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Homoeologous recombination, chromosome engineering and crop improvement

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Abstract

Sears (1956) pioneered plant chromosome engineering 50 years ago by directed transfer of a leaf rust resistance gene from an alien chromosome to a wheat chromosome using X-ray irradiation and an elegant cytogenetic scheme. Since then many other protocols have been reported, but the one dealing with induced homoeologous pairing and recombination is the most powerful, and has been extensively used in wheat. Here, we briefly review the current status of homoeologous recombination-based chromosome engineering research in plants with a focus on wheat, and demonstrate that integrated use of cytogenetic stocks and molecular resources can enhance the efficiency and precision of homoeologus-based chromosome engineering. We report the results of an experiment on homoeologous recombination-based transfer of virus resistance from an alien chromosome to a wheat chromosome, its characterization, and the prospects for further engineering by a second round of recombination. A proposal is presented for genome-wide, homoeologous recombination-based engineering for efficient mining of gene pools of wild relatives for crop improvement.

Introduction

Wild relatives and related species are important resources for broadening the genetic variability of crop plants. Crop plants are often bred for specific quality attributes, such as high yield potential and plant type suited to specific agronomic practices. Therefore, the amount of wild genetic material introduced into an elite cultivar has to be carefully controlled. On the other hand, wild relatives of crops, depending upon the time of evolutionary divergence, may have highly differentiated homoeologous genomes. In hybrids between a crop plant and a wild relative, genetic recombination is usually restricted to homoeologous chromosomes, and in the case of polyploid wheat it is completely suppressed. As a result the transfer of a target gene from a wild relative (often referred to as alien) to a crop plant is difficult, and often accompanied by unacceptable

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wild traits due to genes also present in the transferred chromosome segment. Once present in the wheat background the entire chromosome segment is inherited as a unit. This association is often referred to as linkage drag. Generally, the linkage drag effect is more severe in crops with diploid genetic systems, because their genomes are more sensitive to genetic imbalance compared to relatively more buffered polyploid genomes. The practical consequence has been that few exotic genes in alien germplasm have been exploited in agriculture (Friebe *et al.* 1996).

Various procedures for chromosome manipulation, generally referred to as 'chromosome engineering', have been developed to overcome linkage drag and by reducing the size of the alien chromosome segment transferred to a crop plant genome. Most of the pioneering research in chromosome engineering was carried out in allohexaploid wheat (*Triticum aestivum* L., 2n = 6x = 42, AABBDD) (Sears 1972, also see review by Jiang *et al.* 1994). Common wheat arose recently (6000–8000 years ago) from hybridization of tetraploid wheat *T. turgidum* L. (2n = 4x = 28, AABB) and diploid *Aegilops tauschii* Coss. (2n = 2x = 14, DD), and these two species constitute the primary gene pool. Transfers from these species can be made by homologous recombination either by direct crosses of these species with common wheat or by the production of synthetic wheat (McFadden & Sears 1946, Gill & Raupp 1987). For all practical purposes the genomes of the several hundred Triticeae species to which wheat belongs, have homoeologous genomes, including diploid A genome carriers *T. monococcum* L. and *T. urartu* Tumanian *ex* Gaudily; tetraploid wheat *T. timopheevii* Zhuk. (2n = 4x = 28, AAGG); the D genome cluster of polyploid *Aegilops* species; and *Ae. speltoides* Tausch (2n = 2x = 14, SS), which is closely related to the B genome of polyploid wheat. These species with genomes closely related to the A, B, and D genomes of polyploid wheat constitute the secondary gene pool. Transfers from the secondary gene pool can be achieved by direct crosses and backcrosses with varying levels of homologous recombination. Any techniques that enhance recombination are helpful (Dubcovsky *et al.* 1995). All other Triticeae species that carry genomes other than A, B, and D constitute the tertiary gene pool. Gene transfers from the tertiary gene pool cannot be achieved by homologous recombination, and other strategies for gene transfer need to be exploited. Both physical and genetic methods that cause random chromosome breaks, or promote homoeologous pairing and recombination, have been used in engineering transfers from the tertiary gene pool.

The first step in transfer is the production of an amphidiploid (or partial amphidiploid) between wheat and the alien species followed by the production of individual alien chromosome addition lines (O'Mara 1940). For transferring whole alien chromosome arms to wheat, the centric breakage–fusion behavior of univalents can be exploited (Sears 1952). Once the homoeologous relationship of the alien chromosome carrying a target gene has been established (previously by analyzing its sporophytic and gametophytic compensation ability, and today by molecular marker analysis), the alien chromosome and a homoeologous wheat chromosome are isolated in monosomic condition. In such double-monosomic plants both monosomes stay univalent at meiotic metaphase I. Univalents have a tendency to break at the centromeres, followed by fusion of the broken arms, giving rise to Robertsonian whole arm translocations (Robertson 1916). Depending on the chromosomes involved and the environmental conditions, the desired compensating wheat–alien Robertsonian translocations can be recovered at fairly high frequencies, ranging from small to almost 20% (Davies *et al.* 1985, Lukaszewski 1993, 1994, 1997, Friebe *et al.* 2005).

Several strategies have been used for transferring alien segments that are smaller than complete chromosome arms. Sears (1956) used radiation treatment to transfer a leaf rust resistance gene (*Lr9*) from *Ae. umbellulata* Zhuk. to wheat. Masoudi-Nejiad and co-workers (2002) exploited the action of gametocidal genes to transfer alien chromosome segments to wheat. Both ionizing radiation treatment and gametocidal genes induce random chromosome breakage and fusion of the broken segments resulting in translocation chromosomes. In these instances the majority of translocations is between non-homoeologous chromosomes, and involves duplication/deficiencies; and thus are of genetically non-compensating type and, in general, agronomically undesirable.

Methods related to the manipulation of homoeologous recombination producing genetically compensating transfers are the main focus of this article. Genes known to affect homoeologous recombination have been known for a long time, and have been exploited in chromosome engineering. Riley and co-workers (1968a,b) used a high pairing accession of *Ae. speltoides* to induce recombination between wheat and *Ae. comosa* Sm. in Sibth. & Sm. chromosomes to transfer stripe rust and stem rust resistance genes (*Yr8* and *Sr34*) to wheat.

Later analysis revealed that the targeted chromosome in *Ae. comosa* was structurally rearranged and only a small part of the alien chromosome participated in homoeologous recombination. As a result this transfer was non-compensating (presence of the alien chromosome contributed to segmental duplications and deficiencies), had enormous linkage drag (translocation chromosome mostly consisted of alien chromosome), and was never used in agriculture (Nasuda *et al.* 1998). The use of high pairing *Ae. speltoides,* apart from the targeted chromosome, also introduced non-target genes from *Ae. speltoides* (Friebe *et al.* 1996). Chen *et al.* (1994) introduced high pairing *Ae. speltoides* genes into wheat, and this stock was used in chromosome engineering (Aghaee-Sarbarzeh *et al.* 2000, 2002).

A classic example of a pairing regulator factor widely used in chromosome engineering is the *Ph1* (pairing homoeologous) gene in wheat (Okamoto 1957, Riley & Chapman 1958, Sears & Okamoto 1958, Sears 1977). In hexaploid wheat the *Ph1* gene suppresses homoeologous pairing and controls diploid-like meiosis. In the absence of *Ph1*, homoeologous recombination occurs except in those cases where it is hindered by structural rearrangements. Sears (1977) used radiation treatment to produce a *ph1b* mutant stock in Chinese Spring (CS) wheat. The CS *ph1b* mutant has a deletion at the *Ph1* locus with the deleted segment of chromosome 5B in *ph1b* being about 70 Mb (Dunford *et al.* 1995). In homozygous *ph1b* genotypes, chromosome pairing and recombination also occurs between homoeologous wheat and alien chromosomes, and since then this technique has been the method of choice for directed transfer of alien genes to wheat.

Classically, homoeologous recombination and the amount of introgression were measured by scoring chiasmate associations between cytologically marked chromosomes (Sears 1973). Later, C-banding was applied to determine the identity of the paired homoeologues at meiotic metaphase I (Gill & Chen 1987, Naranjo *et al.* 1987). With the advent of molecular markers and genomic or fluorescence *in-situ* hybridization (GISH or FISH), the extent and pattern of homoeologous recombination compared to homologous recombination was studied in a diverse group of plants (Kamstra *et al.* 1999, Zwierzykowski *et al.* 1999, King *et al.* 2002, Jenczewski *et al.* 2003, Ji & Chetelat 2003, Khrustaleva *et al.* 2005). In certain interspecific hybrids, such as *Lolium*/*Festuca*, *Allium roylei*/*A. fistulosum,* and *Alstroemeria aurea*/*A. inodora*, the frequency of homoeologous recombination visualized by GISH is rather high and up to three crossovers per chromosome were observed (Kamstra *et al.* 1999, King *et al.* 2002, Khrustaleva *et al.* 2005).

In wheat and Triticeae species separated by up to 12 million years of evolutionary divergence (Huang *et al.* 2002), homoeologous recombination frequency may vary from less than 1% to 15% (Islam & Shepherd 1992, Khan 1999, Lukaszewski *et al.* 2004). In addition, the pattern of homoeologous recombination can vary from species to species, between different chromosomes of a species, and between the short and long arms of a chromosome. Lukaszewski *et al.* (2004) studied homoeologous recombination between wheat and rye chromosomes 2B and 2R and found a 30-fold variation in recombination frequency between short (2RS and 2BS; 0.48%) and long (2RL and 2BL; 14.67%) arms. Furthermore, most of the homoeologous recombination was restricted to an intercalary segment of 65% relative length in the 2RS and 2BS arms and to the distal 25% in the 2RL and 2BL arms. Rogowsky *et al.* (1993) identified only two breakpoints detected by a single RFLP marker in a sample of eight wheat–rye recombinants for the 1RL arm of rye probed with 36 RFLP markers. Indeed homoeologous recombination appears to be highly localized and the transfer of a target gene without linkage drag by chromosome engineering appears to be a daunting task.

Several ways exist to enhance the efficiency of chromosome engineering protocols for detecting critical recombinants. Initially, the alien targeted gene must first be introduced in the form of a translocation chromosome into the crop genome to enhance the frequency of its gametic transmission for the recovery of rare recombinants. Next, highly diagnostic yet cost-effective markers must be available to screen a large number of progeny. Lukaszewski (2000) used a diagnostic telomeric C-band and a high-throughput C-banding procedure (Lukaszewski & Xu 1995) to screen 20 234 progeny, and recovered 139 (0.07%) primary recombinants for the rye 1RS and wheat 1S short arms. Pairs of primary recombinants sharing targeted genes were intercrossed to isolate secondary recombinants, which in turn were intercrossed to isolate tertiary recombinants containing a desirable combination of wheat storage protein and rye disease resistance genes. The engineered T1BL·1RS chromosome may potentially overcome the inferior bread-making quality of wheat associated with the original Robertsonian translocation.

Many other alien chromosomes lack such diagnostic cytological markers. Improved genomic *in-situ*

hybridization (GISH) techniques were used to isolate recombinant chromosomes, but the resolution of GISH is a problem (Lukaszewski *et al.* 2005). DNA-based molecular markers offer a method of choice provided they are cost-effective and amenable to high-throughput procedures. However, most of the molecular resources for marker development are available in the crop plant and not in the gene donor. Although much effort has been spent in developing PCR-based SSR (simple sequence repeat) markers that are usable across related groups of species, there has been only limited success (Yu *et al.* 2004, Mullan *et al.* 2005, Peng & Lapitan 2005).

For over 50 years wheat streak mosaic virus (WSMV) vectored by wheat curl mite has been a devastating disease of wheat. Breeders in the 1950s discovered that the only source of resistance was the perennial grass species *Thinopyrum ponticum* (Podp.) Barkworth & D.R. Dewey (syn. *Agropyron elongatum*, 2n = 10x = 70) and *Th. intermedium* (Host) Barkworth & D.R. Dewey (syn. *Agropyron intermedium*, 2n = 6x = 42). Hybrids of these grasses with wheat were used to produce WSMV-resistant lines containing alien chromosomes as additions and translocations. However, all lines proved to be agronomically inferior because of linkage drag. As the tools of chromosome C-banding and GISH became available, we identified a WSMV-resistant, compensating translocation line T4DL·4Ai#2S (Friebe *et al.* 1991). In this chromosome the 4Ai short arm of *Th. intermedium* with the *Wsm1* resistance gene replaced the 4DS arm of wheat and was translocated to the long arm of wheat chromosome 4D. An improved germplasm line WGRC27 containing the T4DL·4Ai#2S translocation was released (Gill *et al.* 1995). WGRC27 has been widely used in breeding, but no cultivars have been released because of an adverse effect on yield potential. Over the last several years we have attempted, but failed, to engineer this chromosome to produce a more agronomically desirable germplasm. This led us to systematically explore the available wheat molecular marker resources for scoring the *Ph1* locus, developing robust PCR-based markers for detecting and identifying recombinant progenies, verifying the recombinants using GISH, and the prebreeding of recombinants into elite background. We believe that we now have a sound strategy for a streamlined procedure to introgress and engineer alien genes in wheat. The principles underlying this strategy should be applicable to other crops.

Materials and methods

Plant materials

We used an array of wheat aneuploids (Sears 1954, 1966, Endo & Gill 1996) to identify molecular markers for the targeted chromosome segments. The materials used in this study included four nullisomic–tetrasomic (N4AT4D, N4BT4D, N4DT4B, N5BT5D) and five ditelosomic (Dt4AS, Dt4AL, Dt4BS, Dt4DS, Dt4DL) stocks, three deletion lines of the short arm of chromosome 4D (del4DS-1, FL 0.53; del4DS-2, FL 0.82; del4DS-3, FL 0.67), and three deletion lines of the long arm of chromosome 5B (del5BL-6, FL 0.29; del5BL-1, FL 0.55; del5BL-14, FL 0.75). The fraction length (FL) value of a deletion stock identifies the position of the breakpoint from the centromere relative to the length of the complete arm (Endo & Gill 1996). Other materials included *T. aestivum* cultivars ‘Karl 92’, ‘Agent’, ‘Overley’, and ‘Chinese Spring’ (CS), CS *ph1b*, the CS-*Th. intermedium* translocations T4DL·4Ai#2S (WGRC27, Wells *et al.* 1982, Friebe *et al.* 1991) and T4AL·4Ai#2S (CI17766 selection number B-6-37-1, Liang *et al.* 1979, Friebe *et al.* 1991), and a CS-*Th. intermedium* disomic substitution (DS) 4Ai#2(4D) (CI17882, Friebe *et al.* 1991). The elite wheat cultivar Overley was crossed to the recombinant lines to develop germplasm with good agronomic performance. All materials are maintained by the Wheat Genetic and Genomic Resources Center (WGGRC) at Kansas State University, Manhattan, USA.

Cytogenetic analysis

C-banding and chromosome identification were according to Gill *et al.* (1991) and GISH followed the protocol of Zhang *et al.* (2004).

STS primer design and analysis

We developed PCR-based STS markers to expedite the screening of large numbers of progeny for rare recombinant chromosomes. A sample of 106 wheat EST (expressed sequence tags) previously mapped to the short arm of chromosome 4D from the centromere to the telomere were selected from the wheat EST mapping project (http://wheat.pw.usda.gov/NSF/project/mapping_data.html). The sequences of these EST were used to design EST-specific primers using the software Primer 3 (Rozen & Skaletsky

2000). In addition, three PCR-based markers, PSR574, WGP90, and PSR2120, were used to detect the *ph1b* mutation (Segal *et al.* 1997, Roberts *et al.* 1999). Amplifications of PCR markers were done in a 25 µl volume containing 2 mM $MgCl_2$, 0.3 mM of each dNTP, 10 pmol of each primer, 50 ng genomic DNA, 1× PCR buffer, and 0.5 units *Taq* polymerase (Bioline, Randolph MA, USA). The PCR reactions were performed in a Peltier thermocycler (MJ-Research, USA) with a touchdown program. The PCR conditions were as follows: 94°C for 10 min; followed by 10 cycles with decreasing annealing temperature by 0.5°C for every cycle at 94°C for 20 s, 63°C for 20 s, and 72°C for 2 min; followed by an additional 35 cycles of 94°C for 20 s, 58°C for 20 s, and 72°C for 2 min. Extension of the amplified fragments was achieved at 72°C for 10 min. To achieve higher rates of polymorphism the amplified products were digested with the four-base cutter enzymes *Alu*I, *Hae*III, *Hpa*II, *Msp*I, and *Rsa*I, fractionated on a 1% agarose gel, and visualized by ethidium bromide staining and UV irradiation.

RFLP analysis

Genomic DNAs were isolated from selected genetic stocks and wheat–*Th. intermedium* recombinant lines and digested with restriction enzymes *Eco*RI, *Eco*RV, *Hin*dIII, and *Dra*I. The RFLP and EST clones used in the present study were kindly provided by Dr A. Graner, Grünbach (now in Gatersleben), Germany (MWG clones); Dr M. D. Gale, Norwich, UK (PSR clones); and Dr O. Anderson (EST clones), USDA-ARS-WRRC, Albany, California, USA. Protocols for Southern hybridization were as described in Qi *et al.* (2003).

WSMV screening

WSMV screening was done using artificial inoculation in a growth chamber according to Martin (1978).

Results

Crossing scheme

Figure 1 shows a flow chart for inducing homoeologous recombination between wheat and alien chromosomes using the *ph1b* mutant. Briefly, WGRC27, which is homozygous for translocation chromosome T4DL·4Ai#2S, was crossed to the homozygous *ph1b* stock. The F_1 was backcrossed to *ph1b*. The BC_1 progenies were screened by molecular markers (see below) to identify plants homozygous for *ph1b* and heterozygous for chromosomes T4DL·4Ai#2S and 4D. In the homozygous *ph1b* condition, the short arm of chromosome 4D is expected to pair with the short arm of T4AL·4Ai#2S. The derived progeny were

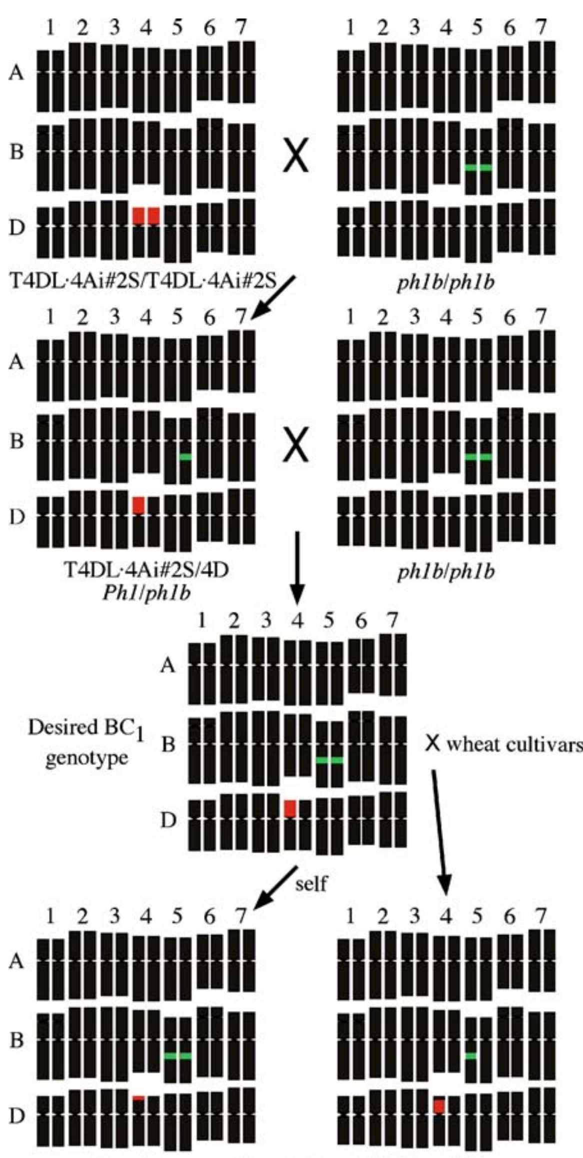

Figure 1. A strategy for producing wheat–alien recombinant chromosomes using induced homoeologous recombination. Green color represents *ph1b* and red color represents a *Th. intermedium* chromosome segment.

screened with molecular markers to recover the recombinant chromosomes.

Marker development and verification

Identifying homozygous *ph1b* genotypes: three PCR-based markers, WPG90, PSR2120, and PSR574, previously developed and mapped within the *ph1b* deletion, allowed the identification of homozygous *ph1b* genotypes (Segal *et al.* 1997, Roberts *et al.* 1999). The physical mapping of these three markers was further verified in the present study. The presence of a 154-bp PSR574 fragment in CS, del5BL-1, and del5BL-14 and its absence in del5BL-6 mapped PSR574 to the deletion bin 5BL6-0.29-0.55 (Figure 2a). Similarly, a 232-bp PSR2120 and a 230-bp WPG90 fragment were present in CS and del5BL-14 and absent in del5BL-6 and del5BL-1, and were mapped to the deletion bin 5BL1-0.55-0.75 (Figure 2a). The three fragments span two chromosome bins and map close to the deletion breakpoint of 5BL1-0.55. The diagnostic WPG90, PSR2120, and PSR574 fragments are missing

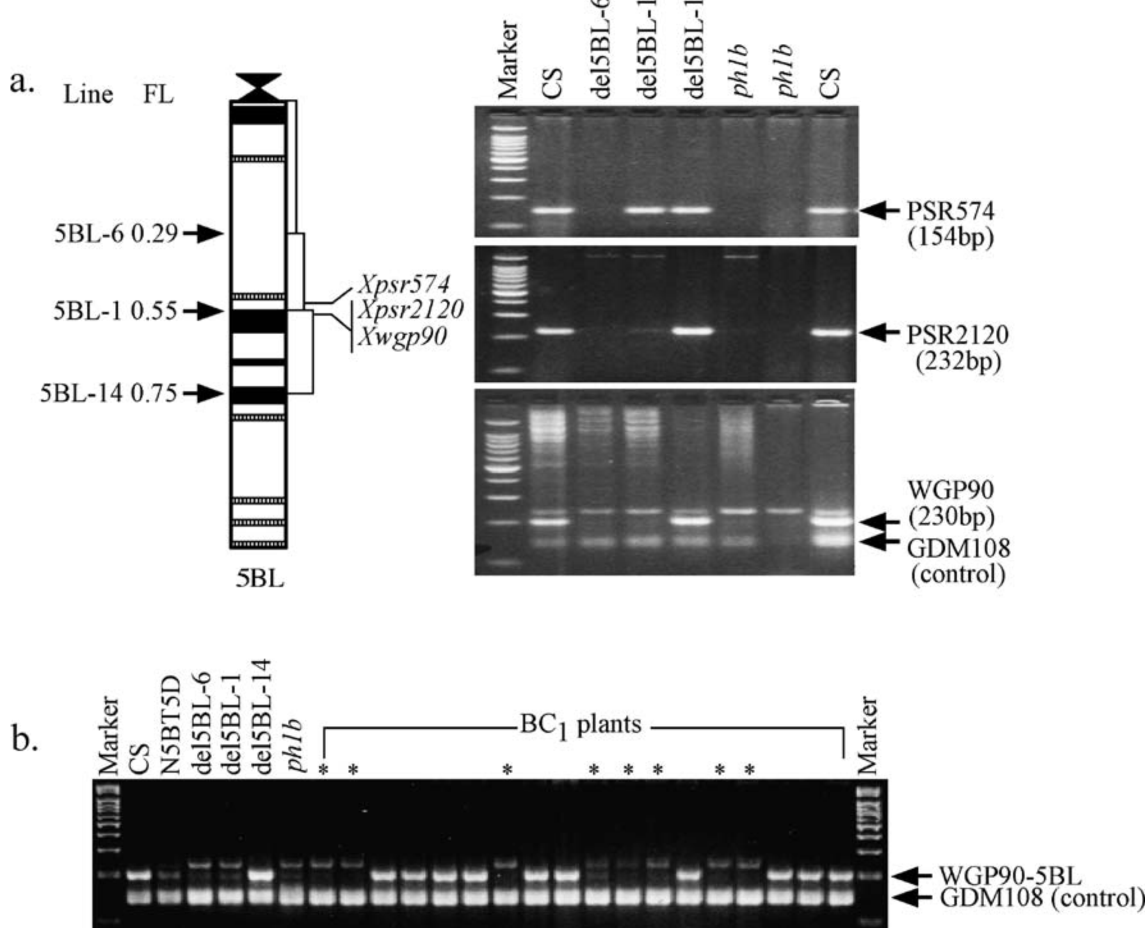

Figure 2. Physical mapping of markers PSR574, WGP90, and PSR2120 to chromosome bins in the long arm of chromosome 5B. The markers were used to detect homozygous *ph1b* plants in BC_1 progeny. (**a**) PCR patterns of markers PSR574, WGP90, and PSR2120. A 5BL-specific fragment detected by PSR574 was absent in del5BL-6, but present in del5BL-1 and del5BL-14. Thus, PSR574 mapped to the bin 5BL6-0.29-0.55. The two markers, WGP20 and PSR2120, had 5BL-specific fragments absent in del5BL-6 and del5BL-1 but present in del5BL-14 and, thus, these markers mapped to bin 5BL1-0.55-0.75. These three markers map close to the deletion breakpoint of 5BL-1. The SSR marker GDM108 was used as a positive control. The idiogram of the C-banded 5B chromosome indicates the breakpoints of the deletion lines, and lists FL values and bin locations of the three markers. The 5B C-banding pattern was taken from Gill *et al.* (1991). (**b**) Identification of homozygous *ph1b* plants in BC_1 progeny of the cross T4DL·4Ai#2S X *ph1b*. A WGP90 5BL-specific fragment was absent in N5BT5D, del5BL-6, del5BL-1, *ph1b*, and in eight BC_1 plants. * BC_1 plants homozygous for *ph1b*.

in the *ph1b* mutant stock, allowing identification of homozygous *ph1b* genotypes.

Development of STS markers polymorphic between the wheat 4DS and *Th. intermedium* 4Ai#2S arms: a sample of 106 EST-based primers were used to screen the two parents, CS *ph1b* and Karl 92, a backcross parent used to produce WGRC27, and the two translocation stocks WGRC27 (T4DL·4Ai#2S) and CI17766 (T4AL· 4Ai#2S). The PCR products were separated on a 1% agarose gel. Only primers of EST BG263898 amplified a polymorphic band in T4DL·4Ai#2S. To increase the level of polymorphism the PCR products were digested with the four-base cutter restriction enzymes *Alu*I, *Hae*III, *Hpa*II, *Msp*I, and *Rsa*I before size separation on a 1% agarose gel. Eight additional primers detected polymorphisms between the wheat parents and translocation stocks. The primer/enzyme combinations are listed in Table 1. These polymorphic primers spanned the entire 4Ai#2S arm. Nullisomic–tetrasomic and ditelosomic stocks of group 4 chromosomes were used to test these polymorphic primers and to identify 4DS-specific fragments. Of the nine polymorphic primers, two (BE444811 and BG263898) are co-dominant markers. The 4DS fragments detected by these two markers can be distinguished from those of chromosomes 4A and 4B (Figure 3). The other seven primers produced dominant markers where the 4DS band co-migrated with those of 4A and 4B, but 4Ai#2S was polymorphic (Figure 3).

It is worth noting that the PCR primer of BG263898 consistently amplified *Th. intermidium* fragments from DS4Ai#2(4D) and T4DL·4Ai#2S. However, we found that a 4DS-specific fragment was missing in DS4Ai#2(4D), but a fragment with a size similar to 4DS fragment seemed to be present in the homozygous translocation of T4DL·4Ai#2S, which was expected to be the missing 4DS fragment (Figure 3). To avoid a PCR error, 22 plants from the original germplasm with T4DL·4Ai#2S were checked with primer BG263898. All had a similar 4DS fragment. However, the RFLP analysis with BG263898 as a probe showed that the 4DS-specific fragment is missing in all of these plants, indicating that the germplasm is homozygous for T4DL·4Ai#2S. Most probably a competitive amplification occurred when the 4DS arm was missing in the translocation stock and the amplified product has molecular weight similar to the 4DS fragment. Competitive amplification is dependent on the constitution of wheat–alien lines and does not occur in DS4Ai#2(4D), which is also nullisomic for the 4DS arm (Figure 3).

Table 1. Primer sequences of STS markers derived from wheat EST on the short arm of wheat chromosome 4D and primer/enzyme combinations producing polymorphic PCR products

Marker	EST	Forward primer 5′-3′	Reverse primer 5′-3′	Annealing temperature (°C)	Enzyme producing polymorphic PCR product
BF483255-STS	BF483255	CGAACGAGAAAGTGCCCTAC	CAGCTTCATGGGGTCAATG	51	*Rsa*I
BE444811-STS	BE444811	GGAATCTCGGGATTTCTGTG	GCGAATCTCAAGCTCCATGT	51	*Hae*III, *Hpa*II *Msp*I, *Rsa*I
BE404401-STS	BE404401	AAGGCCTCTGGACAGTCTGA	AGGAGGTGTGTCCATATCGC	51	*Rsa*I
BG275006-STS	BG275006	GGACCCCTTCTACATCGTCA	GCTTGGATCTGCCTAGTTCG	51	*Msp*I, *Rsa*I
CD453648-STS	CD453648	CAAGGACCGGTACTCATCGT	ATGGTTGAGGAGGGTTTCAA	51	*Alu*I
BE500311-STS	BE500311	GCTGAAACAAGAGATTGGCTTA	GCCTTATGCCGCGTAATAGAT	53	*Hae*III
BG263898-STS*	BG263898	TGCTCAATAAGAACTGGCAGAACG	GGAATCACAACTCAGGGGAAACAG	56	*Hae*III
BE423257-STS	BE423257	CCTCTAATGCCAGACACCCTTTG	ACAGCAAGTGAAACTCGCCTGC	55	*Msp*I
BF483640-STS	BF483640	AGTGAGGAGATGCACAACCC	TAGCCAAAACCAGCTCCAGT	51	*Hae*III, *Msp*I

* The primer BG263898 produces a polymorphic fragment with or without enzyme digestion.

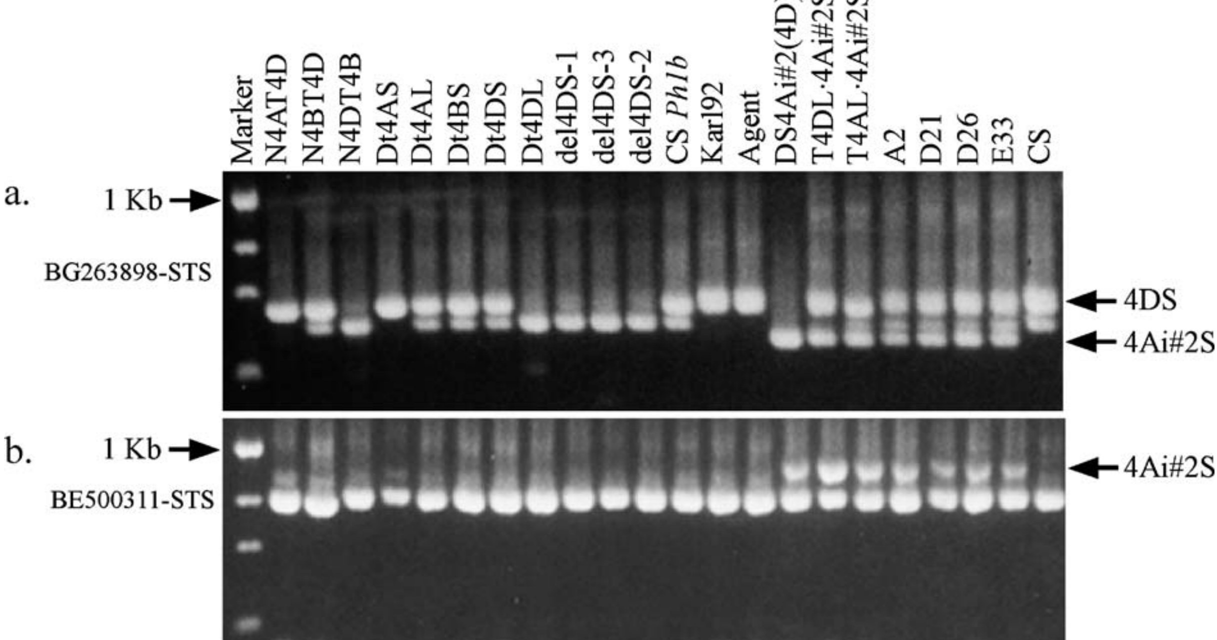

Figure 3. Testing PCR markers with nullisomic-tetrasomic (NT) and ditelosomic (Dt) lines of group 4. (**a**) PCR pattern of BG263898-STS. A 4DS fragment amplified by BG263898 primers could be distinguished from 4A and 4B. The 4DS fragment was absent in N4DT4B, Dt4DL, del4DS-1, del4DS-3, del4DS-2, and DS4Ai#2 (4D). However, another fragment with a size similar to the 4DS fragment was present in homozygous translocation T4DL·4Ai#2S (see text). Plants A2, D21, D26, and E33 were selected from the BC_1 of cross T4DL·4Ai#2S X *ph1b*. These were homozygous for *ph1b* and heterozygous for 4D and T4DL·4Ai#2S. A 4Ai#2S fragment was detected in all wheat–*Th. intermedium* introgression lines. (**b**) PCR pattern of marker BE500311-STS. The 4DS fragment could not be distinguished from 4A and 4B. The 4Ai#2S fragment amplified by the EST primers was present in all wheat–*Th. intermedium* introgression lines.

Identification of recombinants

To further engineer T4DL·4Ai#2S, the translocation stock WGRC27 was crossed with the homozygous *ph1b* mutant stock and the F_1 was backcrossed again with the *ph1b* mutant (Figure 1). Markers WPG90, PSR2120, and PSR574 were used to screen 33 BC_1 plants, and 12 homozygous *ph1b* genotypes were identified (Figure 2b).

The nine polymorphic PCR markers developed for the 4Ai#2S arm were used to screen the 12 homozygous *ph1b* plants. Four plants (A2, D21, D26, and E33) were heterozygous for chromosomes 4D and T4DL·4Ai#2S (Figure 3). GISH analysis on the PMC at meiotic metaphase I in D26 plant was used to determine the amount of chromosome pairing between the 4DS and 4Ai#2S arms. A 4D/T4DL·4Ai#2S ring bivalent was observed in one of 57 PMC analyzed (Figure 4a), indicating that 4DS/4Ai#2S recombinants can be expected at a frequency of about 2%.

The four BC_1 plants homozygous for *ph1b* and heterozygous for chromosomes 4D and T4DL·4Ai#2S were either selfed or backcrossed to elite cultivars to produce wheat–*Th. intermedium* recombinants (Figure 1). A total of 245 progeny were obtained from BC_2 or BC_1F_2. The two co-dominant markers, BE444811 and BG263898, were first used to screen the 245 progeny. Five plants were identified as recombinants (Figure 5) and were further screened by the remaining seven dominant markers to identify the breakpoints in the recombinant chromosomes. Screening for the *ph1b* deletion in these recombinants indicated that three (#36, #64, and #213) were *ph1b* heterozygotes that came from BC_2 progeny, and two (#45 and #87) progeny were homozygous *ph1b* plants from the BC_1F_2. Two types of recombinants were observed (Figure 6). Plants #64, #87, and #213 were homozygous for the proximal 82% of 4DS and heterozygous for the distal 18% of 4DS and 4Ai#2S (Figure 6). Plant #45 was homozygous for the proximal 82% of 4DS arm and appeared to be homozygous for the distal 18% of 4Ai#2S. Plant #36 was heterozygous for the proximal 82% of 4DS and 4Ai#2S and homozygous for the distal 18% of 4DS (Figure 6). All crossovers occurred in the distal chromosome bin 4DS2-0.82-1.00.

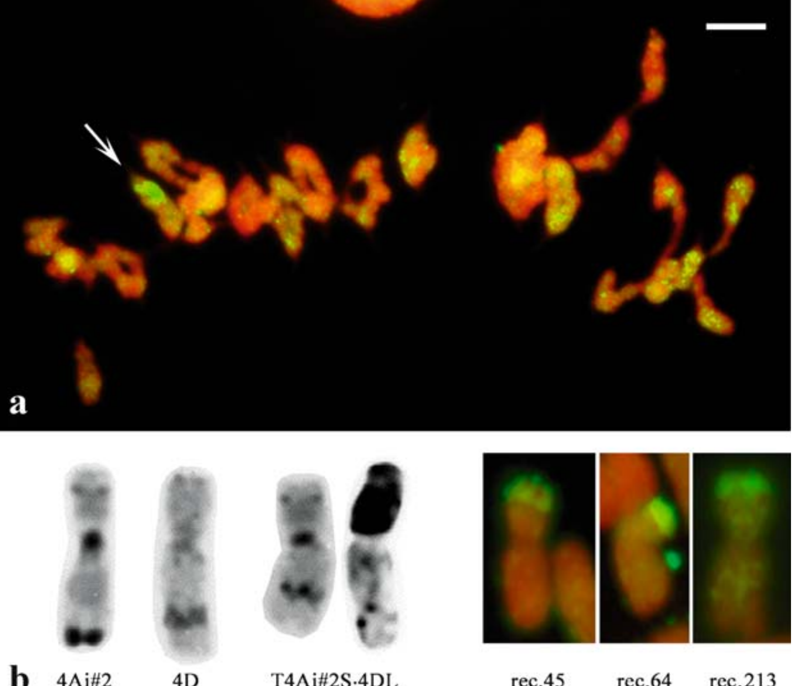

Figure 4. C-banding and FISH patterns of wheat-*Th. intermedium* introgression lines at mitotic metaphase and meiotic metaphase I. (**a**) FISH pattern of a BC_1 plant heterozygous for 4D and T4DL·4Ai#2S and homozygous for *ph1b* at meiotic metaphase I. Homoeologous pairing occurred between wheat and alien chromosomes. Arrow indicates a ring bivalent formed between 4D and T4DL·4Ai#2S. Scale bar = 10 μm. (**b**) C-banding and GISH patterns of the critical chromosomes involved in wheat–*Th. intermedium* introgression lines: from left to right, C-banding pattern of the *Th. intermedium* chromosome 4Ai#2, wheat chromosome 4D, the wheat–*Th. intermedium* Robertsonian translocation chromosome T4DL·4Ai#2S (GISH pattern shown on the right), and GISH patterns of three wheat–*Th. intermedium* recombinant chromosomes.

GISH using *Th. intermedium* genomic DNA as probe was performed on the five different recombinants. The GISH results were consistent with molecular marker data. In plant #45 two chromosomes had distal GISH signals in their short arms, whereas in plants #64 and #213, only one chromosome had a distally located GISH signal in the short arm (Figure 4b). GISH analysis did not detect the distal region of 4DS in the recombinant chromosome of plant #36. The GISH signal covered the entire short arm of the recombinant chromosome in this plant.

To distinguish crossovers in the five recombinant lines, two RFLP markers (Qi unpublished data) and 10 EST markers previously mapped to the distal bin 4DS2-0.82-1.00 (http://wheat.pw.usda.gov/NSF/project/mapping_data.html) were further used to check these recombinants. The crossovers in all five lines were localized to marker intervals within bin 4DS2-0.82-1.00. Lines rec.45 and rec.213 had crossovers between markers CDO453648 and BF291316 (Figure 6); rec.36, rec.64, and rec.87 had crossovers between markers BE403913 and BF229225 (Figures 6 and 7). The *Th. intermedium* segments are longer in rec.45 and rec.213 than in rec.64 and rec.87.

Transmission of recombinant chromosomes and wheat streak mosaic virus resistance evaluation

The four recombinants (rec.45, rec.64, rec.87, and rec.213) with proximal part derived from 4DS and the distal parts from 4Ai#2S, together with the recombinant rec.36, in which the proximal segment was derived from 4Ai#2S and the distal from 4DS, were either backcrossed to Wichita or allowed to self. These progenies were screened with the molecular markers developed in the present study to obtain preliminary data on the transmission of the recombinant wheat–*Th. intermedium* chromosomes and their reactions to wheat streak mosaic virus infection.

All recombinant chromosomes were transmitted through the selfed or BC progenies, but the progeny

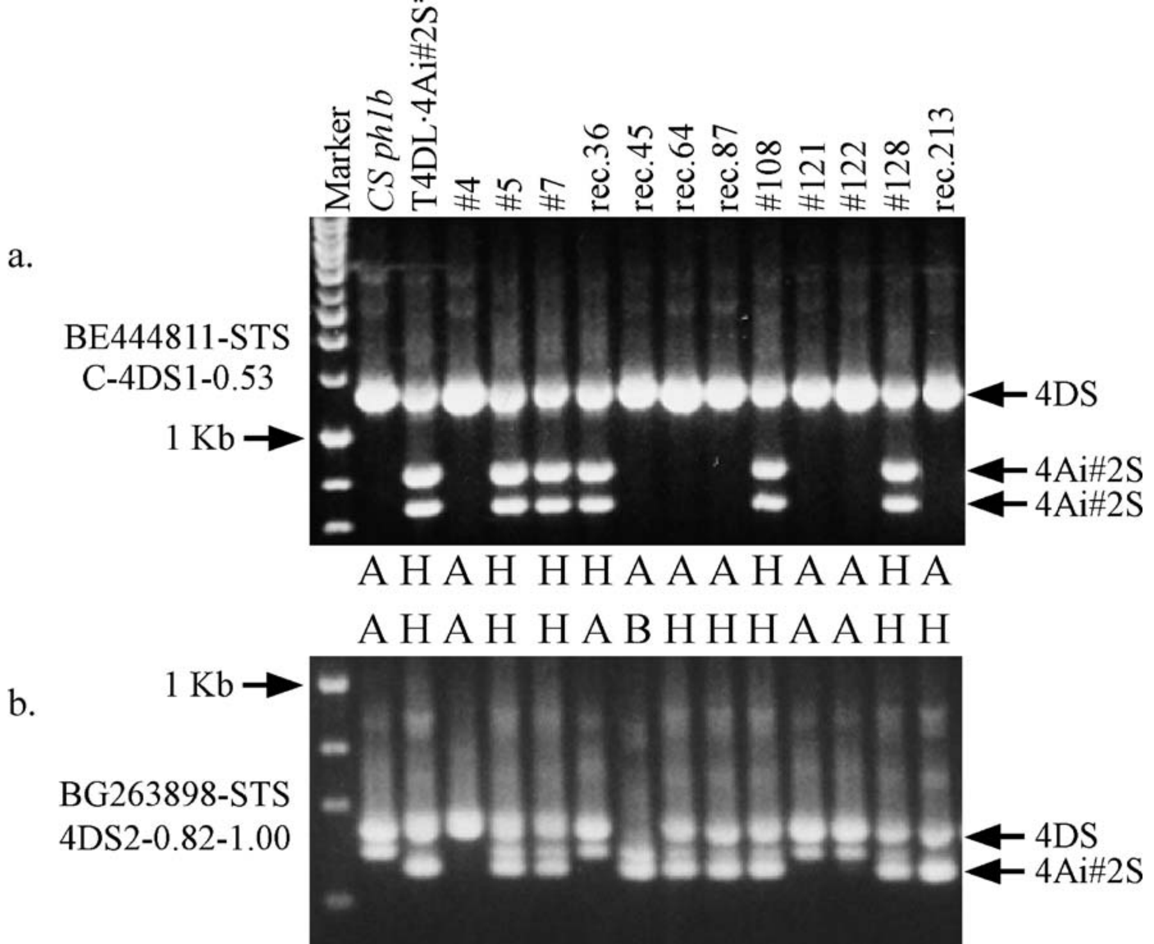

Figure 5. Detection of recombinants in BC_2 and BC_1F_2 progenies derived from plants that were homozygous for *ph1b* and heterozygous 4D and T4DL·4Ai#2S. (**a**) PCR pattern of BE444811-STS, which is a proximal marker. The PCR products were digested with enzyme *Msp*I. (**b**) PCR pattern of BG263898-STS, which is a distal marker. Five recombinants were detected between the two markers. Plant rec.45 appeared to be a homozygous recombinant. A: homozygous 4DS; B: homozygous for the 4Ai#2S *Th. intermedium* chromosome segment; H: heterozygous. * heterozygous translocation T4DL·4Ai#2S.

numbers were too small to detect deviations from expected Mendelian ratios (Table 2). The transmission data of rec.45 were of interest because the parent plant appeared to be homozygous for the recombinant chromosome based on molecular marker and GISH data. Of the 20 progeny from the cross rec.45 × Wichita, nine plants had the recombinant chromosome and 11 were homozygous for 4D, indicating that the parent plant was heterozygous for 4D and rec.4D chromosomes. Two selfed progeny appeared to be homozygous for rec.4D. The most likely explanation of these results is that the parent plant was monosomic for the rec.4D chromosome in germ tissue and chimeric for a pair of rec.4D chromosomes in root tissue.

Based on the preliminary data, only rec.213 appeared to be resistant to WSMV. Nine plants were evaluated for reaction to wheat streak mosaic virus. Two plants were homozygous for 4D and were highly susceptible. Two plants were heterozygous (rec.213/4D) and were highly to moderately susceptible. Four plants were homozygous for the recombinant chromosome 213 and were either moderately or highly resistant, suggesting that *Wsm1* gene was retained in these recombinants. All other recombinant progenies gave susceptible reactions. However, larger numbers of homozygous progenies from all recombinants need to be screened in replicated experiments for a thorough evaluation of their WSMV response.

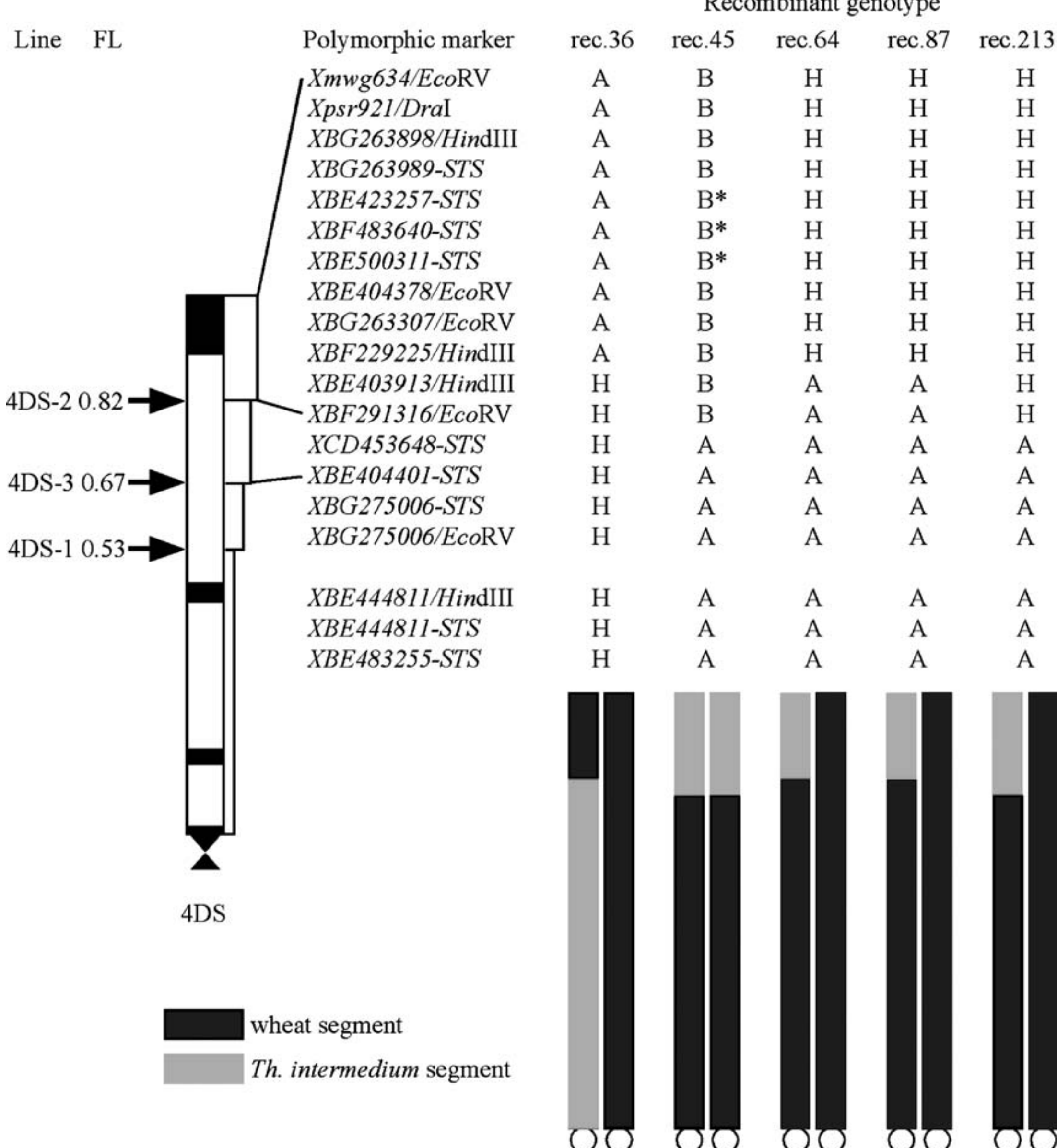

Figure 6. Genotyping the recombinant lines by RFLP and STS markers. Ideogram of C-banded chromosomes of 4DS arm is after Gill *et al.* (1991). The deletion names and breakpoints (indicated as fraction length from the centromere) are on the left and the markers are on the right of chromosome. The position of RFLP markers MWG634 and PSR921 is according to Qi (unpublished data). * Possible homozygous genotype of rec.45, but indistinguishable from heterozygote based on these STS markers.

Discussion

The major objective of this research was to manipulate homoeologous recombination to engineer an alien chromosome segment carrying *Wsm1*. For this purpose an integrated approach combining cytogenetic and molecular resources was used, and five recombinant chromosomes were isolated. One of the recombinants (rec.213), where 82% of the proximal alien chromatin had been eliminated and the distal 18% recovered as a translocation to the distal end of the wheat chromosome 4D, appeared to be resistant to WSMV. Because the recombinant chromosome is the product of a homoeologous recombination event,

it should be a genetically compensating transfer. This was verified by mapping 11 EST-derived markers that co-localized to the alien segment and the missing wheat segment. Further screening, agronomic evaluation, and breeding of the critical recombinant line lie ahead, but the potential contribution of this material in agriculture appears promising. This chromosome engineering experiment was unique because there was no easily scorable cytological marker or disease phenotype. Therefore, the development of molecular marker resources for detecting recombinants was critical to the success of the experiment. The kinds of markers, how many, and the amount of chromosome coverage needed to screen progeny are pertinent questions that need discussion.

More than 16 000 EST loci already mapped in chromosome deletion bins (Qi *et al*. 2004) provide an excellent resource for marker development for specific chromosome regions in wheat. We exploited this resource to develop a set of STS markers. Off 106 bin-mapped 4DS-specific EST markers, only nine STS (seven dominant, two co-dominant) markers were polymorphic between the 4DS and 4Ai#2S arms. However, they provided complete coverage of the 4S arms. This approach provided 9% informative markers.

Table 2. Transmission of recombinant chromosomes in progenies

Recombinant	Material	Genotype of progenies		
		4D/4D	rec./rec.	4D/rec.
	rec.36 selfed	4	2	4
	rec.45 selfed		2	
	rec.45 X Wichita	11		9
	rec.64 selfed	2	2	11
	rec.64 X Wichita	3		7
	rec.87 selfed	2	1	9
	rec.87 X Wichita	2		7
	rec.213 selfed	2	4	3
	rec.213 X Wichita	3		7
	Overley X rec.213	7		5
	Overley/Armadia X rec.213	10		8

wheat; *Thinopyrum*

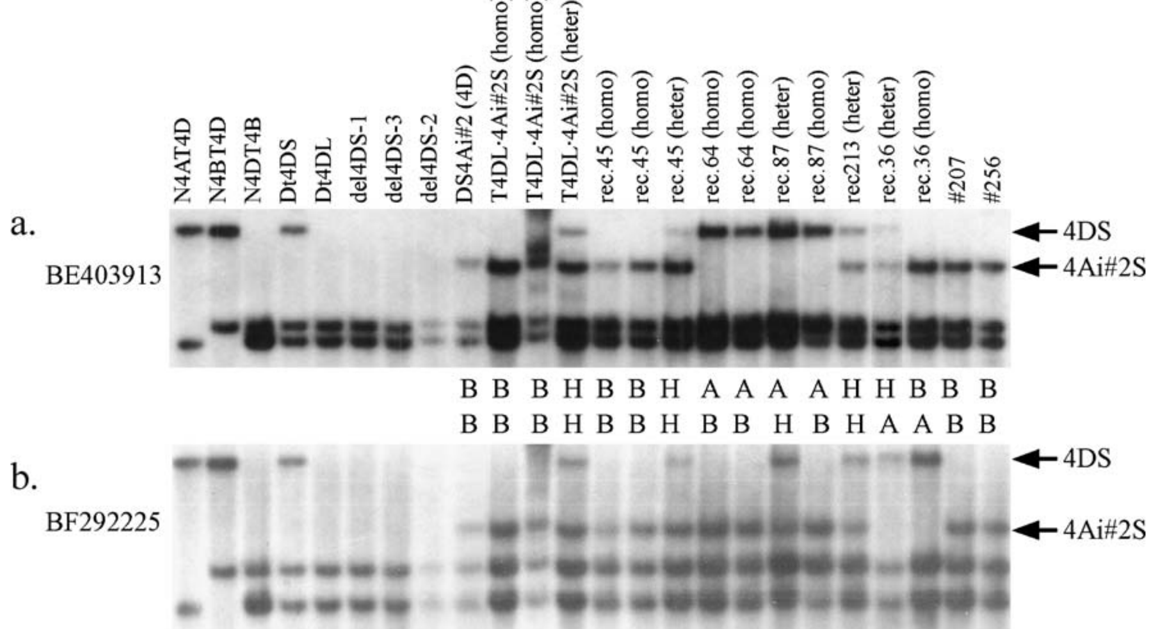

Figure 7. RFLP analysis to detect crossovers among recombinant lines: (**a** and **b**) autoradiographs of Southern hybridizations of genomic DNA of group 4 homoeologous NT, Dt lines, wheat–*Th. intermedium* disomic substitution DS4Ai#2(4D), translocation T4DL·4Ai#2S, and recombinant (rec.) lines. Genomic DNA was digested with restriction enzyme *Hin*dIII and probed with ESTs BE403913 (**a**) and BF292225 (**b**). Crossovers were found between BE403913 and BF292225 in rec.36, rec.64, and rec.87. The homozygous recombinants of rec.36, rec.64, and rec.87 were derived from the selfed progeny of the original recombinants. Heterozygous rec.45 was derived from the progeny of homozygous rec.45 × Wichita. The plants #207 and #256 were derived from BC_2 or BC_1F_2 of the cross T4DL·4Ai#2S X *ph1b* and were homozygous for translocation T4DL·4Ai#2S.

Among the other PCR-based methods, locus-specific genomic SSR (gSSR) and EST-SSR (eSSR) markers are the most widely used classes of markers. However, gSSR markers developed for a crop plant are not transferable to all the wild relatives, especially the most distant genera. In a study of wheat and wheatgrass, only six of 163 wheat gSSR amplified the products in wheatgrass, and it is not known if these were orthologs or paralogs (Mullan *et al*. 2005). However, 41 of 165 wheat eSSR markers produced amplicons in wheatgrass and 28% detected polymorphic loci. Only nine of these markers could be assigned to homoeoloci positions (Mullan *et al*. 2005). Single-nucleotide polymorphisms (SNP) are another versatile class of markers that should be explored.

Among the other classes of markers, RFLP continue to be the most reliable and informative class of markers; but because Southern hybridization methods employed in RFLP detection do not lend themselves to high throughput and entail the use of radioisotopes, they should be strategically used in situations where PCR-based markers provide confusing results, as demonstrated in this study. The BG263898-STS produced an apparent 4DS-similar amplicon in T4DL·4Ai#2S chromosome-carrier progeny and these plants were scored as heterozygous, resulting in many more plants being scored as apparent recombinants. It took many months of careful analysis using specific genetic stocks and additional experiments to overcome this problem. Eventually, RFLP analysis using the same probe clearly showed that the specific plants were actually homozygous for the BG263898 locus. Since several hundred bin-mapped arm-specific RFLP loci are available in wheat, these markers are a crucial and readily available resource for second stage analysis of recombinants.

How many markers should be used to screen for the recombinant progeny? The unlinked codominant markers marking the centromere (BE444811) and telomeric (BG263898) ends were first used to screen all progeny to identify plants with recombinant chromosomes. Although seven additional markers

were used to screen the progeny, no additional recombinants were detected. Our experiment showed that for the primary screening, two (one centromeric and one telomeric-specific) informative co-dominant markers are sufficient to recover all recombinants. It is becoming clear that homoeologous recombination events are rare and result from first-order chiasmata as documented by Lukaszewski (1995). Additional markers then are needed only for secondary screening of the recombinant plants for determining the distribution of recombination sites and estimating the size of the transferred segment. For this purpose, highly informative RFLP markers were used in this study (Figure 6).

While developing molecular markers, it is important to ensure that they provide a complete coverage of the arm. This is best done by selecting bin-mapped markers (http://wheat.pw.usda.gov/NSF/project/mapping_data.html), making sure that at least one informative marker is selected from each bin. Furthermore, the centromeric bin marker should be tightly linked to the centromere and the telomeric bin marker should be the most distal marker on a genetic map. Whether all the recombinants are recovered or not depends on the location of the terminal marker because most recombination in wheat is restricted to the distal ends of chromosomes. From the results we cannot conclude unequivocally that we have recovered all the recombinants, because the exact location of the most terminal marker is not known. The coverage of markers being discussed here is so low that only events related to crossover recombination will be detected. We do not know if non-crossover events (Huang *et al.* 2003) that are detected in high-volume mapping occur in such wide transfers as reported here.

Apart from marker resource development, how do we estimate the size of the progeny (BC_1F_2 or BC_2) needed to isolate a desired number of recombinants? This mostly depends on homoeologous recombination ratio. A direct estimate of the required progeny size is the chromosome pairing in BC_1 plants that are homozygous for *ph1b* and heterozygous for the targeted arm. In this experiment four BC_1 plants were found to be homozygous for *ph1b* and heterozygous for 4D and T4DL·4Ai#2S. Meiotic analysis from one of the plants indicated that the 4DS and 4Ai#2S arms paired at a frequency of 2%. Thus, about 300 progeny would be sufficient to isolate six recombinants. Five recombinants were recovered from 245 BC_1F_2 and BC_2 progenies.

How many recombinants should be obtained to maximize the likelihood of recovering recombinants carrying a target gene, but with the least amount of the alien chromatin? This question has been discussed previously and depends on whether the homoeologous recombination events are random. As observed in rye (Lukaszewski *et al.* 2004) and documented here for wheatgrass, homoeologous recombination is nonrandom. All recombination events were restricted to the distal 18% of the arm. Two occurred between markers CDO453648 and BF291316 at the boundary of del4DS-2 at FL 0.82, and three recombination events occurred between markers BE403913 and BF229225 distal to del4DS-2 at FL 0.82 (see Figure 6). Lukaszewski (1995) provided an elegant explanation of these results. Chromosome interference determines the nature of chiasmate associations. Because pairing is initiated at the chromosome ends, first-order chiasmata occur at the chromosome ends, as is well known in wheat (Sallee & Kimber 1978). The second- and third-order chiasmata occur in progressively proximal regions. Apparently, extreme positive interference between homoeologous chromosomes either eliminates chiasmate associations, hence recombination is entirely or very largely restricted to first-order chiasmata. Therefore, the focus of a chromosome engineering exercise should not be on obtaining a large number of recombinants, which will be wasteful use of resources. Instead, the focus should be on obtaining proximal and distal recombinants spanning the target gene in order to permit a second round of recombination as proposed by Sears (1981) and demonstrated by Lukaszewski (1997). In our experiment we recovered a proximal recombinant (rec.36) that should be resistant based on marker analysis. By crossing this recombinant with distal rec.213 we should be able to recover an interstitial alien transfer with *Wsm1*. Such an experiment will be undertaken if the virus resistance in homozygous rec.36 plants is verified.

One problem that we did not encounter, but reported in other chromosome engineering experiments in wheat, was the high frequency (up to 20%) of recombination between non-designated homoeologues (Lukaszewski 2000 and the references cited therein). This appears to be more frequent for homoeologous arms that show reduced pairing. Depending upon the purpose of the experiment, it may not be a problem as long as the genetic transfer involves homoeologous segments.

Although the impact of nonrandom distribution of homoeologous recombination in chromosome engineering has been discussed, the impact of this

phenomenon on genetic maps based on homoeologous recombination has not. As discussed in the introduction, homoeologous recombination-based maps have been made in *Lolium* and *Allium* (King *et al*. 2002, Khrustaleva *et al*. 2005). It is not known how these maps compare with maps based on homologous recombination. Moreover, recombination sites in homoeologous recombination-based maps are visualized by GISH. As demonstrated by Lukaszewski *et al*. (2005), GISH has limited resolution. We were also unable to detect one out of five recombinants by GISH, although molecular data were unequivocal. These aspects of homoeologous recombination-based maps need further attention in those organisms.

In wheat there are several options for manipulating the *Ph1* gene for induced homoeologous recombination. One class of methods involves the elimination of the *Ph1* gene either through the use of nullisomy for 5B or deletion mutants spanning the *Ph1* locus such as *ph1b* and *ph1c* (Sears 1977, Giorgi 1983). Scoring homozygous *ph1b* (or homozygous *ph1c*) phenotypes is difficult and time-consuming, and methods bypassing this requirement have been developed but they require the skillful use of cytogenetic stocks (Sears 1977). More recently, molecular markers were developed that allow tracking the *ph1b* deletion in segregating populations (Segal *et al.* 1997, Roberts *et al.* 1999). In this experiment we validated three previously reported markers that are proficient for scoring homozygous *ph1b* plants. The homozygous *ph1b* plants are preferable to nullisomic-5B plants, which are subject to reduced fertility.

The second class of methods involves the use of genes that are epistatic to *Ph1* as first reported by Riley *et al.* (1968a,b). The attractive feature of these dominant so-called Ph^I genes (Chen *et al.* 1994) is that high pairing is observed in F_1 hybrids and progeny can be screened for recombinants (Chen *et al.* 1994). However, the Ph^I stock is difficult to maintain and the gene is difficult to follow in segregating populations because of a lack of a tightly linked molecular marker.

By necessity, chromosome manipulation by homoeologous recombination has been limited to a single chromosome or chromosome arm. However, the power of molecular marker analysis is such that we propose a scheme for genome-wide production of recombinant chromosome stocks. This scheme takes into account the knowledge that homoeologous recombination is limited to one or a few sites in each arm, and genes determining most agronomic traits are located in the terminal ends of chromosomes (Qi *et al.* 2004). Also the same number of progenies is needed for the whole genome as is needed for a single chromosome arm manipulation scheme. Thus, for each alien chromosome, four recombination events – two per arm – are needed. A battery of PCR-based codominant centromeric and telomeric markers are required. The difficulty will be devising a crossing scheme to obtain plants that are homozygous for *ph1b* and heterozygous for a set of seven wheat and alien chromosomes. One possibility involves the crossing of the *ph1b* stock ($AAB^{ph1b}B^{ph1b}DD$) with an alien donor species (WW), followed by colchicine-induced chromosome doubling. The resulting ($AAB^{ph1b}B^{ph1b}DDWW$) amphiploid can be crossed with the *ph1c* durum wheat stock ($AAB^{ph1c}B^{ph1c}$) and homoeologous recombination targeted between D- and W-genome chromosomes in the derived $AAB^{ph1b}B^{ph1c}DW$ hybrid. In the absence of GMO (genetically modified organism) wheat, wider exploitation of wheat gene pools may be a necessity for novel traits. All methods that will enhance the efficiency of gene pool mining are worthy of further exploration.

The prospects of chromosome engineering for other crops appear to be quite good because homoeologous pairing is not restricted to the same extent in plants such as *Lolium*, *Festuca*, *Allium*, tomatoes, and *Brassica* (King *et al.* 2002, Jenczewski *et al.* 2003, Ji & Chetelat 2003, Khrustaleva *et al.* 2005) as is the case for the *Ph* system in wheat. As more knowledge about the molecular basis of homoeologous recombination (Griffiths *et al.* 2006) is gained, additional approaches can be devised for its manipulation in a diverse range of crop plants.

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