J^{s}J^{s}J^s) onto the chromosome arm 7DL carrying *Lr19* for resistance to leaf rust, *Sr25* for resistance to stem rust, and *Y* for yellow flour (Sharma and Knott 1966). To verify and/or locate the chromosome position of PCR markers we tested the set of group 7 nullitetrasomic lines: N7AT7D, N7BT7A, N7DT7B (Endo and Gill 1996). All the primers amplifying on chromosome 7D were also tested on the 'Chinese Spring' chromosome arm 7DL deletion stocks obtained from the Wheat Genetics Resource Center, Kansas State University, USA.

The source of *Lr19* used in the recombining population was *Sr2* Batavia*3/C80.1 (Agatha mutant) (source Australian Cereal Rust Control Program, PBI, Cobbitty). This stock was referred as 'Batavia'-19-1-1 and was derived from an EMS mutant of the T4 translocation selected for low yellow pigment in the flour. This modified T4 translocation, called here T4m, did carry the stem rust resistance gene *Sr25* and was indistinguishable in size from T4 with the suite of molecular markers used in this paper.

In order to induce homoeologous pairing the translocations were crossed into stocks with the *ph1b* mutation, a deletion on chromosome 5B. Cultivar 'Angas' with the *ph1b* mutation was obtained from Dr. Ian Dundas, University of Adelaide. The source of the translocations was as follows: cv. 'Batavia' with T4m (*Lr19*); cv. 'Hartog' with TC5 (*Bdv2*). Individuals with both translocations, and homozygous for *ph1b*, were produced following a series of crosses and screening as outlined in Fig. 1. After each round of crosses, DNA was extracted from half seed of the progeny and screened with STSLr19$_{130}$ (Prins et al. 2001) for T4m, BYAgi (Stoutjesdijk et al. 2001) for TC5 and ABC$_{920}$ (Wang et al. 2002) for *ph1b*. The embryo halves of appropriate genotypes were germinated for the next round of crosses. After several intercrosses the target genotypes, containing both translocations and homozygous for *ph1b*, were recovered. These plants were selfed to achieve the first opportunity for recombination under the influence of the *ph1b* genotype. Target individuals were re-selected and

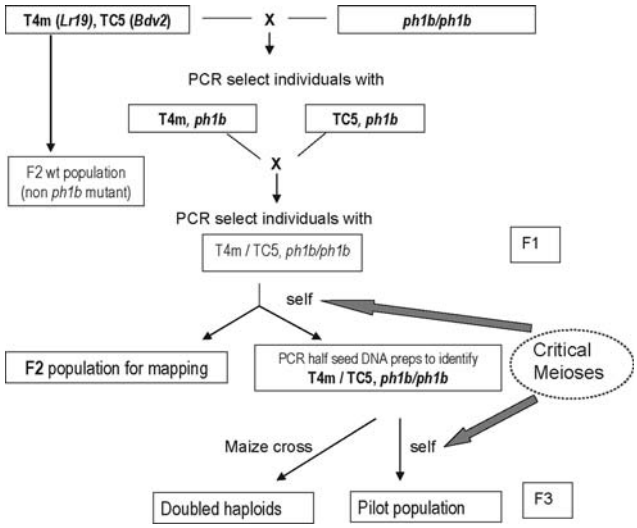

Fig. 1 Procedure to induce recombination between two translocation fragments located at the telomeric region of chromosome 7DL of wheat. The translocations were derived from *Th. intermedium* and *Th. ponticum* and carrying the genes *Bdv2* for BYDV resistance and *Lr19* for leaf rust resistance, respectively

a doubled haploid population was produced using the maize–wheat cross method by SARDI, Adelaide, Australia. Genotyped F_2 half seeds were supplied for growing and crossing with maize pollen.

It should be noted that for both the pilot population and the doubled haploid population (see below) there were two "critical meioses" (Fig. 1), meaning meioses where both translocations could be present under the influence of *ph1b* in homozygosity. Figure 1 outlines the crosses and development of populations.

In the bioassays the wheat varieties 'Cook', 'Hartog', 'Morocco', 'Egret' and 'Trident' were used as leaf rust susceptible controls whereas Agatha was used as the positive control. 'Hartog' and 'Hartog' TC5+ were used as the susceptible and the resistant controls, respectively, for BYDV bioassays.

Molecular markers

Two different ways to find polymorphic markers between the two *Thinopyrum* species and wheat were tested in this study.

EST derived markers

The lithography pictures of wheat ESTs mapping on the chromosome arm 7DL were examined at the NSF Wheat EST Genomics Project's web site http://wheat.pw.usda.gov/ggpages/NSF_Wheat_Resources.html. A visual search was done for ESTs whose hybridization pattern was clearly polymorphic for the chromosome arm 7DL. Sequences of

the chosen ESTs were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/) and PCR primers for each sequence were designed within the EST boundaries, using the program *Primer 3* (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

The primer pairs were tested on: nullitetrasomic lines for group 7; wheat lines with and without *Th. intermedium* and *Th. ponticum* translocations. Four microliters of 10 ng/µl genomic DNA was amplified using the HotStartTaq® DNA polymerase and Master Mix buffer from QIAGEN following the manufacturer's guidelines. Amplification was performed in a ThermoHybaid PX2 and in a PC-9600 cooled thermal cycler as follows: one 15-min cycle at 95°C; 35 cycles including 30 s at 94°C, 30 s at 52–65°C (depending on the individual pair of primers' temperature melting point, Tm) and 30 s at 72°C; and a final 5-min extension step at 72°C. Amplification products were separated on 1, 2 or 3% agarose gels using a mixture of 1:1 Metaphor® and Agarose™ and visualized with ethidium bromide under UV light. Primers amplifying sequences from the chromosome 7D were tested on deletion stocks for the chromosome arm 7DL to determine their locations. By testing these markers on DNA extracted from the translocation lines we were able to differentiate the size of each translocation and the position of several primers.

When a pair of primers amplified a *Thinopyrum* band from the translocation line but not from the corresponding wheat line, the band was considered a dominant marker for the translocation. Likewise, if a pair of primers amplified a band in wheat but not in *Thinopyrum* it was considered dominant for wheat. When a pair of primers amplified a band in *Th. intermedium* but not in *Th. ponticum* or wheat, the marker was considered dominant for *Th. intermedium*; likewise a marker was dominant for *Th. ponticum* if it amplified only in *Th. ponticum*, not in *Th. intermedium* or wheat. Co-dominant markers were those that gave a different size product on two or three of the species tested. One co-dominant marker (BF145935) was particularly useful giving different sized bands for all three parents. Primer pairs showing polymorphism among wheat chromosome 7DL, and translocations TC5 and T4m were selected for testing recombinant populations.

Because few specific bands for *Thinopyrum* genomes were found, a group of 7DL derived ESTs were used for comparison with the rice genome sequences through TIGR Rice Genome Annotation version 5, available at http://www.tigr.org/tigr-scripts/osa1_web/gbrowse/rice/. The orthologous rice genomic sequences were obtained and aligned with the wheat and rice transcripts. Using the genomic rice sequence, primers were designed from the most conserved regions of one exon to the most conserved region on the next exon going through at least one intron, with the *Primer 3* program as described above.

SSR and STS derived markers

Forty-three publicly available SSR and 38 STS primers were reported in the literature to amplify sequences from the chromosome arm 7DL and/or translocations on 7DL. These were tested for polymorphisms between the two *Thinopyrum* translocations and wheat. Polymorphic markers between *Th. intermedium* translocation and wheat, dominant for *Thinopyrum* were: BYAgi (Stoutjesdijk et al. 2001), and gwm37 (Ayala et al. 2001; Roder et al. 1998). Polymorphic markers between *Th. ponticum* translocation and wheat, dominant for *Thinopyrum* were: STS-Lr19$_{130}$ (Prins et al. 2001), S265512 (Gupta et al. 2006) and 3P3/3P4 (Wang and Wei 1995). The primers 3P3/3P4 are distinctive in this study in that they were developed based on an E genome-derived repetitive sequence; Wang and Wei (1995) showed differences in number of repetitive sequences and nucleotide composition in different Triticeae species. The other polymorphic STSs and SSRs were mainly dominant for wheat (Pestova et al. 2000; Roder et al. 1998). Primers were ordered from SIGMA and PrOligo labs.

DNA preparations

DNA from the parental lines was extracted with the DNeasy® Plant Maxi Kit QIAGEN using leaf tissue of young seedlings. When large numbers of individuals were tested, DNA was extracted in a 96 well format using either half seeds or leaf tissue. For the half seed DNA extraction method, each seed was cut across the endosperm cavity, storing the half with the intact embryo in the fridge for subsequent germination if required. The other half seed was powdered in a 96 deep well plate format with one stainless steel ball bearing per well, shaken at a frequency of 29 oscillations per second for 3 min in a mixer mill. Samples were heated at 65°C for a minimum of 1 h with extraction buffer (0.1 M Tris–HCl pH 8.0, 0.05 M EDTA pH 8.0 and 1.25% SDS). After cooling, 150 µl of 6 M ammonium acetate was added to each sample and the plate was spun at 3,000 rpm. DNA was precipitated from 300 µl of supernatant with 180 µl of isopropanol, washed, dried, resuspended in 150 µl of MilliQ water per well and left overnight to dissolve.

For leaf tissue, the sap from 3 cm young leaf was extracted with 600 µl extraction buffer [0.1 M NaCl, 0.10 M Tris–HCl pH 8.0, 0.01 M EDTA and 1% Sarkosyl (*N*-lauroylsarcosine)] in a sap extractor roller. The mixture was collected directly into a tube containing 600 µl of mixture (ratios by volume) phenol (25):chloroform (24):isoamyl alcohol (1), and emulsified on a rotating wheel for at least 5 min but no longer than 30 min, centrifuged 5 min and DNA precipitated with an equal volume of isopropanol

from the recovered aqueous phase, washed dried and re-suspended as described above.

Bioassays

Consecutive bioassays were performed on seedlings to determine the presence of the leaf rust resistance gene *Lr19* and the BYDV resistance gene *Bdv2*.

Leaf rust

Two leaf stage seedlings were sprayed with a fine mist of water to wet the leaf surface, followed by a mixture of rust urediospores of *Puccinia triticina* pathotype 104-1,2,3, (6), (7), 11, 13 and talcum powder. Inoculated plants were sealed in plastic containers, to provide 100% humidity for at least 24 h. Next day a re-inoculation was performed under the same high humidity conditions. Following rust inoculation, BYDV bioassay to test resistance to the virus was performed (see below). When irregular or poor rust infection was obtained, progeny tests were carried out at the University of Sydney Plant Breeding Institute, Cobbitty according to Bariana and McIntosh (1993).

BYDV

The Australian isolate of BYDV-PAV (PAV-Aust.) used in our experiments was maintained in the greenhouse on oats (*Avena sativa* cv. 'Black Coast'). Inoculations with the virus were performed essentially as described in Banks et al. (1995) and Ayala et al. (2001).

At 12–15 days post-inoculation the youngest leaf of each plant was sampled for ELISA testing and the oldest leaves were scored for the presence of rust response (see "Leaf rust" method above). Double antibody sandwich ELISA with polyclonal antibodies against PAV-Aust produced at CSIRO was carried out as described in Ayala et al. (2001). Resistance to the virus was assessed as a reduction in virus titer by dividing the OD value of the infected by the OD of the healthy control (I/H). Genotypes with I/H values under 3 were considered un-infected and not included in further analysis; genotypes with values under 10 were considered to carry resistance. The I/H OD values ranged from 1 to 60. Resistant individuals were transplanted to bigger pots and grown to maturity in the greenhouse. At flowering time, plants were test crossed to 'Hartog' and bagged for selfing.

Data analysis and linkage map

The segregation data for each marker and bioassay results of an F_2 population were compared with the expected segregation ratio for one dominant (Fisher's exact test) or co-dominant gene (Chi-square) using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA). All the molecular and bioassay data fitting the expected criteria were jointly analyzed with the software package MAPMAKER (Whitehead Institute for Biomedical Research). Recombination frequencies between adjacent markers were estimated by multipoint analysis (LOD = 3 and $\theta = 4.0$) and converted to genetic distances (cM) by Kosambi's mapping function.

Results

Development of molecular markers for the wheat chromosome arm 7DL, *Th. intermedium* (TC5) and *Th. ponticum* (T4m) translocations

A total of 229 pairs of primers from different origins were tested using the three genotypes: wheat, 'Hartog' TC5+ (*Bdv2*) and 'Cook' T4m+ (*Lr19*) lines. Twenty two percent of the primer pairs were polymorphic for the three relevant regions of 7D, 7S, and 7J. Polymorphic primer pairs and the results against test genotypes are given in Table 1 and Supplementary Table 1.

Many of the tested ESTs were reported on the NSF Wheat Resources database to hybridize to sequences from more than one chromosome of the wheat genome. Some of the primer pairs designed from those ESTs, amplified multiple bands and the use of nullitetrasomic lines demonstrated that sequences from the different group 7 chromosomes were involved (Fig. 2). Most of the EST-derived primer pairs were monomorphic (data not shown).

Among the polymorphic primers identified using ESTs, SSRs and STSs, the majority amplified a specific band for the wheat chromosome arm 7DL and that band was absent in the homozygous TC5 and T4m translocation lines because the target wheat region was replaced by the translocation fragments. Figure 2b illustrates one such marker BE637476. Although a number of primers amplified a different sized band on *Th. intermedium* and *Th. ponticum*, the frequency of markers which were co-dominant for two or three of the three donor chromosomes (7D, 7S and 7J) was quite low. Four primer pairs amplified different size bands from 7DL, 7S and 7J in the same reaction, but only one, BF145935, was located in the target region and sufficiently robust to be used routinely (Fig. 2a).

Sometimes primers derived from wESTs and developed based on orthologous sequences of rice, failed to give products in wheat; this was the case with the primers BE605194 and BE405646. These primers were designed to span an intron in rice. The failure to amplify in wheat may result from an absence of a corresponding intron in wheat in comparison to the *Thinopyrum* and rice genome.

Table 1 wEST-derived PCR primers amplifying diagnostic bands on *Th. intermedium* and *Th. ponticum* translocations located at the telomeric region of chromosome 7DL of wheat

Primer name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	*Th. ponticum*	*Th. intermedium*	Wheat			Bin on 7DL
					7A	7B	7D	
BE404744	CTCTCTCTTCAGCACGAGTACAATCTC	GTCTCCTTCACGACTGCCTTTAGG	p				w	8
BE405646	GATGCAGCTGTAGGTGACTTTGCT	CGATGTTCAAGAATCCAAACAACC	p					
BE442755	CTTTATGACCGTTCAAACTCGTTC	ATGCGATAAGAGAGATGACCTTCC	p			w	w	4
BE445653	TCTCTTGGACACTGGGTTCGT	ACCTGAGACGGCTGACTTGAC	p				w	8
BE637476	GCAGTGAAGTTCTACACCAGAGAGG	ACATCATCAAAGAGGAAGGTGAAC	p		w	w	w	8
BF145935	CTTCACCTCCAAGGAGTTCCAC	GCGTACCTGATCACCACCTTGAAGG	p	i	w		w	8
BE605194	GGCAGCCTTGAAAAGCATCT	AGACCATGGCATATTAAGGCAAAC		i				
B-21	GGTTGCTTTGGTGACCCTGAAATA	AGATAGGAGCTCAATTGCCGGAAG	p					

Primers amplified also specific bands on the other homoeologous wheat group 7 chromosomes, as indicated above

p Th. ponticum band, *i Th. intermedium* band, *w* wheat band

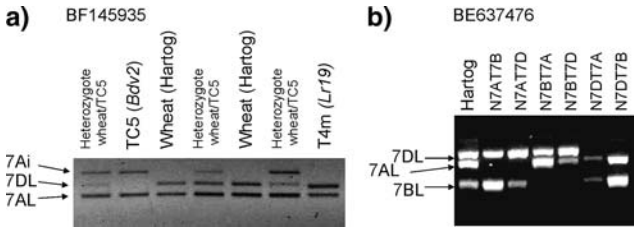

Fig. 2 Example of the PCR banding patterns obtained amplifying genomic DNA with molecular markers derived from wESTs used in this study; **a** co-dominant marker BF145935 primers, on parental lines and hybrids on a 3% agarose gel; **b** BE637476 dominant wheat marker, initial test on nullitetrasomic lines on a 2% agarose gel

Detection of recombinants between *Th. intermedium* TC5 and *Th. ponticum* T4m translocations

In the presence of ph1b *mutation*

Recombination was first observed in a sub-sample of 19 individuals (pilot population) of an F_3 family derived from one F_2 individual confirmed to have the genotype T4m/TC5, *ph1b/ph1b*. This pilot population had been through two meioses in the absence of the *Ph1* gene and was screened using a set of six molecular markers for the target region which were dominant *intermedium*, dominant *ponticum*, co-dominant *intermedium*/wheat, co-dominant *intermedium/ponticum*/wheat and co-dominant wheat/*ponticum* (Table 2). The pilot population was also bioassayed for BYDV and leaf rust.

Recombination was recorded when only one or two of the three expected *ponticum* markers, or one of the two expected *intermedium* markers was present in an individual. Heterozygotes, where apparently no recombination had occurred, were those individuals that showed the presence of all *intermedium* and *ponticum* markers of dominant and co-dominant nature. For example, lines 3, 9, 12, 14, 15, 17, 19 and 21 (Table 2) were considered heterozygotes. Likewise, lines 8 and 11 were considered homozygous for the *intermedium* translocation because all the *intermedium* markers (dominant and co-dominant), were present, and all the *ponticum* markers were absent. Lines 2, 4, 5, 7, 18, 20, 22 and 24 were apparent recombinants for this particular set of markers because some, but not all, of the dominant and co-dominant markers were absent. As anticipated, none of the individuals of this sub-population showed the presence of the co-dominant markers, wheat/*Th. intermedium* (*Xgwm37*) or wheat/*Th. ponticum* (BE637476) in this region (Table 2).

To confirm the presence of recombinant chromosomes and to characterize them further we test crossed surviving F_3 individuals to 'Hartog' and analyzed the progeny. Between two and 14 testcross individuals from each surviving F_3 plant were scored with four molecular markers, depending on seed availability (Supplementary Table 2). As expected the two co-dominant wheat markers were present in all the testcross individuals. In most cases, the genotype of the progeny was segregating but it was possible to distinguish the genotype of the parents. In cases where one particular marker was absent in the parents it was also absent in all the testcross progeny. This exercise confirmed that recombination had occurred and served to isolate putative recombinant chromosomes and to restore the influence of *Ph1*.

To complement the pilot F_3 population analysis, doubled haploid lines with enough seed for bioassays were tested independently for the presence/absence of *Lr19* at Cobbitty and for the presence/absence of *Bdv2* at Canberra. Of 86 lines tested against rust, 37 carried *Lr19* and of 72 lines tested against BYDV, 34 possessed *Bdv2*. In total, of 61 lines tested for both pathogens, 10 carried both *Lr19* and *Bdv2*. In both bioassays monogenic segregation (1:1 for DH population segregating at a single locus) for resistance was observed (Supplementary Table 3).

Table 2 Pilot population of 19 F_3 individuals from one F_2 plant with confirmed TC5/T4m, *ph1b/ph1b* genotype, to search for recombination between the alien segments of TC5 and T4m. Depicted are genotypes of F_3 individuals as tested with molecular markers and bioassays. The rust genotype was determined by bioassay of the derived F_4 families. The last two columns are the results of the two bioassays

Genotypes	3P3/3P4 D-p	BF145935 C-wip	BE637476 C-wp	Lr19-130 D-p	gwm37 C-wi	BYAgi D-i	RUST C-p	BYDV-PAV D-i	
TC-5	–	i	–	–	i	i	S	R	
T4m	p	p	p	p	–	–	R	S	
Wheat	–	w	w	–	w	–	S	S	
B.11.18.2	–	ip	p	p	i	i	ND	R	–[a]
B.11.18.3	p	ip	p	p	i	i	R	R	
B.11.18.4	p	p	p	p	i	i	R/S	S	–[a]
B.11.18.5	p	i	–	–	i	i	S	R	–[a]
B.11.18.7	p	p	p	p	–	i	R	S	–[a]
B.11.18.8	–	i	–	–	i	i	S	R	
B.11.18.9	p	ip	p	p	i	i	R/S	R	
B.11.18.11	–	i	–	–	i	i	S	R	
B.11.18.12	p	ip	p	p	i	i	R/S	R	
B.11.18.14	p	ip	p	p	i	i	R/S	R	
B.11.18.15	p	ip	p	p	i	i	R/S	R	
B.11.18.17	p	ip	p	p	i	i	R/S	R	
B.11.18.18	–	ip	p	p	i	i	R/S	S	–[a]
B.11.18.19	p	ip	p	p	i	i	R/S	R	
B.11.18.20	p	i	–	–	i	i	S	R	–[a]
B.11.18.21	p	ip	p	p	i	i	R/S	R	
B.11.18.22	p	i	–	–	i	–	S	R	–[a]
B.11.18.23	p	p	p	p	–	–	R	S	
B.11.18.24	p	ip	p	p	i	–	ND	R	–[a]

p Th. ponticum, *D-p* dominant *ponticum*, *R* resistant, *i Th. intermedium*, *D-i* dominant *intermedium*, *S* susceptible, *w* wheat, *C-wp* co-dominant wheat and *ponticum*, *R/S* heterozygous, *ND* not determined, – absence

[a] Individuals which are recombinant using these markers

Molecular markers were tested in a sub-set of lines to determine if recombinant chromosomes were present. Twenty-nine selected doubled haploid lines were tested with molecular markers. All the lines showed the presence of *Th. intermedium*, *Th. ponticum* or both genome specific markers and the absence of the markers corresponding to that position in wheat. Clearly single and double crossovers had taken place during the two meioses (Supplementary Figure 1).

In wheat with a normal Ph1 *gene*

Because high numbers of recombinants were found in material containing the *ph1b* mutant, we screened an F_2 population of the cross T4m/TC5 carrying a functional *Ph1* gene (Fig. 1), to confirm suppression of recombination between these two alien translocation segments in wheat under normal conditions.

Eighty-four individuals of a T4m/TC5-derived F_2 population were screened with six molecular markers developed in the first part of this study (Supplementary Table 4). As expected, all the molecular markers showed normal monogenic Mendelian segregation, although there was a small non-significant bias in favor of *ponticum* and against *intermedium* genotypes. With the three-genome co-dominant marker BF145935, the numbers of *ponticum* bands were slightly higher than *intermedium* bands. A segregation bias to the *ponticum* translocation is consistent with the segregation distortion relative to normal 7D noted for this translocation by others (Prins et al. 1997). Importantly no recombination between *Th. intermedium* (TC5) and *Th. ponticum* (T4m) was observed in this population with a normal *Ph1* background (Supplementary Table 4).

Genetic map for recombinant translocations of *Th. intermedium/Th. ponticum* on wheat 7DL

A set of 154 F_2 individuals produced by selfing an *Lr19lr19Bdv2bdv2 ph1bph1b* genotype was used to determine the order of fifteen polymorphic markers on *Th. intermedium* (TC5) and *Th. ponticum* (T4m) translocations located at the distal portion of wheat chromosome arm 7DL. This population had been through only one critical meiosis under the influence of *ph1b* (Fig. 1). Young seedlings of all F_2 individuals were tested for resistance to BYDV and leaf rust in Canberra. In addition, the *Lr19* status of each F_2 individual was confirmed by testing progenies of each F_2 individual as F_3 families for leaf rust response at Cobbitty. Leaf tissue from each F_2 plant was collected for DNA extraction and PCR testing. The segregation ratio obtained for each molecular marker in the population conformed to either 3:1 or 1:2:1 Mendelian

expectation for dominant and co-dominant effects, respectively (data not shown).

In order to exclude the possibility that the alien translocations recombined with 7AL or 7BL, we tested all the individuals in the F_2 population with five polymorphic markers mapping on the long arms of each of the bread wheat group 7 homoeologous chromosomes. Markers BE446475, BF485273, BE637476 produced three bands from one PCR reaction in normal wheat: one each for 7A, 7B and 7D (Supplementary Table 1). Marker BE425305 amplified one band on 7BL; BE442755 amplified two bands, one on 7BL and one on the *Th. ponticum* T4m translocation. All the individuals of the population showed the presence of the 7AL and 7BL markers and the absence of the 7DL distal markers (data not shown). The 7AL and 7BL homoeologous arms and the TC5 or T4m translocations were present, while the equivalent portion of 7DL was absent in all the individuals. We therefore concluded that no recombination in the target region took place with chromosome arms 7AL or 7BL.

All the molecular and phenotypic data were analyzed with the MAPMAKER program. First we constructed a framework map with markers showing high LOD scores of 3 or more (Fig. 3). The most likely positions of other markers with LOD score values of 2 or less, are also indicated in Fig. 3. The resulting map of 85 cM contained mostly dominant markers for the *Th. intermedium* and *Th. ponticum* fragments, two co-dominant markers [BE145935 and the bioassayed resistance to leaf rust (*Lr19*)] plus bioassayed resistance to BYDV (*Bdv2*). The program produced two linkage groups separated by approximately 38 cM. The group proximal to the centromere contained three dominant markers for *Th. ponticum*, whereas the distal one contained the remaining nine markers plus the resistance genes *Lr19* and *Bdv2* separated by approximately 8.3 cM. While a total of 72 recombinant F_2s were identified, 81 individuals (52%) were heterozygotes or had parental genotypes for T4m and TC5, respectively.

By grouping the F_2 individuals of the *ph1b*-derived mapping population with similar genotype based on a set of markers, we obtained 13 distinctive marker combinations (Supplementary Figure 1). Because of the dominant nature of most of the markers, heterozygous loci were resolved by progeny testing. As presented above, the doubled haploids showed unambiguously the occurrence of recombinant *ponticum* and *intermedium* segments (Fig. 4), and it is notable that the F_2 population could be resolved into very similar classes of recombinant chromosomes to the doubled haploid classes.

Inheritance of recombinant *Thinopyrum* fragments in wheat

Individuals derived from testcrosses between recombinant F_3 or doubled haploids and 'Hartog', and confirmed to carry recombinant trigenomic translocations with *Lr19* and *Bdv2*, were selfed and progeny tested again for segregation of molecular markers. Three of these testcross F_2 populations (Table 3), with different initial marker combinations, were chosen for genotyping with seven molecular markers: co-dominant *ponticum*/*intermedium* (3P3/3P4), dominant *ponticum* (B-21, $STSLr19_{130}$), co-dominant *ponticum*/wheat (BE637476), co-dominant *intermedium*/wheat (BF145935), co-dominant *intermedium*/wheat (*Xgwm37*) and dominant *intermedium* (BYAgi) (Table 3).

A total of 143 individuals were analyzed. In all three populations, the seven molecular markers were inherited as

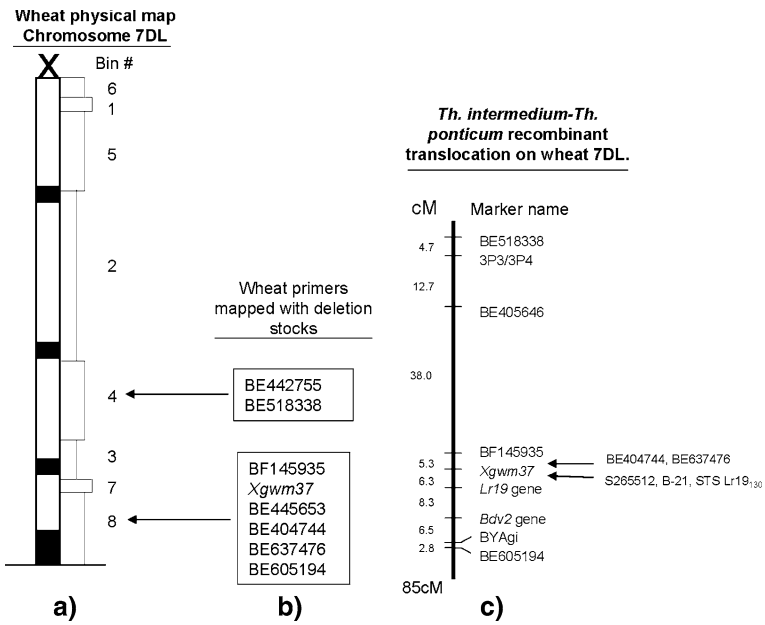

Fig. 3 Linkage map of the TC5/Tm4 7DL wheat translocations recombining in an F_2 population in the presence of *ph1b* mutant gene in homozygosity; (a) physical map of wheat chromosome 7DL for comparison, showing C bands and position of bins; (b) wheat ESTs and SSRs positioned on corresponding bins using deletion stocks, (c) linkage map of recombinant translocations constructed with 154 F_2 segregating lines. At the *extreme right* are depicted, the molecular markers linked to these positions but with LOD score < 2

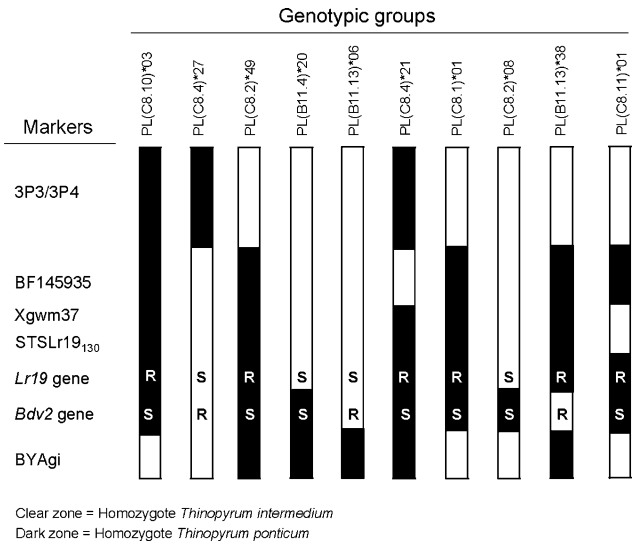

Fig. 4 Recombinants arranged by genotypic classes obtained from the molecular analysis of doubled haploid individuals having experienced two meiosis containing TC5 and T4m translocations under the influence of *ph1b*/*ph1b*. The genomic origin of the distal region of chromosome 7DL is shown using six molecular markers; and the phenotypic responses: resistance (*R*) and susceptibility (*S*). The *clear regions* of each chromosome correspond to *Th. intermedium* and the *dark regions* to *Th. ponticum*

a unit, fitting the Mendelian ratios for segregation at a single locus. The combination of seven markers in each population matched the genotype of the parental recombinant translocation. No new marker combination was observed, confirming the lack of recombination of the fragment once the effect of the *Ph1* gene was restored.

Discussion

Recombination between *Th. intermedium* (TC5) and *Th. ponticum* (T4m) translocated fragments, both on distal positions on chromosome arm 7DL of wheat, was achieved by bringing them together in a homozygous *ph1b* mutant background. Identification of trigenomic recombinant chromosomes was facilitated by the use of molecular markers. The presence of leaf rust and BYDV resistance could be determined on the same individuals by sequential leaf rust and virus bioassays. Recombinants carrying both leaf rust (*Lr19*) and BYDV (*Bdv2*) resistances were found. Since the source of *Lr19* employed in this work was already mutated to reduce the unwanted yellow pigment in the flour, we have not yet attempted to analyze the recombinants for flour color. Preliminary testing for the presence of *Sr25* suggests that some of the recombinants do carry this gene; however, the wheat backgrounds employed in this work carry other stem rust resistance genes which mask the effect of *Sr25*. Further analyses on testcross progenies will be required to assign the presence of *Sr25* to particular recombinant translocations unambiguously.

To dissect and map the alien translocations and possible recombinants it was necessary to develop a new suite of markers. We have taken advantage of homologies and available comparative maps among grass and Triticeae species (Moor et al. 1995; Sorrells et al. 2003) to find candidate sequences from which PCR based markers were developed. Successful reports on the effective exploitation of synteny prompted us to use wheat EST databases to

Table 3 Genomic constitution of the distal region of chromosome 7DL in three individuals containing a different recombinant translocation each. At the right of each chromosome are depicted the segregating ratios corresponding to each F_2 test crossed population to the matching molecular marker in the same row. At the bottom, segregation of the whole recombinant fragment for each population

Molecular Markers			Individual molecular markers F_2 segregating populations		
Name	Specificity	Genotype	B11.18.3.8	B11.18.19.3	B11.106.10
3P3/3P4	Pont (Dom)	p : -	33:10	19:1	-
BF145935	int/wheat(Co-dom)	i : i/w : w	9:24:10	6:13:1	32:32:16
BE637476	pont/wheat (Co-dom)	p : p/w : w	-:34	-:14	-:48
Xgwm37	int/wheat (Co-dom)	i : i/w : w	9:24:10	6:13:1	-:48
B-21	pont (Dom)	p : -	-	-	64:16
STS-Lr19$_{130}$	pont (Dom)	p : -	-	-	64:16
BYAgi	int (Dom)	i : -	-	19:1	64:16

Whole Fragment Segregation	Hmz	Htz	Wheat	Hmz	Htz	Wheat	Hmz	Htz	Wheat
Observed	9	24	10	6	13	1	32	32	16
Expected	11	21	11	5	10	5	20	40	20
P value		0.8ns			0.2ns			0.1ns	

Dark zone Th. ponticum, *clear zone Th. intermedium*, *Hmz* homozygote for the whole translocation, *Hzt* heterozygote whole translocation/wheat, *p* ponticum band, *i* intermedium band, *w* wheat band, – absence of band

search for sequences that would yield markers for the chromosome arm 7DL in wheat and the two homoeologous translocations derived from *Th. intermedium* and *Th. ponticum*. This strategy proved productive.

Since wheat genotypes homozygous for the two alien translocations are completely fertile and phenotypically normal, and high yielding cultivars with the translocations have already been released (Larkin et al. 2002; Singh and Rajaram 1991), we assume that cultivars carrying recombined segments of these translocations will also have high yield potential. Both translocations appear to be sufficiently homoeologous to compensate for the loss of parts of 7DL. This assumption is supported by the number of markers obtained in our work. We identified 53 loci with 31 pairs of primers derived from 7DL wEST sequences. Of those, eight pairs of primers, equivalent to 15%, gave a unique band for *Th. ponticum* and seven pairs (13%) gave a specific band for *Th. intermedium*. The other loci mapped on chromosome arms 7AL (17%), 7BL (21%) and 7DL (34%) of wheat.

The greater number of loci identified on the *ponticum* translocation (T4m), compared to the *intermedium* (TC5) translocation, using primers designed from wheat 7DL ESTs, may suggest that the *ponticum* translocation (a 7J fragment) is more closely related to wheat chromosome arm 7DL than is the *intermedium* translocation (a 7S fragment).

Translocations from *Th. ponticum* and *Th. intermedium* have long been used in wheat breeding but no recombinants between different alien materials have been reported. Knott (1980) demonstrated that alien translocations inherit as a single unit in breeding populations. Attempts to reduce the size of alien translocations have been made in a number of cases using techniques such as: ionizing radiation; tissue culture; removing the function of the *Ph1* gene to induce allosyndetic pairing and homoeologous recombination; and sequential backcrosses of the recombinants to wheat. Despite these efforts and the potential importance, the success rate has been low (Crasta et al. 2000; Khan 2000; Francki et al. 2001; Lukaszewski 2003; Molnar-Lang et al. 2005; Zhang et al. 2005; Jubault et al. 2006). The attainment of recombinants and the number of them probably depends on the phylogenetic distance between the genomes involved. The present paper demonstrates another method of modifying the content of an alien translocation in wheat, namely to recombine it with a second translocation. The approach requires two translocations on the same wheat arm and possibly a closer synteny between the two alien fragments than either one has to the homoeologous wheat chromosome arms.

The relationship between chromosomes of *Th. intermedium* and wheat, or between *Th. ponticum* and wheat was tested by looking at the level of allosyndetic and autosyndetic pairing in hybrids of the two species with wheat (Cai and Jones 1997). Higher autosyndetic pairing frequencies were obtained among *Thinopyrum* chromosomes than among wheat chromosomes in both hybrids suggesting the three genomes of *Th. intermedium* and the five genomes of *Th. ponticum* were more closely related with each other than to the three genomes of wheat (Cai and Jones 1997). A number of cytogenetic studies, have been conducted to determine the genome structure and phylogenetic relationship between *Th. intermedium* and *Th. ponticum*. Based on chromosome pairing and genomic in situ hybridization, the more widely accepted genomic compositions are JJ^sS for *Th. intermedium* and $JJJJ^sJ^s$ for *Th. ponticum* (Chen et al. 1998). The S genome is sometimes called St (Wang et al. 1994). However, alternatives for *Th. intermedium* include EbEeSt (Liu and Wang 1993) and EeSt(V-Eb-R) (Kishii et al. 2005). An alternative for *Th. ponticum* is EEEStSt (Zhang et al. 1996). No chromosome pairing could be observed in hybrids between decaploid *Th. ponticum* and diploid *Th. elongatum* (EE); therefore it seems unlikely that the decaploid possesses an E genome (Dvorak 1981). Likewise various workers have found that E genome markers fail to hybridize or amplify from *Th. ponticum* or *Th. intermedium* (Ayala and Larkin, unpublished). Lyubimova (1970) obtained fertile individuals after crossing *Th. intermedium* and *Th. ponticum*, suggesting shared homoeology among some of their genomes. Chen et al. (1998) reported homology between the J genomes of *Th. intermedium* and *Th. ponticum*, although the two sets of chromosomes appeared distinct at the centromeric region. However genome conclusions based on chromosome pairing in hybrids is compromised by the observation that both *Th. ponticum* and *Th. intermedium* appear to have genes which promote homoeologous pairing (Zhang 1992; Jauhar 1995; Han et al. 2004).

The genome origin of the T4 (*Lr19*) translocation remains uncertain. It was produced by irradiation of a substitution line called $7el_1(7D)$ (Sharma and Knott 1966; Dvorak and Knott 1977). Zhang et al. (2005) refer to it as a 7E chromosome, however this appears to be a designation based only on the fact that the decaploid *Th. ponticum* used to be called *Agropyron elongatum*, and the diploid *Ag. elongatum* species has genome designation E. It is likely that T4 was derived from the chromosome 7J. Likewise the literature is inconclusive about the origin of the L1 chromosome from *Th. intermedium* and its derived translocations, TC5, TC6, TC14 and YW642. Chen et al. (1998) suggested that L1 involved a J chromosome. Fedak et al. (2001) suggested that to be a J^s chromosome. However Zhang et al. (1996) used genomic hybridization to convincingly demonstrate the involvement of a 7S chromosome, albeit slightly modified in the centromeric region. The failure to obtain recombination between TC5 and T4 in a normal *Ph1* genetic background confirmed that they are not from the same genome type (this paper and Ravi Singh, unpublished). Thus

if *Th. ponticum* is JJJJ^{s}J^{s} and *Th. intermedium* is JJ^{s}S, we can be confident that T4 (*Lr19*) and TC5 (*Bdv2*) do not both come from 7J or both from 7J^{s}. Furthermore, the results reported here are consistent with the growing consensus that the L1 (and therefore the derived TC5) involved a 7S chromosome (Zhang et al. 1996; Wang and Zhang 1996) and T4 (*Lr19*) was derived from a 7J chromosome. The recombinants developed and reported in this paper appear therefore to include trigenomic chromosomes that might carry designations such as 7DS·7DL-7JL-7SL. Previously Fedak and Han (2005) have reported multicolor GISH evidence of a chromosome in the addition line Z5 (Larkin et al. 1995) composed of D, A and alien (*Th. intermedium*) genomic portions. However to the knowledge of the present authors, this is the first report of allosyndetic recombination between different alien translocations.

The high recombination frequency observed between TC5 and T4m translocations is likely to be affected not only by the general relatedness of their progenitor genomes but also by the level of synteny specifically between the translocations and by the structure of the surrounding wheat chromatin (Sandhu and Gill 2002; Feuillet and Keller 2002; Akhunov et al. 2003; Hossain et al. 2004; Erayman et al. 2004). Having obtained perfectly fertile progeny from the new recombinants it seems likely the translocated segments from both *Thinopyrum* species were orthologous to the lost wheat region, allowing compensation for the missing wheat chromosome segments. Recombining TC5 and T4m has allowed new combinations of beneficial genes to be assembled, while also avoiding the problem of translocation self-elimination, which arose, when T4 was shortened (Prins and Marais 1998; Marais et al. 2001; Groenewald et al. 2005). Although as explained earlier, there has been no analysis of flour pigmentation in this study, we would expect some *Lr19*-containing recombinants to have eliminated this unwanted locus. The gene promoting yellow endosperm was mapped with molecular markers in deletion mutants closely linked to the *Sr25* gene for resistance to stem rust and away from the *Lr19* gene for resistance to leaf rust (Prins and Marais 1998; Marais et al. 2001; Groenewald et al. 2005).

Since there was a high recombination frequency and Mendelian segregation was evident, we determined the genetic order of markers in the F_2 population. A robust genetic linkage map of 85 cM was constructed with markers appearing in two clusters, three *Th. ponticum* markers mapped on the proximal side while and all the other markers and resistance genes *Lr19* and *Bdv2* congregated at the distal end. This clustering of markers by MAPMAKER was not associated with an absolute physical constraint on where recombination may occur, since crossovers occurred in a number of positions within this linkage group. The marker clustering appears only to be the result of an absence of markers between the two groups.

From a practical perspective it is significant that the new digenomic alien translocations (on trigenomic chromosomes) were inherited as a single linkage block, after restoration of the *Ph1* gene function. All the *intermedium* and *ponticum* markers were inherited as a single Mendelian unit in testcross F_2 populations derived from three recombinants. We anticipate that some of the recombinant translocations will be usefully deployed for wheat improvement, especially those combining leaf rust and BYDV and possibly stem rust resistances. Upon further characterization we will designate and release recombinants with names such as Pontin1 and Pontin2, accompanied by suitable molecular markers.

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