Introgression of chromosome segments from multiple alien species in wheat breeding lines with wheat streak mosaic virus resistance

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ABSTRACT

Pyramiding of alien-derived *Wheat streak mosaic virus* (WSMV) resistance and resistance enhancing genes in wheat is a cost-effective and environmentally safe strategy for disease control. PCR-based markers and cytogenetic analysis with genomic *in situ* hybridization were applied to identify alien chromatin in four genetically diverse populations of wheat (*Triticum aestivum*) lines incorporating chromosome segments from *Thinopyrum intermedium* and *Secale cereale* (rye). Out of twenty experimental lines, ten carried *Th. intermedium* chromatin as T4DL*4Ai#2S translocations, while, unexpectedly, seven lines were positive for alien chromatin (*Th. intermedium* or rye) on chromosome 1B. The newly described rye 1RS chromatin, transmitted from early in the pedigree, was associated with enhanced WSMV-resistance. Under field conditions, the 1RS chromatin alone showed some resistance, while together with the *Th. intermedium* 4Ai#2S offered superior resistance to that demonstrated by the known resistant cultivar Mace. Most alien-wheat lines carry whole chromosome arms, and it is notable that these lines showed intra-arm recombination within the 1BS arm. The translocation breakpoints between 1BS and alien chromatin fell in three categories: 1) at or near to the centromere, 2) intercalary between markers UL-Thin5 and *Xgwm1130*, and 3) towards the telomere between *Xgwm0911* and *Xbarc194.* Labelled genomic *Th. intermedium* DNA hybridized to the rye 1RS chromatin under high stringency conditions, indicating the presence of shared tandem repeats among the cereals. The novel small alien fragments may explain the difficulty in developing well-adapted lines carrying *Wsm1* despite improved tolerance to the virus. The results will facilitate directed chromosome engineering producing agronomically desirable WSMV-resistant germplasm.

KEYWORDS

Fluorescent *in situ* hybridization, molecular markers, wheat, *Thinopyrum intermedium*, rye, *Wheat streak mosaic virus*

INTRODUCTION

Wheat streak mosaic virus (WSMV), transmitted by the wheat curl mite (WCM) *Aceria tosichella* Kiefer, is an important yield limiting disease of wheat (*Triticum aestivum*, 2n=6x=42, AABBDD) (Graybosch *et al*., 2009). First recorded in the United States, it has since spread widely, infecting both winter and spring wheat cultivars. Infected plants show greenish yellow streaks and chlorosis with stunted growth, low root biomass, reduced water uptake efficiency and low yield (Thomas *et al*., 2004; Price *et al*., 2010). Both WSMV and the mite vector survive during the late summer on 'green bridges' provided by volunteer wheat and other susceptible wild and cultivated grasses (Divis *et al*., 2006). Since viral diseases cannot be controlled directly by agrochemicals, management of insect vectors and agronomic conditions must be employed. Some transgenic wheat lines have been developed with WSMV-resistance in controlled environments (Fahim *et al.*, 2010), but have not been tested in the field.

Genetic resistance to WSMV offers the most environmentally and economically desirable strategy for disease control and has been pursued since resistance to WSMV was discovered (Friebe *et al*., 1991; Graybosch *et al.*, 2009; Mutti *et al*., 2011). Today, there is huge interest in both using genomic prediction to access genotype × environment interaction using marker and pedigree information in breeding programmes (Crossa *et al*., 2014), and in enhancement of the genetic variability available in wheat through wide hybridization (see Heslop-Harrison and Schwarzacher, 2012). Among the natural sources, the gene pool of rye *Secale cereale* (2n=14, genomes RR) and the perennial wheat grasses, *Thinopyrum intermedium* (Host) Barkworth and Dewey syn. *Agropyron intermedium* (Host) P. Beauv. (2n=6x=42, JJJ^{s}J^{s}SS) and *Th. ponticum* (Podp.) Barkworth and D.R. Dewey (2n=10x=70, JJJJJJJ^{s}J^{s}J^s) are of particular interest (Kim *et al.*, 2004; Li and Wang, 2009). These sources provide large reservoirs of useful genes including WCM and WSMV-resistance, and chromosomes or chromosome segments from *Thinopyrum* have been transferred into wheat backgrounds (Li *et al*., 2007; Graybosch *et al.*, 2009). While rye is susceptible to WSMV, there is evidence that rye genes in wheat background can delay the spread of the disease (Li *et al*., 2007) through resistance to either the vector or the virus itself. Many wheat lines carry a T1BL*1RS translocation, with the 1RS rye chromosome arm being reported to confer a range of biotic and abiotic stress resistances (Heslop-Harrison *et al*., 1990; Kim *et al.*, 2004). Two genes for WSMV-resistance, *Wsm1* and *Wsm2*, have been used in wheat improvement (Graybosch *et al*., 2009; Haley *et al*., 2011). *Wsm1* is present on the short arm of *Th. intermedium* chromosome 4Ai#2, and offers effective field resistance to WSMV in wheat (Chen *et al.*, 2003; Friebe *et al.*, 2009). The *Wsm2* gene (of unknown origin, but perhaps from bread wheat itself) was mapped to the short arm of wheat chromosome 3B (Lu *et al.*, 2011). A third *Th. intermedium* derived gene, *Wsm3*, was mapped to chromosome T7BS*7S#3L (Liu *et al*., 2011) but it is yet to be exploited commercially.

The *Wsm1*, *Wsm2* and *Wsm3* resistances show temperature dependency (Liu *et al.*, 2011; Seifers *et al*., 2013). Lines with *Wsm1* gene from 4Ai#2S are resistant at 20°C and delay symptoms of the disease up to 25°C but are susceptible at 28°C (Fahim *et al.*, 2012). *Wsm2* resistance was originally ineffective above 18°C but exposure to virus over several generations resulted in *de novo* resistance up to of 28°C (Fahim *et al*., 2012; Seifers *et al*., 2013) while, the *Wsm3* derivatives displayed effective resistance at a temperature of 24°C (Liu *et al*., 2011). Wheat-*Th. intermedium* substitution lines that carried entire chromosomes 4Ai#2 were resistant at 27°C, suggesting the presence of further resistance genes on *Th. intermedium* 4Ai#2L (Fahim *et al*., 2012). *Wsm1* might also be effective against other viruses, as the winter wheat cultivar 'Mace' (Graybosch *et al*., 2009) with *Wsm1* gene resists the co-infection of WSMV and the related *Triticum mosaic virus* (TriMV) up to 19°C, indicating the effectiveness of *Wsm1* selection in disease synergism (Liu *et al.*, 2011).

Transfer of desirable genes and gene combinations into varieties with durable and non-race-specific resistance constitute core objectives of modern plant breeding (Tester and Langridge 2010;

Heslop-Harrison and Schwarzacher 2012). The successful transfer of 4Ai#2S of *Th. intermedium* in the form of the T4DL*4Ai#2S translocation represents the most widely exploited source of WSMV-resistance (Wells *et al.*, 1982; Friebe *et al.*, 2009). The present study used genomic *in situ* hybridization (GISH; Schwarzacher *et al*., 1992) complemented by targeted DNA markers and field evaluation to characterize resistance in Nebraska-adapted winter wheat lines originating from four genetically diverse populations that include the Kansas WSMW-resistant *Th. intermedium* lines in their pedigree, and where resistant and susceptible sister-lines are available within populations (Divis *et al*., 2006).

MATERIALS AND METHODS

Plant material

Table 1 lists the wheat lines used in this study and pedigrees where known. Reference WSMV-resistant (R) lines KS95H102 and KS96HW10-1 both carrying the *Wsm1* gene (Divis *et al.*, 2006; Graybosch *et al*., 2009) and susceptible (S) lines 'Millennium' and 'Tomahawk' were used as controls. Experimental lines previously classified as R or S to WSMV (Divis *et al.*, 2006) were derived from four breeding populations, designated here as populations I, II, III and IV (Table 1). Mace (PI 651043; Graybosch et al 2009) was derived from population III and was tested as N02Y5117. PCR marker screening involved selected experimental lines (Table 1) as well as *Th. intermedium* (cultivars Manaska, Beefmaker, Haymaker, Reliant), *T. aestivum* land race 'Chinese Spring' wheat, 'Beaver' wheat (T1BL*1RS wheat-rye translocation), *S. cereale* 'Petkus' rye, aneuploids Chinese Spring wheat nullisomic-1B-tetrasomic-1A (CS N1B-T1A), nullisomic-4A-tetrasomic-4D (CS N4A-T4D) and nullisomic-4D-tetrasomic-4B (CS N4D-T4B) and the pedigree lines KS91H184, KS91H174, RioBlanco, MO8 Vista, Redland Tam107, Anton (see Supplementary Table S3). DNA was extracted using standard CTAB methods.

Virus screening in greenhouse and field

Lines were originally scored in 2002 for response to natural infection by WSMV at Hays, KS USA and Sidney, NE USA (Divis *et al.*, 2006). Visual subjective assessment of the degree of chlorosis and plant stunting were used (Table 1 and footnotes). Selected lines were further evaluated in the field at the University of Nebraska Agricultural Research and Development Center, Mead, NE. In 2011 individual spikes from each line were harvested and used to seedat least 20, 1.2 m replicate rows of single plant-derived lines in September 2011 as a block surrounded by early planted wheat that served as a 'green bridge' for the development of WCM populations. Multiple plantings of the resistant cultivar Mace and the susceptible line Tomahawk were included as a control. Through the fall, wheat curl mite (WCM) populations migrated from the early planted wheat to the experimental plants, providing a natural source of WSMV inoculation. The level of infestation was higher in 2012 than in 2002. Response to WSMV was rated using the visual scores (Table 1). In addition, at the flag leaf stage, chlorophyll content was assessed using a Soil Plant Analytical Development (SPAD) meter (model 502 Plus, Konica Minolta Sensing, Inc., Osaka, Japan). For each treatment, 10 SPAD readings were taken and averaged. Analysis of variance (Proc GLM, Version 9.4, SAS, Cary, NC) followed by mean separation using Duncan's multiple range test was used to compare mean SPAD readings of each experimental line to each other, and to Mace and Tomahawk; significant and not significant differences at p=0.05 are indicated in Table 1.

Fluorescent *in situ* hybridization (FISH)

Spread preparations of chromosomes were made from both seedling root tips or anthers from all 20 breeding lines and control *Th. intermedium*, normal wheats, and wheats containing the T1BL*1RS

translocation using proteolytic digestions (see Schwarzacher and Heslop-Harrison, 2000). Probes (described in Forsström *et al.*, 2002; Contento *et al.*, 2005; Patokar *et al.*, 2015) included the rDNA sequences pTa71 (9 kb complete repeat unit of 25S-5.8S-18S rDNA of *T. aestivum*) and pTa794 (410 bp fragment of 5S rDNA of *T. aestivum),* and the repetitive DNA sequences pSc119.2 (or CS13, 120 bp tandem repeat isolated from *S. cereale* and dpTa1 or Afa**,** 340 bp tandem repeat from *T. aestivum*). Small insert clones were amplified by PCR using M13 primers. Total genomic DNA from *Th. intermedium, S. cereale* and *Aegilops* (syn. *Triticum*) *tauschii* was sheared to 3-5 kb pieces by autoclaving. For labelling, biotin-16-dUTP and digoxigenin-11-dUTP (Roche Diagnostics, Basel, Switzerland) were incorporated in separate reactions using BioPrime Array CGH Genomic Labeling System (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions.

In situ hybridization followed the technique of Schwarzacher and Heslop-Harrison (2000) with small modifications. A total of 40 µl probe mixture was applied per slide, containing 50% (v/v) formamide, 20% (w/v) dextran sulphate, 2 × SSC (saline sodium citrate: 0.3 M NaCl, 0.03 M sodium citrate), 25-100 ng probe, 0.025 µg of salmon sperm DNA and 0.125% (w/v) SDS (sodium dodecyl sulphate) and 0.125 mM EDTA (ethylenediamine-tetraacetic acid). For genomic *in situ* hybridization (GISH) autoclaved genomic DNA from Chinese Spring (20-30 × of the probe concentration) was added as blocking DNA. Probe and chromosomal DNA was denatured together on a Hybaid Omniblock (Thermo Fisher Scientific) at 75ºC for 7 minutes under plastic cover slips and slowly cooled to the hybridization temperature of 37ºC overnight. Washes were carried out with 20% (v/v) formamide and 0.1 × SSC at 42°C, equivalent to 85% stringency. Hybridization sites were detected with 2 µg/ml streptavidin conjugated to Alexa594 (Molecular Probes, Thermo Fisher Scientific) and 4 µg/ml antidigoxigenin conjugated to FITC (fluorescein isothiocyanate) (Roche Diagnostics). Chromosomes were counterstained with 4 µg/ml DAPI (4´,6-diamidino-2-phenylindole) diluted in McIlvaines buffer (pH 7.0) and mounted in antifade solution (Citifluor, London, UK). Photographs were taken on a Zeiss epifluorescence microscope with single band pass filters equipped with a CCD camera (ProgRes C12, Optronics, Milton Keynes, UK, model S97790) or Nikon Eclipse N80*i* fluorescent microscope equipped with a DS-QiMc monochromatic camera (Nikon, Tokyo, Japan). Each metaphase was captured in three different filter sets and then overlayed and analyzed with Adobe Photoshop CS3 (Adobe Systems, San Jose, California, USA) or NIS-Elements BR3.1 software (Nikon) using only cropping, and functions affecting the whole image equally.

Molecular marker analysis

PCR markers were chosen from literature and appropriate databases, and the sequences along with their melting temperature, source, references and expected product size are given (Supplementary Table S1). PCR markers specific for 4Ai#2S chromatin were used to confirm the presence of the *Wsm1* gene. 1BS or 1RS specific markers were employed to detect the presence of rye chromatin and identify molecular breakpoints along the 1BS wheat arm. Primer sequences of all markers except UL-Thin5 were obtained from published sources. Nucleotide sequences for some of the publically unavailable Gatersleben Wheat Microsatellites (GWM) markers were kindly provided by Marion S. Röder (IPK, Gatersleben, Germany). DNA amplification was carried out in a 15 µl reaction mixture containing 100 ng of template DNA, 1 × Kapa Biosystems buffer A, 1.5 mM MgCl, 200 µM of dNTPs (Bioline, London, UK), 0.6 µM of each primer and 0.5 U of Kapa Taq DNA polymerase (Kapa Biosystems, Wilmington, Massachusetts, USA). PCR conditions were: 94°C for 4 minutes, followed by 30 cycles at 94°C for 1 minute, 50-60°C respectively (Supplementary Table S1) for 45 seconds, 72°C for 2 minutes, and final extension of 72°C for 7 minutes. PCR amplification and polymorphism of the products were analysed on 1.5-3% agarose gels.

Table 1. Pedigrees of reference lines and four populations of derived lines (from C. James Peterson, USDA-ARS) studied here with results of WSMV-resistance field tests at different locations and years and summary of FISH/GISH results obtained with genomic *Th. intermedium,* rye and repetitive DNA probes.

Reference/ population	Pedigree/Source	Line/variety	Wheat chromosome with alien segment			Field Response to WSMV[c)]			
						2002[d)]		2012, Mead, NE	
			4D	1B	?D	Sidney, NE	Hays, KS	SPAD[e)] Mean (std)	Visual Score
KS102[a)]	KS91H184/KS89H20// TAM 107	KS95H102	+	-		R	R		
KS10-1[a)]	KS91HW29//RioBlanco/ KS91H184	KS96HW10-1	+	-		R	R		
Millenium[a)]	Arapahoe/Abilene//NE86 488	Millennium	-	-		S	S		
Tomahawk[b)]	Agripro, WI88-083	Tomahawk						14.5 d (2.1)	S
Population I	CO850034//T-57/5 *TAM107/3/(KS91H184/ RioBlanco/KS91HW29// VISTA)	N02Y5018	+	BPI		R	R		
		N02Y5019	-	BPI		S	S	23.5 c (2.3)	MS
		N02Y5021	-	-		S	S		
		N02Y5025	+	-		R	R		
		N02Y5003	-	BPIII		R	R	24.0 c (2.5)	MR
Population II	Yuma//T-57/3/LAMAR/4/4 *Yuma/5/(KS91H184/ArlinS/ KS91HW29//NE89526)	N02Y5057	+	-		R	R		
		N02Y5075	+	-		R	R		
		N02Y5078	+	-		R	R		
		N02Y5082	-	-		S	S		
		N02Y5096	-	-		S	S		
Population III	Yuma // T-57 /3/CO850034/4/4 *Yuma/5/(KS91H184/ ArlinS/KS91HW29)// NE89526)	N02Y5105	-	-		S	MR		
		N02Y5106	+	-		R	MR		
		N02Y5109	-	-	+	R	R	15.4 d (1.7)	S
		N02Y5117 (Mace)	+	-		R	R	28.5 b (6.9)	R
		N02Y5121	-	-		S	S		
Population IV	MO8/Redland//KS91H184/3 *RioBlanco	N02Y5149	+	BPIII		R	R	36.3 a (3.5)	R
		N02Y5154	+	-		R	R		
		N02Y5156	-	BPIII		S	S		
		N02Y5163	-	BPII		S	S		
		N02Y2016	+	BPI		R	R		

Abbreviation: WSMV, Wheat streak mosaic virus.
Recombinant chromosomes were disomic in all cases and included the T4DL*4Ai#2S Th. intermedium translocation (4D; see Figure 3a); an alien fragment on the short arm of 1B of classified into three breakpoint (BP) types by FISH and molecular markers (see Table 2 and Figures 3b and 4): BPI very small, BPII small and BPIII whole arm alien fragment; and a small alien fragment on an unknown D-genome chromosome (?D; see Supplementary Figure S1).

Other Sources: Wells et al., 1982; Divis et al., 2006; Graybosch et al., 2009 and personal communication.

a) R=resistant or no symptoms, MR=moderately resistant or slight symptom severity, MS=moderately susceptible or moderate symptom severity, S=susceptible or severe. In terms of plant phenotype, a score of 1=no loss of chlorophyll, and no stunting; 2=random and occasional yellow foci or streaks, 3=50% loss of chlorophyll and 4=leaves complete yellow and plants severely stunted.
b) From Divis et al., 2006. Scores are visual ratings, based on degree of streaking/chlorosis and stunting.
c) Soil Plant Analytical Development metre readings for leaf chlorophyll content; Higher SPAD readings indicate higher intensity of greenness of leaf tissue (no or mild symptoms), while lower SPADreadings indicate increasing severity of virus symptoms. Means followed by the same letter were not significantly different at P=0.05.
d) Reference lines previously known for presence/absence of Th. intermedium chromatin and validated here.
e) Tomahawk was used as reference susceptible to WSMV in the 2012 trial only; it was selected from a bulk population of crosses of adapted parents.

RESULTS

Twenty experimental lines from four breeding populations (Table 1) previously classified as resistant or susceptible to WSMV (Divis *et al.*, 2006) were rescored in the field and analysed in detail by molecular cytogenetics and PCR markers to identify their genomic constitution, the alien chromatin present and nature of WSMV-resistance.

Phenotypic responses to WSMV infection

Lines classified as resistant to WSMV in the 2002 season (Divis *et al*., 2006) were found to carry the T4DL*4Ai#2S translocation (Table 1 and below) except for N02Y5003 and N02Y5109. These two lines, along with the resistant Mace, the susceptible control Tomahawk and two other lines, N02Y5019 and N02Y5149, were selected for further phenotypic characterization in 2012 (Table 1). The level of infection in 2012 was much higher than that seen in either location in 2002, due both to increased mite pressure, and unseasonably warm fall and spring growing conditions. N02Y5109 clearly was susceptible in 2012 (Table 1); its symptom development as measured by SPAD readings was not significantly different from that of the known susceptible Tomahawk. Some wheat lines possess tolerance to low levels of mite and virus infection, but, under higher viral loads, or under more severe environmental conditions, as observed in 2012, this tolerance breaks down, and may explain the inconsistent response of N02Y5109 that sometimes shows limited resistance. N02Y5003 and N02Y5109 were scored MR and MS, respectively, in 2012. Early in the season, both appeared resistant, but as temperatures increased, resistance diminished. Nonetheless, mean SPAD readings were significantly higher in these two lines than in Tomahawk (Table 1). Finally, N02Y5149 was scored as R in this trial, and had significantly higher SPAD readings than the R control line Mace (Figure 1, Table 1)

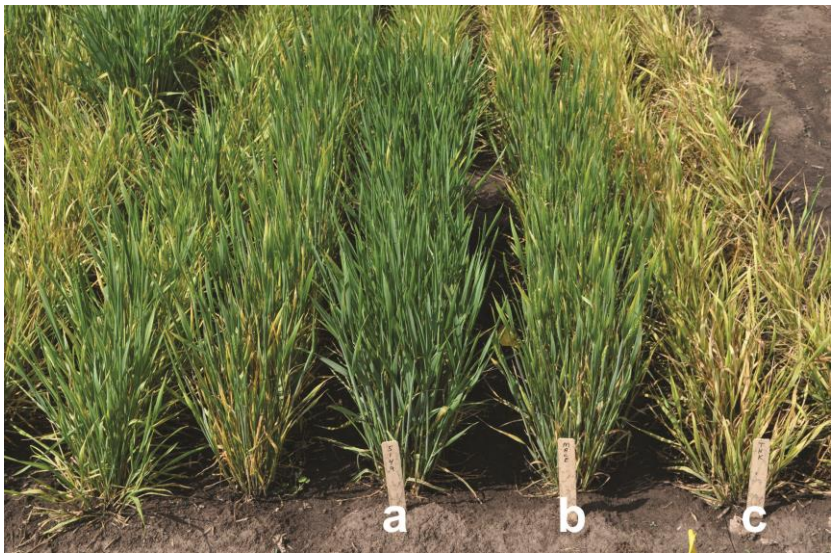


Figure 1: Field response of highly resistant line N02Y5149 (a), resistant cultivar Mace (b) and susceptible check Tomahawk (c) to natural infection with WSMV, Mead, NE, 2012, showing yellow leaves due to loss of chlorophyll and stunting in susceptible plants and full green leavs in resistant plants.

Molecular cytogenetic characterization

Fluorescent *in situ* hybridization (FISH) with total genomic *Th. intermedium* DNA revealed a number of alien wheat translocation and recombinant chromosomes in the twenty lines analysed (Table 1; Figures 2 and 3, Supplementary Figures S2 and S3). The characteristic banding patterns of dpTa1/Afa (mainly on the D-genome), pSc119.2 (abundant in the B-genome with some sites on A and D-genome chromosomes), 5S rDNA (on the short arms of group 1 and 5 chromosomes) and 45S rDNA probes (on 1B, 6B, 5D, 1A and sometimes detected on 7D) were used and characteristic banding patterns (Forsstrom *et al.*, 2002; Contento *et al.*, 2005; Patokar *et al.*, 2015) compared in normal chromosomes and those carrying alien segments. The recipient wheat chromosomes were identified as 4D (Figure 3a) and 1B present in some lines of Populations I and IV (Figure 3b); translocation and recombinant chromosomes are described in more detail below. Total genomic rye DNA was also used as probe to identify rye chromosome segments.

Genomic *in situ* hybridization (GISH) with *Th. intermedium* genomic DNA

GISH to the resistant reference lines, known to include *Th. intermedium* DNA, KS96HW10-1 and KS95H102 (Figure 2a, Supplementary Figure S3a and Table 1), revealed a pair of small chromosome arms labelled with total genomic *Th. intermedium* DNA that also hybridized with weaker signal to the D genome chromosomes of wheat (see also Figures 2b and h, Supplementary Figures S3b, e and g). This *Th. intermedium* chromatin has a strong terminal band with the repetitive DNA probe pSc119.2 (Figure 2a; see also in the derived line N02Y5106, Figure 2e, and N02Y2016, Supplementary Figure S3h) and was present as a centric translocation. The wheat chromosome arm was identified as 4DL by its hybridization pattern with the repetitive DNA probe dpTa1/Afa (Figure 3a and shown in the derived line N02Y5057, Figure 2h, and Mace, Graybosch *et al* 2009 and Supplementary Figure S3f). Millennium, the susceptible reference line, did not show any detectable *Th. intermedium* chromatin (Table 1).

Population I. The R-lines, N02Y5018 (Figure 2b) and N02Y5025 (Supplementary Figure 3Sc, have the T4DL*4Ai#2S translocation (Table 1), but a further small fragment with strong *Th. intermedium* DNA hybridization was detected at the distal end of the short arm of a large chromosome pair in line N02Y5018 (Figure 2b). This recombinant chromosome was identified as 1B due to its hybridization pattern with 5S rDNA, 45S rDNA and the repetitive probe pSc119.2 (Figure 3b; the origin of this recombinant chromosome is discussed below). Further alien fragments on 1B were detected in lines N0Y5019 (Supplementary Figure S3b) and N02Y5003 (Figure 2c) that both lack the T4DL*4Ai#2S translocation (Table1). These two lines varied in their WSMV response in 2002, with line N0Y5019 showing more symptoms; however, SPAD readings in 2012 were similar. No *Th. intermedium* chromatin was detected in the S-line N02Y5021 (Table 1).

Population II and III. All R-lines, N02Y5057 (Figure 2h), N02Y5075 (Supplementary Figure S3d), N02Y5078 (Supplementary Figure S3e), N02Y5106 (Figure 2e) and Mace (N02Y5117; Supplementary Figure 2f), showed *Th. intermedium* chromatin in the form of the T4DL*4Ai#2S chromosomal translocation (while *Th. intermedium* origin chromatin was not detected in either S-lines N02Y5082 or N02Y5096 using FISH and no large *Th. intermedium* fragments were detected in N025105, N02Y5109 and N02Y5121 (Table 1). Alien chromatin was detected on another D-genome chromosome identified by GISH with *T. tauschii* DNA (Figure S2) in line N02Y5109 of population III that demonstrated inconsistent phenotypic response to WSMV (Table 1).

Population IV. The R-lines, N02Y5149 (Figure 2f), N02Y5154 (Supplementary Figure S3g) and N02Y2016 (Supplementary Figure S3h) incorporated *Th. intermedium* chromatin in the form of T4DL*4Ai#2S chromosomal translocation (Table 1). Alien fragments on 1B hybridizing with *Th. intermedium* DNA were also detected in R-line N02Y5149, N02Y2016 as well as in S-lines N02Y5156 (Figure 2g) and N02Y5163 (Figure 2d).

Characterization of lines with the 1B translocation or recombinant chromosomes

The parental R-lines (KS91H184 and KS91H174) carrying *Th. intermedium* chromatin in the form of T4DL*4Ai#2S translocation (Divis *et al*., 2006), have never been described as carriers of alien chromatin on 1B. However, labelled genomic *Th. intermedium* DNA probe identified seven 1B recombinants or translocations in populations I and IV (Table 1). Two approaches were followed to identify the nature and origin of the 1B alien fragments. Firstly, FISH experiments carried out using labelled genomic DNA from *Th. intermedium* and the probe pSc119.2 on lines having the 4D translocation with and without the 1B recombinant chromosome were analysed, and a possible reciprocal translocation between the T4DL*4Ai#2S and wheat 1BS chromosome was ruled out as the pSc119.2 site remained constant (Figures 2a, 2e and 3b). Secondly, the 1BS recombinants were screened with GISH using labelled rye genomic DNA probe along with use of molecular markers characterising 1BS or 1RS (e.g. Figure 2c,Table 2 and below).

GISH confirmed lines N02Y5003, N02Y5149 and N02Y5156 to have a centric T1BL*1RS (Type III) translocation, as revealed by the bright fluorescence of rye 1RS chromatin (e.g., Figures 2c andg). Strong fluorescence of the terminal heterochromatic region of the alien rye chromatin was evident and also was detected by the *Th. intermedium* genomic probe (Figures 2c and f) indicating cross hybridization between rye and *Th. intermedium* DNA. N02Y5156 (susceptible) and N02Y5003 (moderately susceptible; Table 1) both lack the 4D-alien translocation. N02Y5149 (resistant, displaying the exceptionally high SPAD value) carries the *Wsm1* gene of 4D and the 1RS arm, suggesting 1RS in this line enhances WSMV-resistance (Table 1). Among the other resistant lines, N02Y5018 (Figure 2b) and N02Y2016 (Supplementary Figure S3h) also carried the additional small, Type I, 1BS alien chromatin along with *Wsm1* of 4D (Table 1) but their WSMV field resistance response is typical of *Wsm1* only. Small distal 1B alien fragments were detected in N02Y5018 (Figure 2b), N02Y5019 (Supplementary Figure S3b), N02Y5163 (Figure 2d) and N02Y2016 (Supplementary Figure S3h) with labelled *Th. intermedium* genomic DNA (Table 1), but GISH with rye DNA did not detect rye chromatin in these lines.

Figure 2: *In situ* hybridization of example root-tip metaphase and meiotic pachytene chromosomes from WSMV-resistant (a, b, c, e, f, h) and WSMV-susceptible wheat lines (d, g). Hybridization signal of total genomic DNA is shown in red and of repetitive DNA probes in green. Wheat chromosomes fluoresce blue with DAPI. Relevant chromosomes are identified. Whole alien chromosome arms are indicated with arrows and small segments or cross-hybridization are indicated with arrow heads. Bar 10μm (a, b, c, d, e, f, h) and 20μm (g). In situ hybridization to further lines with alien fragment are shown in Supplementary Figure S3.

a) KS96HW10-1**, d)** N02Y5163 and **e)** N02Y5106: genomic *Th. intermedium* DNA (biotin, Alexa594, red) and 120bp repeat pSc119.2 (digoxigenin, FITC, green)

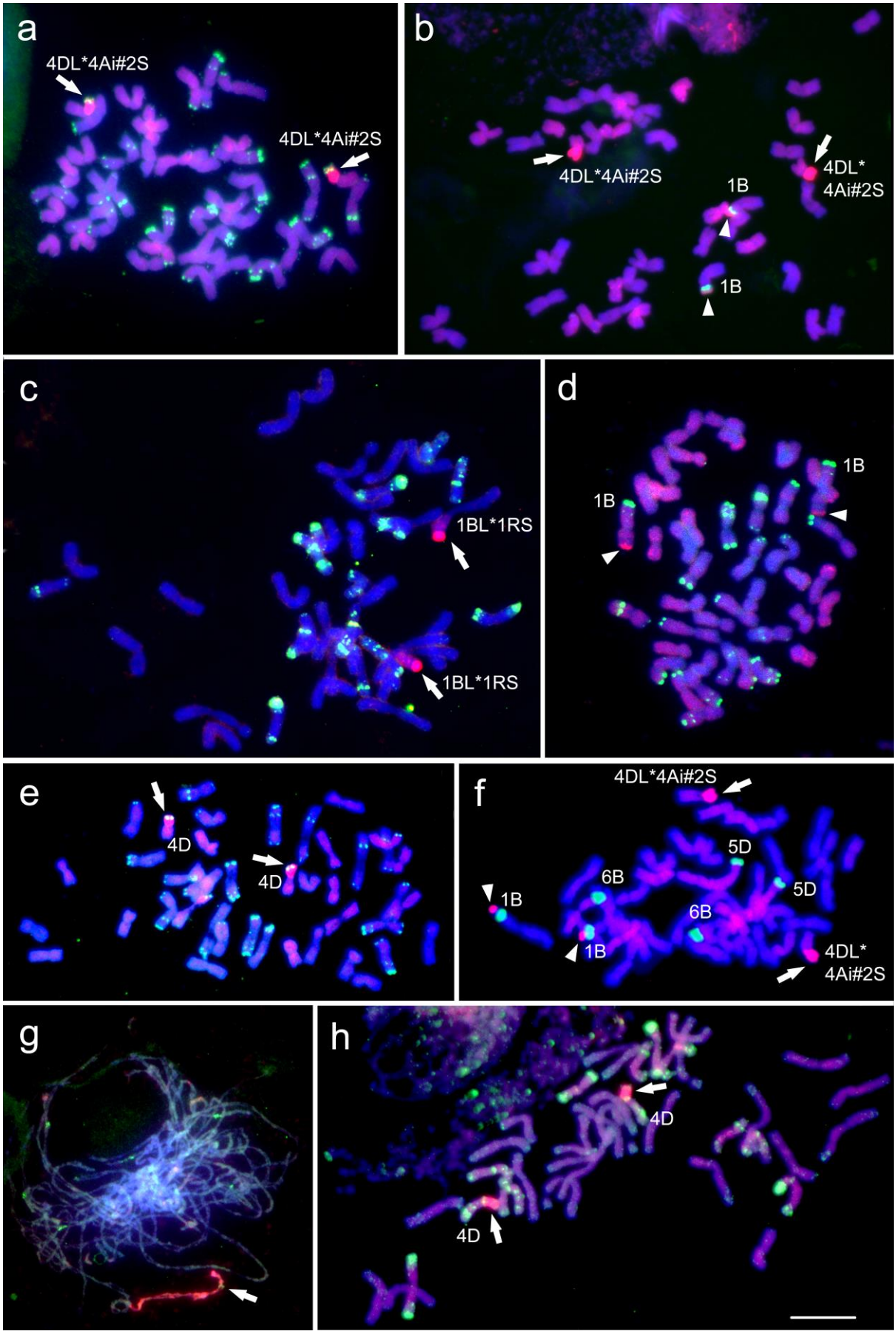
b) N02Y5018: genomic *Th. intermedium* DNA (biotin, Alexa594, red) and 5S rDNA (digoxigenin, FITC, green)

c) N02Y5003: genomic rye DNA (biotin, Alexa594, red) and 340bp repeat dpTa1/Afa (digoxigenin, FITC, green)

f) N02Y5149: *Th. intermedium* (digoxigenin, FITC shown in red) and 45S rDNA (biotin, Alex594 shown in green)

g) N02Y5156: Pachytene chromosomes: genomic rye DNA (biotin, Alexa594, red) and 5S rDNA (digoxigenin, FITC, green)

h) N02Y5057: genomic *Th. intermedium* DNA (biotin, Alexa594, red) and 340bp repeat dpTa1/Afa (digoxigenin, FITC, green)


a
4DL*4Ai#2S
4DL*4Ai#2S
b
4DL*4Ai#2S
1B
4DL*
4Ai#2S
1B
c
1BL*1RS
1BL*1RS
d
1B
1B
e
4D
4D
f
4DL*4Ai#2S
5D
6B
5D
1B
6B
1B
4DL*
4Ai#2S
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h
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4D

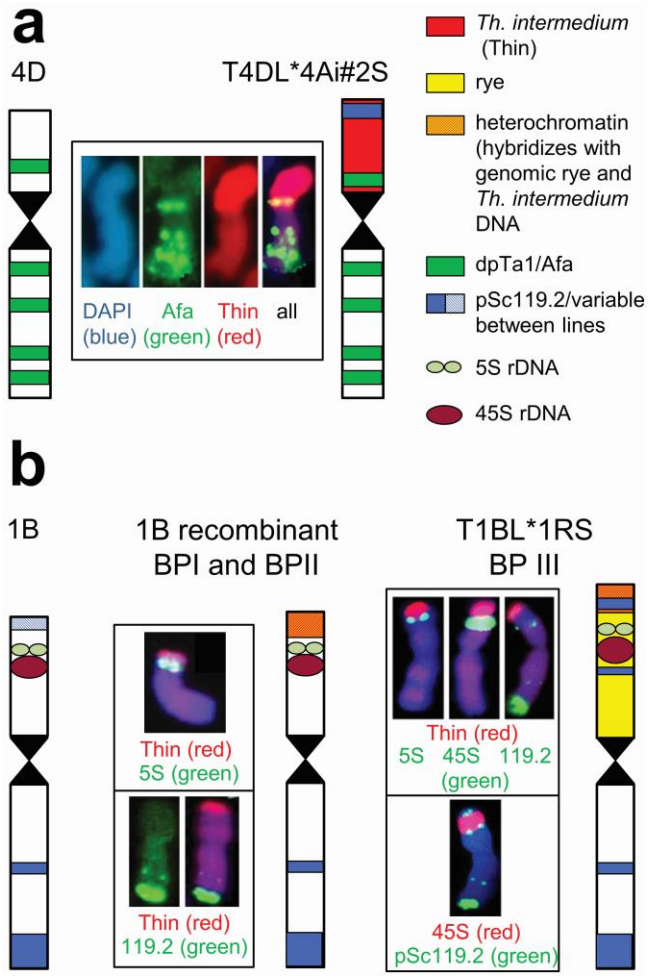

Figure 3: Diagram of alien translocation and recombinant chromosomes. Examples of FISH signals of two probes each (green and red) on blue DAPI stained chromosomes are given.
a) Wheat 4D and T4DL*4Ai#2S chromosomes showing the dpTa1/Afa, pSc119.2 and genomic DNA hybridization patterns. The *Th. intermedium* arm has a terminal pSc119.2 site (see Figure 2e) and the dpTa1 site is more proximal than on 4D.
b) Wheat chromosome 1B, 1B recombinant (BPI and PBII) and T1BL*1RS showing the characteristic arrangement of 5S (light green), 45S rDNA (brown) and pSc119.2 sites (blue). Chromosome arm 1BS has one telomeric pSc119.2 site that is not present in all lines, while 1RS is slightly longer and has two pSc119.2 sites distal and proximal of the NOR region. The BPI and BPII recombinant chromosomes characterised by molecular markers cannot be distinguished cytologically. Note that the subtelomeric heterochromatin hybridizes strongly with both rye and *Th. intermedium* genomic DNA.

PCR marker screening

Detection of *Th. intermedium* chromatin

PCR markers STS-J15 and WSR11 produced characteristic amplicons of approximately 420 bp and 200 bp respectively (Talbert *et al.*, 1996; Fahim *et al.*, 2012). Both markers detected polymorphism among the resistant and susceptible lines and the presence of a marker band related to the *Wsm1* resistance gene (Supplementary Figure S1). Neither marker could identify the resistance mapped to chromosomes other than T4DL*4Ai#2S, as detected in line N02Y5003 (Table 1). Interestingly, WSR11 amplified a DNA band of the same size from two WSMV-susceptible lines N02Y5019 and N02Y5156 carrying the 1B recombinant chromosome (Table 1).

Detection of rye chromatin

1B recombinant lines (Table 1) along with positive and negative controls were analysed with nine 1RS PCR markers (Table 2; for references and conditions see Supplementary Table S1). The rye telomeric repetitive DNA marker pAW161, amplified indiscriminately a monomorphic band of about 350 bp in all samples. Markers AW2-5, SCM9, pAWS5/S6, *Xiag95*, 1B-267, O5, Pr20H and *Xrems1303* produced diagnostic bands from rye DNA and confirmed the presence of 1RS chromatin in lines N02Y5003, N02Y5149, N02Y5156, N02Y5163 and the control T1BL*1RS Beaver wheat, but not from lines N02Y5018, N02Y5019 and N02Y2016 (Table 2). The O5 marker also amplified DNA fragments of the same size from all lines with 4Ai#2S.

Breakpoint mapping of the 1B alien chromosome fragment

A total of 36 PCR markers from wheat 1B were applied for mapping the size of the alien chromatin seen on 1B in population I and IV and all markers successfully amplified DNA from wheat and/or rye chromatin (Supplementary Table S2). Only 18 markers (50%) produced polymorphic bands that could be assigned to the 1BS chromosome of wheat and were applied in breakpoint mapping (Table 2). Few of these polymorphic markers amplified multiple loci but only, bands for the expected product size were scored (Supplementary Table S2). The breakpoint between wheat and rye chromatin was identified by the appearance of wheat markers on the recombinant 1BS arm of wheat after taking into account its presence or absence in the control Chinese Spring wheat-nullisomic-1B-tetrasomic-1A (CS N1B-T1A) line, Beaver and rye. The order of markers and breakpoint localization along the 1BS recombinants is based on published genetic and physical maps (Table 2). Overall the molecular markers results revealed good agreement with the original sources for marker size and relative positions along the 1BS (Table S1).

Based on *in situ* hybridization data (Table 1) and wheat 1BS specific markers, the seven 1BS recombinants identified were divided into three groups by their breakpoint (BP; Table 2) that identify three types of 1B recombinants. Line N02Y5018, N02Y5019 and N02Y2016 incorporated the smallest, Type I; N02Y5163 with intermediate, Type II; and N02Y5003, N02Y5149 and N02Y5156 have the complete 1RS chromatin, Type III (Table 1, Figure 4). Lines N02Y5018 (Figure 2b), N02Y2016 and N02Y5019 have lost only two distal wheat markers *Xfc618, Xgwm0911* and are grouped in BPI. The size of the lost 1BS arm in N02Y5163 is larger than BPI having lost five distal wheat markers *Xfc618, Xgwm0911, Xbarc194, Xgpw7059* and XBF474204 but retains *Xpsp3000* a marker linked to *Gli-1* gene and is placed in BPII (Table 2). GISH with rye genomic DNA revealed the centromeric breakpoint in line N02Y5003 (Figure 2c), N02Y5149 and N02Y5156 (Figure 2g) and all tested 1BS specific wheat markers were absent placing the lines in BPIII (Table 2). Physical and genetic map based position of the 1BS and 1RS polymorphic markers is based on comparative map position from published sources and the most likely order of these markers here is given (Figure 4).

Origin of rye fragments in wheat-*Th. intermedium* hybrid lines

The origin of the 1B alien chromatin was investigated by analysing available lines in the pedigree of populations I and IV with specific markers for 1BS and 1RS. Parental WSMV-resistance lines KS91H174 and KS91H184 have an intact 1BS of wheat origin, while RioBlanco and M08 tested positive for 1B alien chromatin. Further, wheat 1BS specific markers *Xfc618* and *Xpsp3000* revealed the size of the 1B alien chromatin to be smaller in RioBlanco compared to the M08 (Supplementary Table S3). Marker profile and negative PCR with rye marker of RioBlanco was similar to N02Y5018, N02Y5019 and N02Y2016 that may be the donor parent of 1BS alien chromatin of these lines (Table 1). Both RioBlanco and M08 are among the parents of population IV (Table 1), but marker profile and FISH results make M08 the potential donor of the 1RS chromatin seen in lines N02Y5149 and N02Y5156 (Figure 2g). The origin of the whole arm 1RS chromatin in R-line N02Y5003 (Figure 2c) and medium size in S-line N02Y5163 (Figure 2d) could not be assigned to M08 or RioBlanco (compare Table 1 and Table 2). Seed of three parents of population I (CO850034, T-57 and KS91HW29) was not available and thus origin and introgression of the whole 1RS in N02Y5003 (Figure 2c) from these lines could not be tested. TAM107 with a T1AL*1RS translocation (Villareal *et al.*, 1996) is also in the pedigree of Population I, but no alien chromatin was found on the 1A chromosomes in the lines investigated here, but it cannot be excluded that the 1RS has transferred to 1B.

Table 2. Results of 1BS and 1RS polymorphic markers applied in breakpoint mapping of lines of Population I (bold) and Population IV (italic) carrying 1B alien chromatin. Markers are arranged in their most probable order from telomere to centromere based on published physical and genetic maps. Chinese Spring or Anton wheat and the Chinese Spring nullisomic-1B-tetrasomic-1A line, rye and the known T1BL*1RS variety Beaver were used as controls. For PCR conditions, full list of markers and sources see Tables S1 and S2.

	Wheat 1BS markers[a)] **Telomere ----------→ Centromere**																		**Rye 1RS markers[a)]** **Telomere ----------→ Centromere**							
	BPI		**BPII**			**BPIII**																				
	Xfc 618	*Xgwm 0911*	*Xbarc 194*	*Xgpw 7059*	XBF4 74204	*Xgwm 1100*	*Xgwm 4435*	UL-Thin5[b)]	*Xgwm 1130*	*Xpsp 3000*	*Xwmc 230*	*Xgpw 363*	*Xwmc 406*	*Xwmc 49*	*Xucr_ 6*	*Xgwm 4144*	*Xgwm 1078*	*Xucr_ 3*	1B-267	O5	Xiag 95	*Xrems 1303*	Pr 20H	AW 2-5	SCM 9	PAW S5/S6
Experimental lines and controls																										
Chinese Spring	+	+	+	+	+?	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-?	-
N02Y5018	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+[c)]	-	-	-	-	-	-
N02Y5019	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
N02Y2016	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+[c)]	-	-	-	-	-	-?
N02Y5163	-	-	-	-	-	+?	-?	¢	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+?
N02Y5003	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
N02Y5149	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+[c)]	+	+	+	+	+	+
N02Y5156	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+?	-	+	+	+	+	+	+	+	+
Beaver (1BL*1RS)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Rye	-									-								-	+	+	+	+	+	+	+	+
CS N1B-T1A	-	-	-	-	-		-	-	-	-	-	-	-		-	-	-	+	-	-	-	-	-	-	-	-

Markers are arranged in their most probable order from telomere to centromere based on published physical and genetic maps. Chinese Spring or Anton wheat and the Chinese Spring nullisomic-1B-tetrasomic-1A line, rye and the known T1BL*1RS variety Beaver were used as controls. For PCR conditions, full list of markers and sources see Tables S1 and S2.

a) Marker allele presence is indicated with +: presence; -: absence; +?: most probably present; -?: most probably absent
¢: PCR failed to amplify for unknown reason; empty fields mean no data obtained.

b) UL-Thin5 is a new EST marker designed during this study.

c) O5 also amplifies the same size DNA fragment from lines that carried the 4Ai#2S chromosome, therefore presence does not necessarily mean presence of the rye allele.

DISCUSSION

Many current WSMV-resistant wheat lines including the hard winter wheat variety Mace (Figure 1) have benefited from the *Wsm1* gene of 4Ai#2 origin. Some of the initial lines carrying *Wsm1* were associated with undesirable traits such as reduced yield and poor bread-making quality, but subsequent crosses and selection eliminated many negative effects from the introgressed *Th. intermedium* chromatin (Divis *et al.*, 2006; Graybosch *et al.*, 2009). We could demonstrate that the reference Kansas resistance lines KS95H102 and KS96HW10-1 have alien material in the form of T4DL*4Ai#2S translocation (Figures 2 and 3, Supplementary Figures S2 and S3). Further, in four populations of the Nebraska USDA-ARS crossing programme (Divis *et al*., 2006) that gave rise to Mace, the 4Ai#2S *Wsm1* origin resistance was identified in ten (including Mace) of the adapted winter wheat lines. Sensitive sister control lines in each population did not carry the T4DL*4Ai#2S translocation (Table 1). The source of resistance in all R-lines is most likely either KS91H184 or KS91H174, both being selected from populations that had been randomly mated for several generations and derived from CI 17884, a WSMV-resistance line carrying the *Wsm1* gene on a chromosome arm translocated from *Th. intermedium* (Wells *et al*., 1982).

Additionally, we detected rye chromatin transferred to chromosome 1B in some of the lines and resistance-enhancement associated with rye was identified in the *Th. intermedium* alien wheat introgression lines tested. It is generally thought that rye itself is probably susceptible to WSMV and that 1RS in wheat-rye translocation does not confer resistance, but that rye can resist colonization of both greenbug and wheat curl mite (WCM), the vector of WSMV (Thomas *et al*., 2004; Divis *et al*., 2006). Our results revealed that the parental WSMV-resistance lines KS91H174 and KS91H184 have intact 1B chromosomes and the 1RS fragments identified in the lines investigated must originate from other lines within the pedigrees. The pedigree of population IV contains the line M08 a 1R(1B) substitution or T1BL*1RS translocation line (Zhang *et al*., 1998) derived from anther culture after hybridization of hexaploid wheat and hexaploid *Triticale* and identified as having a recombinant 1B (Supplementary Table S3). More importantly, M08 was noted in preliminary observations made at the University of Nebraska (P.S. Baenziger, personal communication 2008) as potentially harbouring genes conferring some tolerance to WSMV. The 1RS arm of population IV is most likely from M08 and together with the *Wsm1* gene of T4DL*4Ai#2S translocation in line N02Y5149 resulted in field resistance to WSMV superior to any other line (Figure 1, Table 1). This enhanced resistance to WSMV in the 2012 field study might very well have arisen from increased tolerance to the vector, WCM. On its own, 1RS in line N02Y5163 was however not able to confer WSMV-resistance. The pedigree of Population I includes TAM107 with T1AL*1RS (Villareal *et al*. 1996) and RioBlanco with an incomplete 1RS arm (Supplementary Table S3), and thus the origin of the whole 1RS arm of N02Y5003, probably different to the one of N02Y5149, is not clear, however it seems to be responsible for the enhanced resistance of this line that lacks the T4DL*4Ai#2S translocation (Tables 1 and 2).

Variable behaviour of 1RS resistance supports different origins, but also emphasizes the potential role of epigenetic modification and chromatin remodelling in gene expression (see Slotkin and Martienssen, 2007). Our lines (Table 1) have undergone interspecific hybridization stress that may cause heritable changes to epigenetic marks. Development of resistance in originally WSMV-susceptible lines has shown that WSMV-resistance genes in wheat cultivars might be present but unexpressed (Lu *et al*., 2011; Seifers *et al*., 2013). The expression of these genes could be modulated and controlled by genes, transcription factors or chromatin-regulators from the alien chromosome segments.

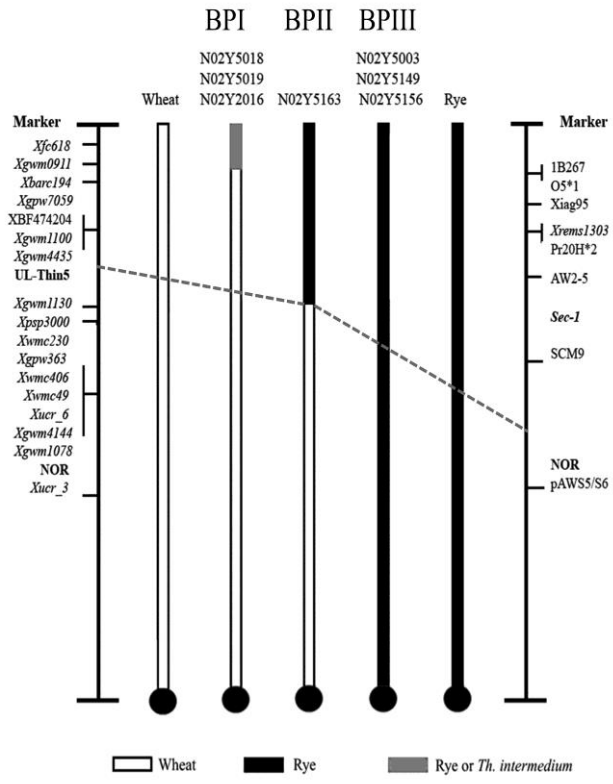

Figure 4: Diagrammatic representation of most likely marker positions and translocation breakpoints (BPs) along the recombinant 1B lines N02Y5018, N02Y5019, N02Y2016, N02Y5163, N02Y5003, N02Y5149 and N02Y5156 based on published marker order for 1BS and 1RS (Anugrahwati *et al*., 2008; Reddy *et al*., 2008; and references and data from Table S1). White bars represent wheat 1BS, black represents rye 1RS and red bars represent the distal 1B alien chromatin that may be rye or *Th. intermedium* chromosomal segments; centromeres are represented by dark circles. *Sec-1* and NOR position are based on Lei *et al*. (2012). Asterisks (*) represent repetitive DNA markers (Tang *et al*., 2011) for which chromosomal positions are proposed here, UL-Thin5 is a new marker. Line N02Y5018, N02Y5019 and N02Y2016 has the translocation BP between *Xgwm0911* and *Xbarc194*. Physical length of this alien chromatin may not be the same but this BP is identified by the same markers (Table 2). Line N02Y5163 has the translocation BP between UL-Thin5 and *Xgwm1130*, while line N02Y5003, N02Y5149 and N02Y5156 has a centric T1BL*1RS translocation (Table 1).

Within the lines studied, a very small distal alien fragment was identified on 1B in some lines (Table 2 and Figures 2-4) although it was not able to confer WSMV resistance. Wheat-alien translocations with minimal alien chromatin *per se* are of importance, as they are predicted to have less likelihood of linkage drag (Forsström *et al.*, 2002; Niu *et al.*, 2011) although linkage drag is less pronounced or buffered in polyploid wheat compared to the diploid progenitors. Not all large alien fragments are disadvantageous; they may potentially introduce more variation and assure a wider variety of resistances (Friebe *et al.*, 2009). The presence of cryptic alien chromatin, as discovered here on chromosome 1B, and the associated loss of some important wheat genes might explain some of the negative effects and undesirable traits in the lines studied and we cannot exclude that the small alien fragments interfered in developing novel well adapted lines with *Wsm1*.

Genomic *in situ* hybridization (GISH) is a powerful technique that allows identification of alien-wheat recombinant chromosomes, particularly when combined with repetitive DNA probes (e.g. Forsstrom *et al.*, 2002; Sepsi *et al*., 2008), and thus subsequent selection of molecular markers could concentrate on the chromosome arm (or segment) identified, rather than a genome wide marker screen (Niu *et al.*, 2011). The markers used here classified the seven 1BS recombinants into three breakpoint categories (Figure 4, Table 2). The order and location of these markers along the 1BS was largely as reported, although a few e.g. *Xgwm1130* and *Xgwm0911* (Ganal and Röder, 2007) were inverted. This discrepancy is not a rare phenomenon when wheat cultivars from different pedigrees are compared (Akhunov *et al.*, 2013). The recombination breakpoints were all distal to the NOR on 1BS (Figure 4) suggesting the presence of recombination hot-spots in this region (Reddy *et al*., 2008).

While the T1BL*1RS whole arm translocation (BPIII) was identified clearly by GISH in N02Y5003, N02Y5149 and N02Y5156, and rye specific markers distal to the NOR were present (Table 2), both *in situ* hybridization with rye genomic DNA and 1RS specific PCR markers failed to identify the small alien BPI fragments on 1BS in lines N02Y518, N02Y5019 and N02Y2016 that lost only the most distal wheat 1BS specific markers *Xfc618* and *Xpsp3000* (Table 2 and Figure 4). However, the alien fragment is not below the resolution of GISH, as genomic *Th. intermedium* DNA hybridized to these fragments consistently (Table 1 and Figure 2). Therefore, the possibility of a non-rye alien fragment, possibly from *Th. intermedium* itself cannot be ruled out although *Th. intermedium* genomic DNA can cross-hybridize to the distal tandem repeats present at the telomere of a normal 1RS (Figure 2d, 2f). Detection of very small alien chromatin segments can be difficult both cytologically and with molecular markers: GISH screening using labelled rye genomic DNA probe and wheat DNA as a block failed to visualize the presence of rye chromatin in known recombinants that carried the *SrR* gene (Anugrahwati *et al.*, 2008) and negative PCR results have been reported for several wheat-rye translocation lines that contained 1RS chromatin (Mago *et al.*, 2002; Anugrahwati *et al*., 2008).

The variable PCR results with some 1RS markers (Table 2) could be due to DNA changes as a consequence of intensive crossing and back-crossing involved in modern wheat lines, since rearrangements, shuffling or even loss of marker positions is not a rare phenomenon and previously undetected interstitial deletions can occur; mutation in the priming sites may also result in no PCR amplification. Alternatively, the small alien fragment is not of rye origin as is indicated by the GISH or the markers amplify from *Th. intermedium* chromatin not on 1B (Table 1).

The presence of rye material and the value of 1RS in combination with *Th. intermedium* WSMV-resistance is new (Tables 1 and 2). The short arm of rye chromosome 1 (Figure 2c) is the most widespread alien chromatin in wheat breeding programmes and has been used to incorporate new genes for stress tolerance and yield potential into wheat including several important genes, such as *Lr26, Sr31, Yr9, Pm8* and *Pm17* (Heslop-Harrison *et al.*, 1990; Forsström *et al.*, 2002; Mago *et al.*, 2002; Reddy *et al.*, 2008). *Th. intermedium* is a member of the tertiary gene pool of wheat and since the 1960s at least 15 genes for fungal or viral resistance have originated from *Th. intermedium* as chromosomal segments (Li and Wang, 2009). The 4Ai#2S alien arms, which carry WSMV-resistance also harbours resistance for the fungal pathogen *Tapesia yallundae* (Chen *et al.*, 2003). In wheat backgrounds, the 4Ai#2S does not cause meiotic instability, and there is considerably more potential for exploitation of *Th. intermedium* introgression in wheat breeding.

Significant yield losses are associated with WSMV infections (Graybosch *et al.*, 2009) and at least three additional viruses have been reported as being transmitted by the WCM vector (Navia *et al*., 2013). There is huge interest in deploying multiple effective genes in combination as a means of disease control and improving the durability of resistance (Liu *et al.*, 2011; Heslop-Harrison and Schwarzacher, 2012). A good means to improve resistance would be to stack the novel 1RS enhancer in germplasm carrying other known WSMV-resistances to achieve the desired goals of deploying combinations of effective genes. Line N02Y5149 is potentially the first R-line with multiple WSMV-resistance affecting genesand the direct utilization of this selection should improve the durability of WSMV-resistance. Therefore, the rye chromatin and increased WSMV-resistance are important, especially for gene pyramiding approaches integrating genomic and molecular cytogenetic approaches with traditional breeding (Tester and Langridge, 2010; Heslop-Harrison and Schwarzacher, 2012).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA ARCHIVING

Relevant data are given in the Supplementary Information and in the Leicester Research Archive http://hdl.handle.net/2381/10951. It is also available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.82f5q

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Supplementary Information accompanies this paper on Heredity website (http://www.nature.com/hdy)

Supplementary information

Introgression of chromosome segments from multiple alien species in wheat breeding lines with wheat streak mosaic virus resistance

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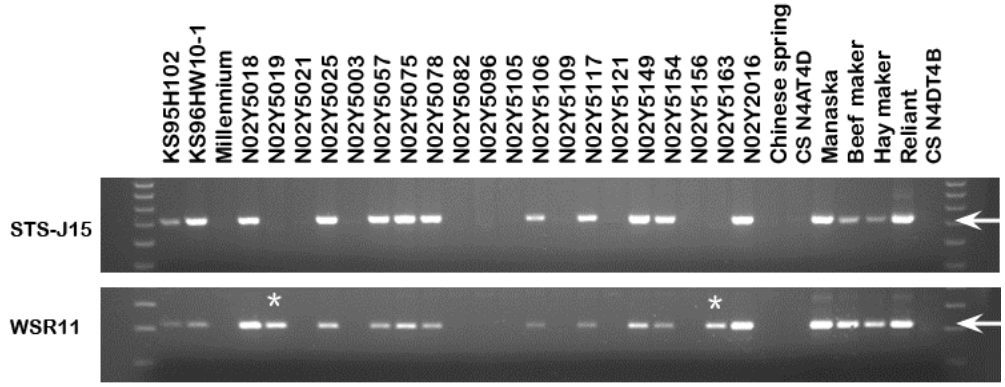

Figure S1. PCR amplification pattern of STS-J15 and WSR11 markers from wheat-*Th. intermedium* hybrid lines. Arrows indicate to the 420bp and 200bp amplicons produced by *Th. intermedium* and the WSMV-resistant lines with 4Ai#2S chromosomal translocation (Table 1). WSR11 also produced this 200bp band from WSMV-susceptible lines N02Y5019 and N02Y5163 (asterisks). On either side of the agarose gel (2%) is a DNA length marker Q-Step 2.

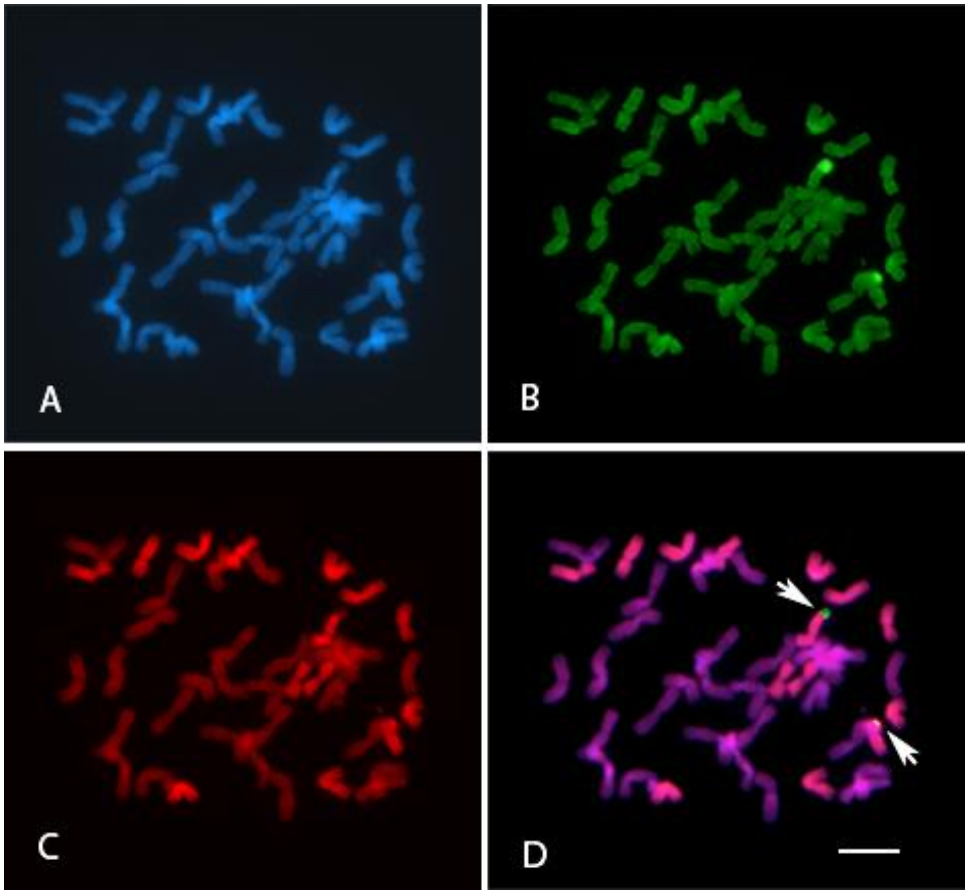

Figure S2. Root-tip metaphase chromosomes of the WSMV-resistant line N02Y5109 (2n=42) after fluorescent *in situ* hybridization (FISH). (A) Chromosomes fluoresces blue with DAPI. (B) *In situ* hybridization of the total genomic DNA from *Th. intermedium* labelled with digoxygenin 11-dUTP (detected in green) that allows the detection of *Th. intermedium*-origin chromosome segments (C) *In situ* hybridization of the total genomic DNA from *Ae. tauschii* labelled with biotin 16-dUTP (detected in red) allows the detection of D-genome chromosome of wheat. (D) Overlay of A, B and C images, alien chromosomal segments are indicated by arrows. Bar represents 10μm.

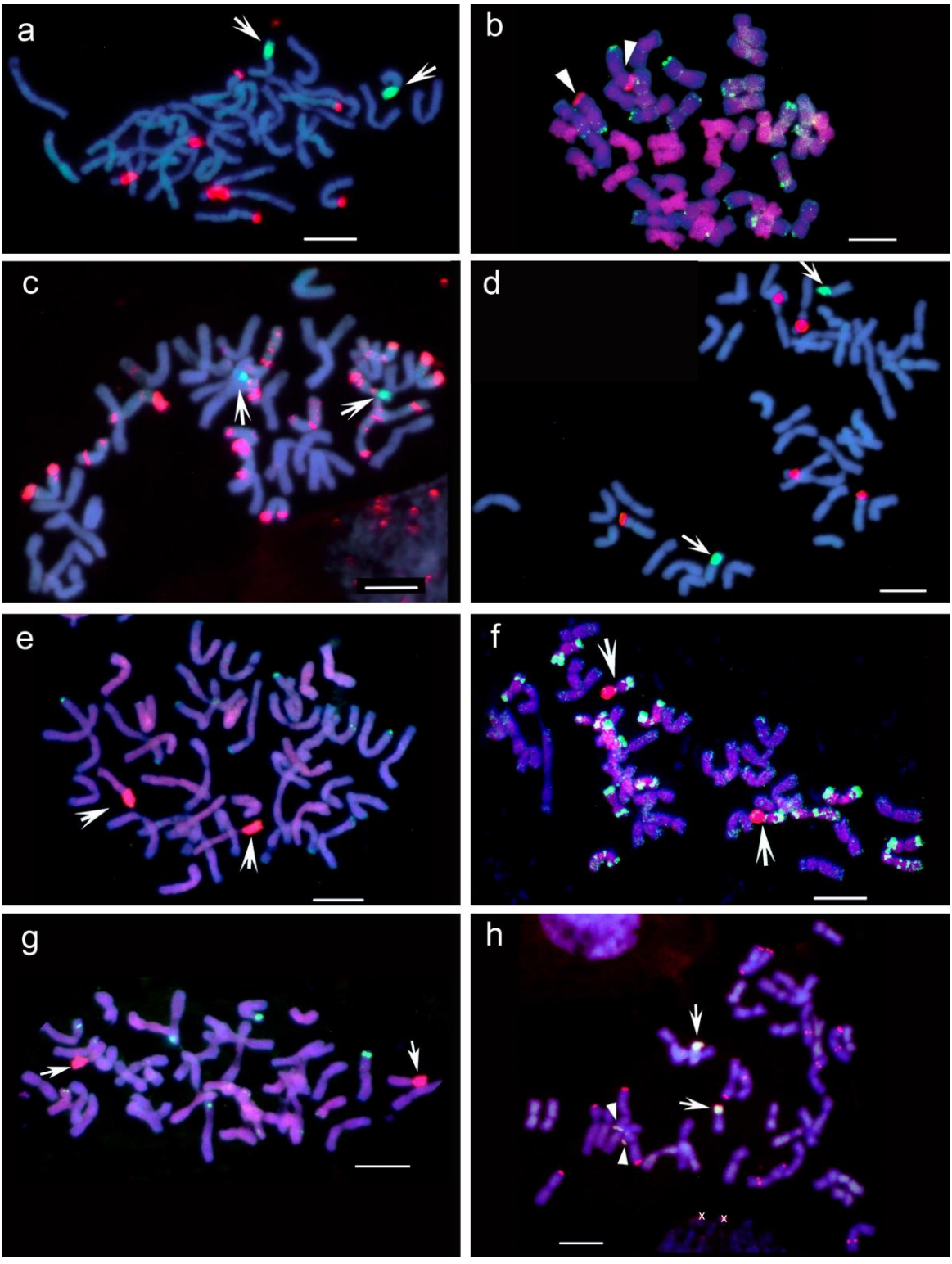

Figure S3: *In situ* hybridization to root-tip metaphase chromosomes from WSMV-resistant wheat lines. Hybridization signal of total genomic DNA is shown in green (a,c), red (b,e,f,g) or white (h), and of repetitive DNA probes in complementary colour red or green. Wheat chromosomes fluoresces blue with DAPI. Whole alien chromosome arms are indicated with arrows and small

segments with arrow heads. D genome chromosomes of wheat also show hybridization with the genomic *Th. intermedium* DNA (b, e, g). Bar 10μm.
a) KS95H102 **d)** N02Y5075 : genomic *Th. intermedium* DNA (digoxigenin, FITC, green) and 45S rDNA (biotin, Alex594, red)
b) N02Y5019, **e)** N02Y5078: genomic *Th. intermedium* DNA (biotin, Alexa594, red) and 120bp repeat pSc119.2 (digoxigenin, FITC, green);.
c) N02Y5025: genomic *Th. intermedium* DNA (digoxigenin, FITC, green) and 340bp repeat dpTa1/Afa (biotin, Alexa594, red)
f) N02Y5117 'Mace' : genomic *Th. intermedium* DNA (biotin, Alexa594, red) and 340bp repeat dpTa1/Afa (digoxigenin, FITC, green)
g) N02Y5154: genomic *Th. intermedium* DNA (biotin, Alexa594, red) and 5S rDNA (digoxigenin, FITC, green)
h) N02Y2016; genomic *Th. intermedium* DNA (digoxigenin, FITC, white) and 120bp repeat pSc119.2 (biotin, Alexa594, red); two signals from an interphase at the bottom of the panel are marked by crosses.

Table S1 PCR markers used to detect alien chromatin and breakpoint mapping of the 1BS recombinant chromosome, melting temperature (T_m), product sizes and source of marker shown.

Sr#	Marker name	Type	Primer sequences	Tm (°C)	Expected product size (bp)	Source
1	STS-J15	STS	F: GTAGCAGGGGAAGCTGAAGA R: CCGAGCTCACACGCTAATTT	60	420	Talbert et al. 1996.
2	WSR11	EST	F: TCCCGGTACTTATCGAGGTG R: CCGCAAGTCTTACTGCAACA	60	200	Fahim et al. 2012.
3	AW2-5	RFLP	F: GAATCCCATTGTTCAGCAAGT R: TAGCACTCCAGCAGACTCCAC	56	~500	Langridge et al. 1998.
4	SCM9	SSR	F: TGACAACCCCCTTTCCCTCGT R: TCATCGACGCTAAGGAGGACCC	60	220	Saal and Wricke 1999.
5	pAW161	Rye repetitive DNA sequence	F: TGAGGGCCCAGACGGCCCTTTTTG R: TTATCGCAATTACAACTCAAATTT	60	350-480	Guidet et al. 1991.
6	pAWS5/S6	R173 family repeats	F: AACGAGGGGTTCGAGGCC R: GAGTGTCAAACCCAACGA	60	220 and 320	Rogowsky et al. 1992.
7	1B-267	STS	F: GCAAGTAAGCAGCTTGATTTAGC R: AATGGATGTCCCGGTGAGTGG	60	220	Mago et al. 2002.
8	*Xiag95*	STS	F: CTCTGTGGATAGTTACTTGATCGA R: CCTAGAACATGCATGGCTGTTACA	60	1000	Mago et al. 2002.
9	O5	Repetitive DNA	F: CCCAGTCACTACAACGAGAGT R: GCTACAAGAGCTTCGTGCAG	60	393	Tang et al. 2011.
10	Pr20H	SCAR	F: GTTGGAAGGGAGCTCGAGC R: GTTGGGCAGAAAGGTCGACATC	60	750	Tang et al. 2011.
11	*Xrems1303*[*1]	EST-SSR		60	309	Khlestkina et al. 2004.
12	Glu-1Dy10	STS	F: GTTGGCCGGTCGGCTGCCATG R: TGGAGAAGTTGGATAGTACC	60	750	Smith et al. 1994.
13	*Xpsp3000*	SSR	F: GCAGACCTGTGTCATTGGTC R: GATATAGTGGCAGCAGGATACG	55	252-286	Bryan et al. 1997.
14	*Xwmc49*	SSR	F: CTCATGAGTATATCACCGCACA R: GACGCGAAACGAATATTCAAGT	60	206	Somers et al. 2004
15	*Xwmc500*	SSR	F: ATAGCATGTTGGAACAGAGCAC R: CTTAGATGCAACTCTATGCGGT	60	185	Somers et al. 2004
16	*Xfc618*	SSR	F: TCTACATACGGACTGAAATGGATAC R: CCTGATTGAGACTCTGGTTACATAAGACTACTC	60	250	Reddy et al. 2008.
17	XBF293222	RFLP	F: GGTTTGCTTTTGCCAATTGTTCTTG R: TATATGTTGGATGGGAGCAAAATCC	50	~400[*3]	Reddy et al. 2008.

Table S1 continued

Sr#	Marker name	Type	Primer sequences	Tm (°C)	Expected product size (bp)	Source
18	XBF474204	EST	F: AATCACACGACCCAGTAAGTTCTC R: CTCAAGTACCTCTGCTTCAACTTC	52	~480[*3]	Reddy et al. 2008.
19	Xpsp2530.1	EST	F: CCTAAACCCTAAACCCTAGAC R: TTCTCACCCAACCACCAGCAGCT	55	~200[*3]	Mao et al. 1997.
20	*XksuD14a*	RFLP	F: CCAAAGAGCATCCATGGTGT R: CGCTTTTACCGAGATTGGTC	50	~550[*3]	Talbert et al. 1994.
21	*Xwmc85*	SSR	F: GGAGTAAGAGAAACATGCCGAA R: GTGCATGCATGAGAATAGGAAC	61	228	Somers et al. 2004
22	*Xgwm0550*	SSR	See *2	55	150 ~300[*4]	Ganal and Röder 2007.
23	*Xgwm0911*	SSR	See *2	55	272	Ganal and Röder 2007.
24	*Xgwm1028*	SSR	See *2	50	116	Ganal and Röder 2007.
25	*Xgwm1078*	SSR	See *2	55	144	Ganal and Röder 2007.
26	*Xgwm1130*	SSR	See *2	60	116	Ganal and Röder 2007.
27	*Xgwm1100*	SSR	See *2	50	227	Ganal and Röder 2007.
28	*Xgwm3035*	SSR	See *2	60	225	Ganal and Röder 2007.
29	*Xgwm4144*	SSR	See *2	60	191	Ganal and Röder 2007.
30	*Xgwm4435*	SSR	See *2	60	214	Ganal and Röder 2007.
31	*Xwmc230*	SSR	F: AGAAGCGAGCAGGTGTGTTTGA R: CTGCTTCCTCCCACAACAGATG	60	213 ~230[*4]	Somers et al. 2004.
32	*Xbarc119*	SSR	F: CACCCGATGATGAAAAT R: GATGGCACAAGAAATGAT	55	208	Developed by P. Cregan and Q. Song (available at http://wheat.pw.usda.gov)
33	*Xgpw1170*	SSR	F: AGATCGTTCATCCGATCTGC R: CAATCTCAGTTTGATGTCCTTCAG	60	166	Sourdille et al. 2004.
34	*Xgpw363*	SSR	F: GTGTGTGGTTGGAGGGAACT R: ATAAGAACATCGAGCGACCG	60	242	Sourdille et al. 2004.
35	*Xbarc194*	SSR	F:CGCAATCATGTTCCTAAGAATATTTGTCCA R: CGCATGTCCCGCTAACCAATAGTCT	50	166	Developed by P. Cregan and Q. Song (available at http://wheat.pw.usda.gov)
36	*Xgwm264*	SSR	F: GAGAAACATGCCGAACAACA R: GCATGCATGAGAATAGGAACTG	60	160	Röder et al. 1998
37	*Xucr_6*	EST	F: TCGAAGGAGAATACGCTGGT R: GCCCATAAGATTTTGCAACG	60	1100	Sharma et al. 2009.
38	*Xucr_8*	SSR	F: CCTGCTCTGCCATTACTTGG R: TGCACCTCCATCTCCTTCTT	60	165	Sharma et al. 2009.
39	*Xucr_3*	EST	F: TGCCTCTCTTGCACTTAGCA R: TGGGCTGCTAAAAGGATCAC	60	500	Sharma et al. 2009.

Table S1 continued

Sr#	Marker name	Type	Primer sequences	Tm (°C)	Expected product size (bp)	Source
40	*Xucr_4*	EST	F: CAAGGAGGTTGGTTTCCTGA R: CGAATACAAGCCGTTCATCA	60	575	Sharma et al. 2009.
41	*Xgpw1143*	SSR	F: CTGTTGTGGGGTGTGCATGT R: CCCCAGCAGCATGAATAAGT	60	206	Sourdille et al. 2004.
42	*Xwmc329*	SSR	F: ACAAAGGTGCATTCGTAGA R: AACACGCATCAGTTTCAGT	54	118	Somers et al. 2004.
43	*Xwmc406*	SSR	F: TATGAGGGTCGGATCAATACAA R: CGAGTTTACTGCAAACAAATGG	60	217	Somers et al. 2004.
44	*Xgpw7059*	SSR	F: AACACCAATGACCTGATCGC R: TCCTCAACAGCTCCAGTGC	60	~220[*2]	Sourdille 2009.
45	*Xgwm374*	SSR	F: ATAGTGTGTTGCATGCTGTGTG R:TCTAATTAGCGTTGGCTGCC	60	180	Röder et al. 1998.
46	*Xbarc128*	SSR	F: GCGGGTAGCATTTATGTTGA R: CAAACCAGGCAAGAGTCTGA	60	250	Developed by P. Cregan and Q. Song (available at http://wheat.pw.usda.gov)
47	**UL-Thin5**	EST (EF570121.1)	F: ACGACATGGTCGTCTACGG R: CCTCGCCTCTCTTCCTTCTT	60	552	Developed in this study

Serial 1-2: for detection of *Th. intermedium* DNA; 3-11: for detection of 1RS; 12-47: for detection of BPs along 1BS of wheat
*1: synthesized oligos were used from Viktor Korzun (KWS, Germany)
*2: SSR marker for 1BS, mentioned in Ganal and Röder 2007. The nucleotide sequence of these markers was provided by Marion S. Röder (IPK, Gatersleben Germany)
*3: size was estimated from the result here and size was not found in author's paper
*4: obtained product size differed from the published size.

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Table S2 PCR markers used for detection of 1BS or 1RS chromatin. Underlined markers are the polymorphic markers informative in detecting 1BS or 1RS chromatin. For primers and references for the markers see Supplementary TableS1.

Line	GISH analysis[*1]			*Xpsp3000*	*Xwmc49*	*Xwmc500*	*Xfc618*	XBF293222	XBF474204	Xpsp2530.1	*XksuD14a*	*Xwmc85*	*Xgwm0550*	*Xgwm0911*	*Xgwm1028*	*Xgwm1078*	*Xgwm1130*	*Xgwm1100*	*Xgwm3035*
	Rec. 4D	Rec. 1B	Rec. ?D																
KS96HW10-1	+/+	-/-	-/-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Millennium	-/-	-/-	-/-	1	1?	1	1	1	1	1	1	1	1	1	1	1	1	0	1
N02Y5018	+/+	+/+	-/-	1	1	1	0	1	1	1	0	1	1	0	1	1	1	1	1
N02Y5019	-/-	+/+	-/-	1	1	1	0	1	1	1	1	1	1	0	1	0	1	1	1
N02Y5025	+/+	-/-	-/-	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1
N02Y5003	-/-	+/+	-/-	0	0	1	0	1	0	1	0	1	1	0	1	0	0	0	1
N02Y5096	-/-	-/-	-/-	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1
N02Y5109	-/-	-/-	+/+	1	0	1	1	1	0	1	0	1	1	1	1	1	1	1	1
N02Y5117	+/+	-/-	-/-	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
N02Y5149	+/+	+/+	-/-	0	0	1	0	1	0	1	1	1	1	0	1	0	0	0	1
N02Y5156	-/-	+/+	-/-	0	0	1	0	1	0	1	1	1	1	0	1	1?	0	0	1
N02Y5163	-/-	+/+	-/-	1	1	1	0	1	0	1	1	1	1	0	1	1	1	1?	1
N02Y2016	+/+	+/+	-/-	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
Beaver	-/-		-/-	0	0	1	0	1	0	1	1	1	1	0	1	0	0	0	1
Manaska	+/+	+/+	+/+	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
CS N4A-T4D	-/-	-/-	-/-	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
CS N4D-T4B	-/-	-/-	-/-	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
Chinese Spring	-/-	-/-	-/-	1	1	1	1	1	?	1	0	1	1	1	1	1	1	1	1
CS N1B-T1A	-/-	-/-	-/-	0	#	#	0	#	0	#	#	1	#	0	#	0	0	0	1

Table S2 continued

Line	GISH analysis[*1]			*Xgwm 4144*	*Xgwm 4435*	*Xwmc 230*	*Xbarc 119*	*Xgpw 1170*	*Xgpw 363*	*Xbarc 194*	*Xgwm 264*	*Xucr_ 6*	*Xucr_ 8*	*Xgpw 1143*	*Xwmc 329*	*Xwmc 406*	*Xgpw 7059*	*Xgwm 374*	*Xbrac 128*
	Chr. (4D)	Chr. (1B)	Chr. (?D)																
KS96HW10-1	+/+	-/-	-/-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Millennium	-/-	-/-	-/-	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
N02Y5018	+/+	+/+	-/-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
N02Y5019	-/-	+/+	-/-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
N02Y5025	+/+	-/-	-/-	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
N02Y5003	-/-	+/+	-/-	0	0	0	1?	1	0	0	1	0	1	0	1	0	0	1	1
N02Y5096	-/-	-/-	-/-	1?	1	1	1	1	0	1	1	1	1	1	¢	1	1	1	1
N02Y5109	-/-	-/-	+/+	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
N02Y5117	+/+	-/-	-/-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
N02Y5149	+/+	+/+	-/-	0	0	0	1	1	0	0	1	0	1	0	1	0	0	1	1
N02Y5156	-/-	+/+	-/-	0	0	0	1	1	0	0	1	0	1	0?	1	0	0	1	1
N02Y5163	-/-	+/+	-/-	1	0?	1	1	1	1	0	1	1	1	1	1	1	0	1	1
N02Y2016	+/+	+/+	-/-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Beaver	-/-		-/-	0	0	0	1	1	0	0	1	0	1	1	1	0	0	1	1
Manaska	+/+	+/+	+/+	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1
CS N4A-T4D	-/-	-/-	-/-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CS N4D-T4B	-/-	-/-	-/-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Chinese Spring	-/-	-/-	-/-	1	1	1	1?	1	1	1	1	1	1	1	1	1	1	1	1
CS N1B-T1A	-/-	-/-	-/-	0	0	0	1	1	0	0	1	0	1	0?	1	0	0	1	1

Table S2 continued

Line	GISH analysis[*1] Chr. (4D)	Chr. (1B)	Chr. (?D)	**UL-Thin5**	*1B-267*	*SCM9*	*Xucr_3*	*Xucr_4*	*Xiaq-95*	PAW S5/S6	AW2-5	PAW161	O5	Pr20H	*Xrems1303*
KS96HW10-1	+/+	-/-	-/-	1	0	0	1	1	0	0	0	0	1	0	0
Millennium	-/-	-/-	-/-	1	0	1	1	1	0	0	0	0	0	0	0
N02Y5018	+/+	+/+	-/-	1	0	0	1	1	0	0	0	1	1	0	0
N02Y5019	-/-	+/+	-/-	1	0	0	0	1	0	0	0	1	0	0	0
N02Y5025	+/+	-/-	-/-	1	0	0	1	1	0	0	0	1	1	0	0
N02Y5003	-/-	+/+	-/-	0	1	1	0	1	1	1	1	1	1	1	1
N02Y5096	-/-	-/-	-/-	1	0	0	1	1	0	0	0	1	0	0	0
N02Y5109	-/-	-/-	+/+	1	0	0	1	1	0	0	0	1	0	0	0
N02Y5117	+/+	-/-	-/-	1	0	0?	1	1	0	0	0	1	1	0	0
N02Y5149	+/+	+/+	-/-	0	1	1	0	1	1	1	1	1	1	1	1
N02Y5156	-/-	+/+	-/-	0	1	1	0	1	1	1	1	1	1	1	1
N02Y5163	-/-	+/+	-/-	¢	1	1	1	1	1	1?	1	1	1	1	1
N02Y2016	+/+	+/+	-/-	1	0	0	1	1	0	0?	0	1	1	0	0
Beaver	-/-		-/-	0	1	1	0	1	1	1	1	1	1	1	1
Manaska	+/+	+/+	+/+	0	0	0	0	1	0	0	0	1	1	0	1
CS N4A-T4D	-/-	-/-	-/-	1	0	0	1	1	0	0	0	0	0	0	0
CS N4D-T4B	-/-	-/-	-/-	1	0	0?	1	1	0	0	0	0	0	0	0
Chinese Spring	-/-	-/-	-/-	1	0	0?	1	1	0	0	0	1	0	0	0
CS N1B-T1A	-/-	-/-	-/-	0	0	0?	1	0	0	0	0	0	0	0	0
Rye DNA				#	0?	1	0	0	1	1	1	1	1	1	1

*1: presence or absence of *Th. intermedium* or rye DNA fragments revealed by GISH
4D: indicates 4Ai#2S chromatin of *Th. intermedium*; 1B indicates to alien fragments seen on wheat chromosome1B; ?D indicates to an unidentified D-genome recombinant chromosome, hybridizing to *Th. intermedium* genomic DNA.
+/+: alien fragments of similar size seen (homozygous); -/- when no alien fragments seen.
1: presence of the marker allele; 0 absence of the marker allele; 1? most probably present; 0? most probably absent; # when DNA was not available for PCR; ¢ PCR not successful for technical reasons.
Lines in bold indicate carriers of 1B recombinant chromosome.

Table S3 Pedigree lines used to identify potential 1B carriers using 1BS or 1RS specific markers

Pedigree line	Population	Wheat marker*2		Rye marker*3				Remarks
		Xfc618	*Xpsp3000*	*Xiag95*	1B267	SCM9	pAWS5/S6	
KS91H174*1	I	1	1	0	0	0	0	Normal wheat 1BS
KS91H184*1	II, III, IV	1	1	0	0	0	0	Normal wheat 1BS
Rio Blanco	I, IV	0	1	0	0	0	0	Lost distal wheat marker, and potentially 1BS is recombinant
MO8	IV	0	0	1	1	1	1	1RS present
Vista	I	1	1	0	0	0	0	Normal wheat 1BS
Redland	IV	1	1	0	0	0	0	Normal wheat 1BS
TAM107	I	1	1	0	0	0	0	Normal wheat 1BS
Anton	Control	1	1	0	0	0	0	Normal wheat 1BS
Beaver (1BL*1RS)	Control	0	0	1	1	1	1	1RS present
Rye	Control	0	0	1	1	1	1	

*1: are parental WSMV-resistance lines carrying 4Ai#2S chromosomal arm of *Th. intermedium* and *Wsm1* resistance gene
*2: *Xfc618* is one of the most distal 1BS marker (Reddy et al. 2008); *Xpsp3000* a closely linked marker to *Gli-1* locus (Bryan et al. 1997)
*3: amplifies diagnostic 1RS DNA
1: presence of the marker allele; 0 absence of the marker allele