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ORIGINAL PAPER

Development and characterization of wheat lines carrying stem rust resistance gene *Sr43* derived from *Thinopyrum ponticum*

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Abstract

Key message **Wheat lines carrying Ug99-effective stem rust resistance gene *Sr43* on shortened alien chromosome segments were produced using chromosome engineering, and molecular markers linked to *Sr43* were identified for marker-assisted selection.**

Abstract Stem rust resistance gene *Sr43*, transferred into common wheat (*Triticum aestivum*) from *Thinopyrum ponticum*, is an effective gene against stem rust Ug99 races. However, this gene has not been used in wheat breeding because it is located on a large *Th. ponticum* $7el_2$ chromosome segment, which also harbors genes for undesirable traits. The objective of this study was to eliminate excessive *Th. ponticum* chromatin surrounding *Sr43* to make it usable in wheat breeding. The two original translocation lines KS10-2 and KS24-1 carrying *Sr43* were first analyzed using simple sequence repeat (SSR) markers and florescent genomic in situ hybridization. Six SSR markers located on wheat chromosome arm 7DL were identified to be associated with the *Th. ponticum* chromatin in KS10-2 and KS24-1. The results confirmed that KS24-1 is a 7DS·$7el_2$L Robertsonian translocation as previously reported. However, KS10-2, which was previously designated as a $7el_2$S·$7el_2$L-7DL translocation, was identified as a 7DS-$7el_2$S·$7el_2$L translocation. To reduce the *Th. ponticum* chromatin carrying *Sr43*, a BC_2F_1 population (Chinese Spring//Chinese Spring *ph1bph1b**2/KS10-2) containing *ph1b*-induced homoeologous recombinants was developed, tested with stem rust, and genotyped with the six SSR markers identified above. Two new wheat lines (RWG33 and RWG34) carrying *Sr43* on shortened alien chromosome segments (about 17.5 and 13.7 % of the translocation chromosomes, respectively) were obtained, and two molecular markers linked to *Sr43* in these lines were identified. The new wheat lines with *Sr43* and the closely linked markers provide new resources for improving resistance to Ug99 and other races of stem rust in wheat.

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Introduction

Wheat (*Triticum aestivum* L., $2n = 6x = 42$, genome AABBDD) stem rust, caused by *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn (*Pgt*), is one of the major diseases of wheat. In the past 30 years, stem rust has been effectively controlled in most of the wheat-growing regions by eradicating the alternate host (barberry; *Berberis vulgaris* L. and *B. canadensis* Mill.) and deployment of stem rust resistance (*Sr*) genes (Singh et al. 2006; Zhong et al. 2009). However, stem rust recently became a serious threat

to wheat production due to the emergence of Ug99 races in Africa (Singh et al. 2011). The first Ug99 race, identified in Uganda in 1999 (Pretorius et al. 2000), was designated as TTKSK based on the North American stem rust nomenclature system (Jin et al. 2007a; Wanyera et al. 2006). TTKSK has broad virulence to currently deployed *Sr* genes including *Sr31*, which is derived from rye (*Secale cereal* L.) and present in many cultivars worldwide (Singh et al. 2006).

In addition to its broad virulence, TTKSK has rapidly moved out of Africa and evolved new virulent variants. TTKSK appeared in Yemen and Iran in 2006 and 2007, respectively (Nazari et al. 2009; Singh et al. 2008). Two new variants, TTKST and TTTSK, which are virulent to *Sr24* and *Sr36*, respectively, were identified in Kenya in 2006 and 2007 (Jin et al. 2007a, 2008, 2009; Wanyera et al. 2006). Recently, four stem rust races, including PTKSK (detected in Kenya and Ethiopia), PTKST (Kenya and South Africa), TTKSF (South Africa and Zimbabwe), and TTKSP (South Africa), have been verified under the Ug99 lineage (Park et al. 2011; Visser et al. 2011). To date, seven Ug99 races have been identified in the eastern African highlands, as well as Zimbabwe, South Africa, Sudan, Yemen, and Iran (Singh et al. 2011). Their epidemic in Africa and spread to other continents seriously threaten wheat production worldwide. Thus, there is an urgent need to accelerate the identification and deployment of effective resistance genes against Ug99 races into commercial cultivars.

Sr43 is an effective resistance gene against Ug99 races (Xu et al. 2009). This gene was originally transferred from *Thinopyrum ponticum* (Host) D. R. Dewey ($2n = 10x = 70$) into common wheat through chromosome substitution and translocation between a *Th. ponticum* group 7 chromosome ($7el_2$) and wheat chromosome 7D (Knott et al. 1977; Kibiridge-Sebunya and Knott 1983). The original translocation lines, KS10-2 and KS24-2, carry a pair of translocation chromosomes identified as $7el_2S{\cdot}7el_2$-7DL and 7DS·$7el_2$, respectively (Kim et al. 1993). The $7el_2S{\cdot}7el_2L$-7DL chromosome in KS10-2 consisted of the short arm and a large portion of the long arm of $7el_2$ and the distal one-half of 7DL, while the 7DS·$7el_2L$ chromosome in KS24-2 was a Robertsonian translocation in which the long arm of chromosome $7e1_2$ replaced 7DL (Kim et al. 1993). In both translocation lines, the *Th. ponticum* chromatin carrying *Sr43* is too large to be directly used for breeding due to linkage drag. Most importantly, the *Th. ponticum* chromatin carrying *Sr43* also carries the *Y* gene for yellow flour color, which is an undesirable quality trait in common wheat (Knott et al. 1977; Kibiridge-Sebunya and Knott 1983).

To make the rust resistance gene usable in wheat breeding, Kim et al. (1993) tried to break the linkage between *Sr43* and *Y* in KS10-2 by crossing it with another wheat–*Th. ponticum* 7D/$7el_1$ translocation line, K11695, which has a different breakpoint from KS10-2 (Kim et al. 1993). However, the attempt was not successful. The objective of this study was to eliminate or reduce the deleterious linkage drag associated with *Sr43* by reducing the *Th. ponticum* chromatin using *ph1b*-induced homoeologous pairing (Qi et al. 2007; Marais et al. 2010) and marker-assisted selection for homoeologous recombinants (Niu et al. 2011).

Materials and methods

Plant materials

Two wheat–*Th. ponticum* 7D/$7el_2$ translocation lines, KS10-2 and KS24-1 (Kim et al. 1993; Friebe et al. 1996), carrying *Sr43* on the *Th. ponticum* segments in common wheat 'Thatcher' background were used as the donor of the stem rust resistance gene. The translocation chromosomes in KS10-2 and KS24-1 were previously designated as $7el_2S{\cdot}7el_2L$-7DL and 7DS·$7el_2L$, respectively (Kim et al. 1993). The original seed of the two translocation lines were kindly provided by Dr. D. R. Knott, Department of Crop Science and Plant Ecology, University of Saskatchewan, Saskatoon, Canada. Wheat cultivar 'Chinese Spring' (CS) and the CS *ph1b* mutant were used as parents for crosses and backcrosses. Wheat cultivars Thatcher and CS, CS nulli-tetrasomic lines N7AT7D (nullisomic for 7A and tetrasomic for 7D), N7BT7D (nullisomic for 7B and tetrasomic for 7D), CS N7DT7B (nullisomic for 7D and tetrasomic for 7B), and a wheat line ISr6-Ra carrying a temperature-sensitive stem rust resistance gene *Sr6* (Knott and Anderson 1956) were used as checks for stem rust evaluation and molecular marker analysis. *Th. ponticum* accession AESR1 was used as the source of DNA probe for fluorescent genomic in situ hybridization (GISH). A wheat line LcSr25Ars (Jin et al. 2007b) and cultivar Wheatear (Liu et al. 2010) carrying *Sr25* derived from *Th. ponticum* chromosome $7el_1$ were used in marker haplotype analysis. Thirty-two common wheat lines and cultivars (Niu et al. 2011) were used for the validation of one newly developed STS marker and one SSR marker.

Development of *Sr43*-carrying wheat lines with reduced amounts of alien chromatin

The two original translocation lines, KS10-2 and KS24-1, and the controls (Thatcher and CS) were first evaluated for seedling reaction to multiple races of stem rust to identify a race that could differentiate *Sr43* from the *Sr* genes in Thatcher. They were then analyzed using SSR markers on chromosome 7D and GISH to precisely locate the *Th. ponticum* chromatin and identify the SSR markers that were polymorphic between *Th. ponticum* chromatin and

wheat chromosome 7D. Based on the stem rust tests and marker/GISH analyses, we chose KS10-2 as the donor of *Sr43* to develop new wheat lines with reduced *Th. ponticum* chromatin using the chromosome engineering procedure described by Niu et al. (2011). KS10-2 was crossed as male to the CS *ph1b* mutant. The F_1 plants were then backcrossed to CS *ph1b*. The BC_1F_1 plants were tested for reaction to a stem rust race and genotyped with *Ph1* molecular markers to select resistant individuals that were homozygous for *ph1b*. The selected BC_1F_1 plants were backcrossed to CS. The BC_2F_1 populations were tested for stem rust resistance and the resistant BC_2F_1 plants were genotyped with the SSR markers located within the wheat chromatin that was replaced by the *Th. ponticum* segment in KS10-2. Based on marker analysis, the BC_2F_2 progenies derived from the BC_2F_1 plants with potentially shortened *Th. ponticum* chromosome segments were tested for stem rust resistance and analyzed using GISH to determine the physical size of the shortened alien chromosome segments. Confirmed homozygous wheat lines with shortened alien segments derived from the BC_2F_2 were further tested with multiple stem rust races including TTKSK.

Stem rust resistance evaluation

To identify a local race that could detect *Sr43* in the backcross populations (BC_1F_1, BC_2F_1 and BC_2F_2), Thatcher, CS, KS10-2, and KS24-1 were evaluated for reactions to races TMLKC, TPMKC, TCMJC, THTSC, HKHJC, LBBLB, MCCFC, and JCMNC. The *Pgt* races were designated based on the North American stem rust nomenclature system (Roelfs and Martens 1988) expanded to five differential sets (Jin et al. 2008). Based on the tests, we selected the race TMLKC, which has an avirulent/virulent formula of 6 8a 9a 9b 17 24 30 31 38/5 7b 9d 9e 9g 10 11 21 36 McN Tmp and can differentiate *Sr43* from the *Sr* genes in Thatcher, to evaluate the backcross populations. The final introgression lines carrying shortened *Th. ponticum* fragments were verified for their resistance to TTKSK at the USDA-ARS Cereal Disease Laboratory, St. Paul, MN. They were also tested with TMLKC and seven additional local races, including MCCFC, QCCJB, QFCSC, QTHJC, RHFSC, TPMKC, and TPPKC, at two temperature regimes (21 and 26 °C). Stem rust inoculation and evaluation were performed as described by Niu et al. (2011). Infection types were scored using the scale described by Stakman (1962), where 0 = immune, ; = necrotic flecks, 1 = small necrotic pustules, 2 = small to medium-sized chlorotic pustules with green island, 3 = medium-sized chlorotic pustules, and 4 = large pustules without chlorosis. Plants with infection type 3 or over were considered susceptible, and plants with an infection type less than 3 were considered resistant.

Molecular marker analysis

Molecular marker analysis was used to characterize the original translocation lines, detect homozygous *ph1b* BC_1F_1 plants, genotype the BC_2F_1 population containing *ph1b*-induced homoeologous recombinants, and compare the difference between *Sr43* and *Sr25*. The original translocation lines carrying *Sr43* (KS10-2 and KS24-1), Thatcher, CS, CS N7AT7D, CS N7BT7D, and CS N7DT7B were analyzed for polymorphisms using SSR markers mapped to chromosome 7D in the wheat SSR consensus map (Somers et al. 2004). DNA isolation and polymerase chain reaction (PCR) amplification were performed as described by Yu et al. (2009, 2010a). The PCR products were electrophoresed on 6 % polyacrylamide gels using the procedure of Yu et al. (2009). The gels were stained with Gel-Red and scanned with a Typhoon 9410 imager (Molecular Dynamics, Ithaca, NY, USA). The SSR markers that were polymorphic between the *Th. ponticum* chromosome segment in KS10-2 and wheat chromosome 7D in CS and Thatcher were used to genotype the BC_2F_1 population using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) as described by Tsilo et al. (2009) and Niu et al. (2011). The detection of the *ph1b* allele in the BC_1F_1 plants was performed using multiplex touchdown PCR with three markers, AWJL3, PSR128, and PSR574 (Roberts et al. 1999) as described by Niu et al. (2011). The marker haplotype analysis for comparing the wheat lines carrying *Sr43* and *Sr25* was performed using polyacrylamide gel electrophoresis as described by Klindworth et al. (2012).

Fluorescence genomic in situ hybridization

Genomic in situ hybridization was performed using a similar protocol as described by Yu et al. (2010a). In this study, CS genomic DNA was used as block DNA, and the genomic DNA from *Th. ponticum* accession AESR1 was used as a probe. The somatic chromosome images from GISH were examined under a Zeiss Axioplan 2 Imaging Research Microscope and captured using an Axiocam HRm CCD camera (Carl Zeiss Light Microscopy, Germany). The length of interchanged chromosomes and *Th. ponticum* chromosome segments were measured in about 20 cells with good spread of mitotic metaphases. The sizes of the *Th. ponticum* chromosome segments were calculated as the average percentage of the length of *Th. ponticum* chromosome segment divided by the total length of the interchanged chromosome.

Identification and validation of molecular markers linked to *Sr43* on shortened *Th. ponticum* chromosome segments

Genomic DNA used to identify and validate molecular markers linked to *Sr43* on shortened *Th. ponticum*

chromosome segments in the new wheat lines was extracted from seedling plants as described by Niu et al. (2011). Molecular marker and GISH analysis localized the shortened *Th. ponticum* chromosome segments carrying *Sr43* on the distal region of chromosome arm 7DL in the new wheat lines. Therefore, the wheat EST sequences mapped to the deletion bin 7DL3-0.82-1.00 (http://wheat.pw.usda.gov/cgi-bin/graingenes/report.cgi?class=breakpointinterval;name=7DL3-0.82-1.00;show=locus) in the distal region of 7DL were selected to design STS primers using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) under general settings. These primers were used to screen for polymorphism among Thatcher, CS, KS10-2, and a bulk of four resistant plants and a bulk of four susceptible plants from the BC_2F_2 (CS//CS *ph1b-ph1b**2/KS10-2) population.

To validate the molecular markers linked to *Sr43* on the shortened *Th. ponticum* chromosome segments, a polymorphic STS marker that was identified following the procedure described above and an SSR marker (*Xcfa2040*) associated with the shortened *Th. ponticum* chromosome segments, were used to genotype the BC_2F_2′s and 32 common wheat cultivars. Marker analysis was done as described by Niu et al. (2011). PCR were carried out as follows: 95 °C for 5 min, 95 °C for 40 s, 56 °C for 40 s, and 72 °C for 1 min, repeated for 36 cycles, with a final extension at 72 °C for 10 min. The PCR products were separated on an 8 % non-denaturing polyacrylamide gel.

Analysis of wheat quality characteristics and flour color

Grain samples of the two new wheat lines (RWG33 and RWG34) with the shortened *Th. ponticum* chromosome segments and their parents KS10-2, Thatcher, and CS were used for a preliminary quality test to determine if the new wheat lines still carried the gene *Y* for yellow flour color. A bulk grain sample of each of KS10-2 and RWG33 harvested from a greenhouse, two samples of each of CS and RWG34 (greenhouse), and three samples of Thatcher (greenhouse and field), respectively, were used in the test. Wheat kernel characteristics were analyzed according to the AACCI Method 55-31 (American Association of Cereal Chemists International (AACCI) 2010) using a Single Kernel Characterization System (SKCS 4100, Perten Instruments, Hägersten, Sweden). Grain samples (about 20 g per sample) were milled in a Brabender Quadrumat Jr. mill (C.W. Brabender Instrument Inc., South Hackensack, NJ, USA) after tempering to 16.0 % moisture content. Flour ash content was determined according to the AACCI Method 08-01 (American Association of Cereal Chemists International (AACCI) 2010). Flour protein content was determined using the nitrogen combustion method (Method 46-30, American Association of Cereal Chemists International (AACCI) 2010). Flour color was evaluated using a Minolta CR-200 Chroma meter (Minolta Camera Co., Ltd, Ramsey, NJ, USA). Individual color rating scales were as follows: *L** value for whiteness (100 white, 0 black), *a** value for red-green chromaticity (+60 red, −60 green), and *b** value for yellowness (+60 yellow, −60 blue). Flour yellow pigment concentration was analyzed as described by Santra et al. (2003). A 0.125 g flour sample was mixed with 1.25 mL of water-saturated butanol in a 1.5-mL microcentrifuge tube by vortexing. The mixture was kept in the dark for 16–18 h and then centrifuged at 10,000*g* for 10 min. Yellow pigment concentration (YPC) was expressed as the absorbance of supernatant measured at 440 nm (Abs_{440nm}) on a spectrophotometer. All quality data were analyzed using the GLM procedure in SAS statistical software version 9.2 (SAS Institute, Cary, NC, USA). The means of each of the quality parameters were separated by least significant difference (LSD).

Results

Characterization of the original translocation lines with molecular markers showed that six co-dominant SSR markers (*Xbarc172*, *Xwmc150*, *Xbarc121*, *Xwmc797*, *Xbarc111*, and *Xcfa2040*) previously mapped to the long arm of chromosome 7D (Somers et al. 2004) were polymorphic between the two translocation lines (KS10-2 and KS24-1) and the wheat checks (Thatcher and CS) (Fig. 1). All six markers produced the same amplicons from both KS10-2 and KS24-1 (Online Resource Fig. S1). The GISH results confirmed that the interchanged chromosome in KS24-1 is a 7DS·7el$_2$L Robertsonian translocation, but the

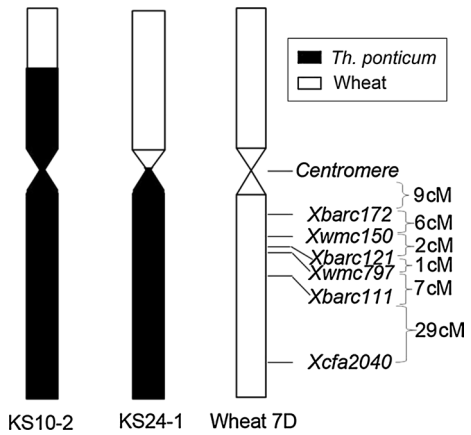

Fig. 1 Schematic representation of wheat–*Thinopyrum ponticum* 7D/7el$_2$ translocation lines KS10-2 and KS24-1 and the positions of six SSR markers based on the marker information described by Somers et al. (2004). The *Th. ponticum* chromatin is represented in *black*. Wheat chromatin is represented in *white*

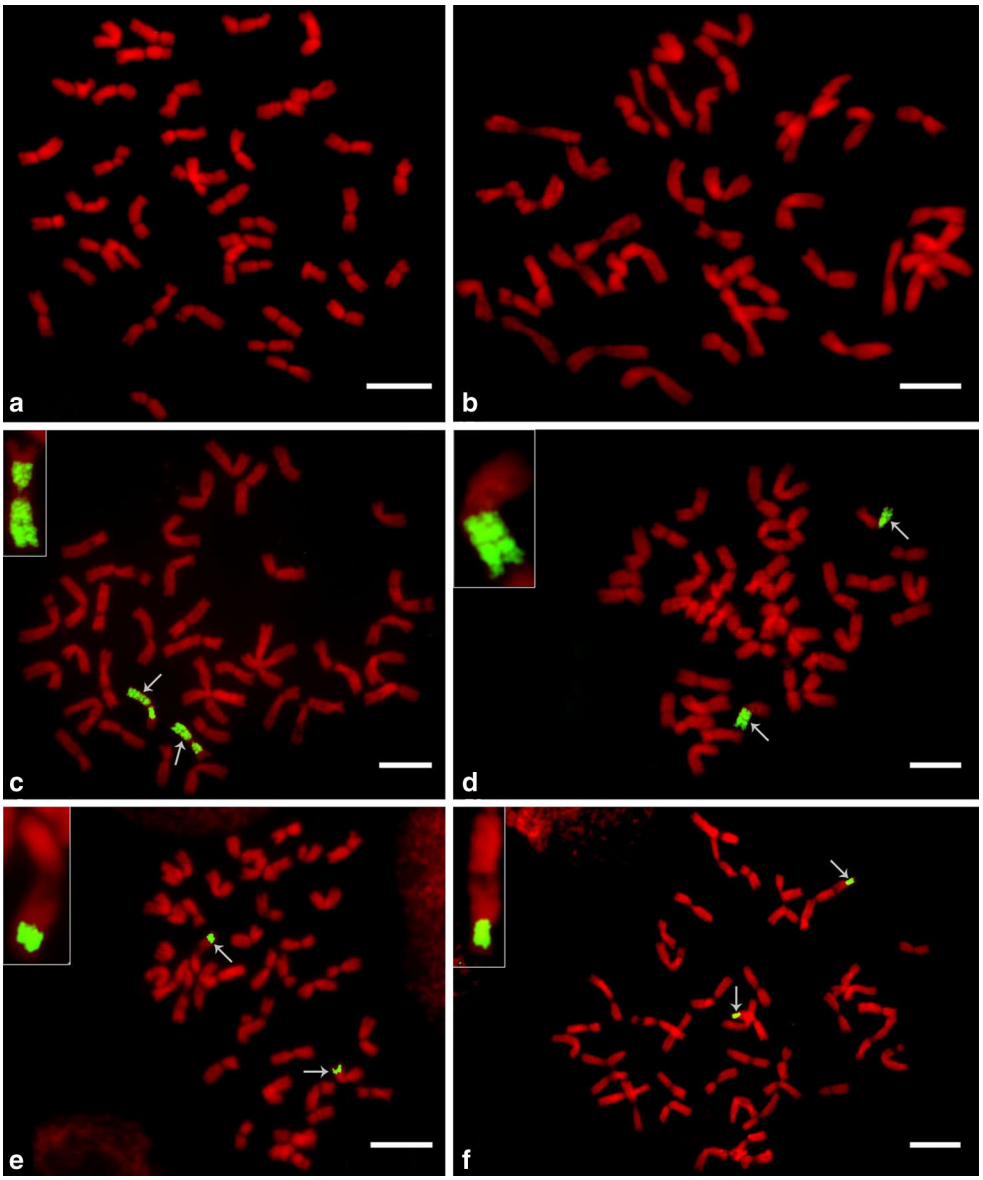

Fig. 2 Images from GISH analysis of wheat-*Thinopyrum ponticum* translocation lines carrying stem rust resistance gene *Sr43*. GISH was performed using *Th. ponticum* (AESR1) genomic DNA as the probe and Chinese Spring (CS) genomic DNA as the block on Thatcher (**a**), CS (**b**), original wheat–*Th. ponticum* translocation lines KS10-2 (**c**) and KS24-1 (**d**), and two new wheat lines RWG33 (**e**) and RWG34 (**f**) with *Sr43* on shortened *Th. ponticum* chromatin. The alien chromatin from *Th. ponticum* (*green color*) is indicated by *arrows*. *Bar* = 10 μm

interchanged chromosome in KS10-2 included the whole long arm and approximately 60 % of the short arm of *Th. ponticum* chromatin, with only a distal segment on the short arm being wheat chromatin (Fig. 2c, d). Therefore, we re-designated KS10-2 as a 7DS-7el$_2$S·7el$_2$L translocation.

To develop *ph1b*-induced homoeologous recombinants, 89 BC$_1$F$_1$ plants (CS *ph1bph1b**2/KS10-2) were produced and tested with stem rust race TMLKC. The segregation ratio of resistant to susceptible was 27:62; and, this ratio did not fit a 1:1 ratio ($\chi^2 = 13.8$, $P < 0.001$), indicating segregation distortion at this gene locus. The 27 resistant plants were analyzed with molecular markers PSR128, PSR574, and AWJL3 to detect the presence of *ph1b*. Ten resistant plants were identified as homozygotes for *ph1b* and were backcrossed to CS to develop ten families composing a large BC$_2$F$_1$ population. A total of 706 plants in the eight families were tested with stem rust race TMLKC, and there were 270 resistant and 436 susceptible plants (Table 1). The χ^2 test indicated that segregation in only four of the eight families (84-14, 84-27, 84-48 and 84-83) fit a 1:1 ratio (Table 1), indicating significant segregation distortion among the families.

Table 1 Segregation for resistance to stem rust race *Pgt*-TMLKC in eight BC_2F_1 families derived from the backcross of Chinese Spring (CS) to eight plants having the pedigree CS *ph1bph1b**2/KS10-2

Family no.	Resistant	Susceptible	χ^2 (1:1)	Probability
84-2	26	64	16.0	<0.001
84-14	48	42	0.4	0.527
84-20	29	61	11.4	<0.001
84-27	37	53	2.8	0.094
84-48	36	52	2.9	0.089
84-52	27	61	13.1	<0.001
84-54	32	52	4.8	0.028
84-83	35	51	3.0	0.083
Total	270	436	39.0	<0.001

To identify new wheat lines carrying *Sr43* on reduced alien segments, the 270 BC_2F_1 plants resistant to TMLKC were screened for dissociation of *Sr43* from one or more of the six co-dominant SSR markers (*Xbarc172*, *Xwmc150*, *Xbarc121*, *Xwmc797*, *Xbarc111*, and *Xcfa2040*) located on 7DL (Online Resource Fig. S1). Except that 2 plants had CS alleles at three SSR loci but had missing data at other three SSR loci (Plant Types 5 and 6; Table 2), 51 of 270 plants carried CS alleles at all six SSR loci (Plant Type 1; Table 2), 13 retained *Th. ponticum* alleles at 1–2 marker loci (plant types 2, 3, 4, 7, 8, and 9; Table 2), and the remaining 204 plants carried *Th. ponticum* alleles at a minimum of three SSR loci (plant types 10 through 15; Table 2). To identify the lines carrying shortened *Th. ponticum* segments, five to six BC_2F_2 plants derived from each of the BC_2F_1 plants carrying all wheat alleles at the six marker loci and the plants having *Th. ponticum* alleles at 1–2 marker loci were tested with TMLKC (Table 2). All progenies derived from the BC_2F_1 plants carrying the *Th. ponticum* allele at the *Xcfa2040* locus segregated in their reactions to stem rust. However, the progenies from the BC_2F_1 plants without the *Th. ponticum* allele at this marker locus were susceptible regardless of the genotype at other marker loci (Table 2). This result indicated that *Xcfa2040* is the most closely linked marker to *Sr43* among the six tested SSR markers.

We then analyzed the resistant BC_2F_2 plants derived from the BC_2F_1 plants carrying the *Th. ponticum* allele at the *Xcfa2040* locus using GISH. The resistant BC_2F_2 plants from two families, 84-14 and 84-83, were identified to carry a T7DS•7DL-7el$_2$-7DL translocated chromosome with shortened *Th. ponticum* chromosome segments (Table 1; Fig. 2f, e). The individuals homozygous for the shortened translocations derived from families 84-14 and 84-83 were designated RWG33 and RWG34, respectively. The shortened *Th. ponticum* chromosome segments in both RWG33 and RWG34 are interstitially located in the subterminal region of 7DL. Compared to KS10-2 in which the large *Th. ponticum* segment was calculated as 83.3 % (20 cells) of the translocated chromosome, RWG33 and RWG34 were calculated as 17.5 % (27 cells) and 13.7 % (20 cells) of the translocated chromosomes, respectively. Therefore, about 79.0 and 83.6 % of *Th. ponticum* chromatin surrounding *Sr43* were removed in the two new wheat lines by *ph1b*-induced homoeologous recombination.

The GISH patterns and the physical position of the SSR marker *Xcfa2040* on chromosome 7D suggested that *Sr43* was most likely located in deletion bin 7DL3-0.82-1.00. To

Table 2 SSR marker analysis of 270 BC_2F_1 plants derived from backcross Chinese Spring (CS)//CS *ph1bph1b**2/KS10-2

Plant type	No. of plants	Haplotypes of SSR markers[a]					
		Xbarc172	*Xwmc150*	*Xbarc121*	*Xwmc797*	*Xbarc111*	*Xcfa2040*
1	51	W	W	W	W	W	W
2	2	W	W	W	W	W	T
3	2	W	W	W	W	T	W
4	1	T	W	W	W	W	W
5	1	–	–	–	W	W	W
6	1	–	W	–	W	W	–
7	1	W	W	T	W	W	T
8	4	–	W	T	W	W	T
9	3	T	W	W	W	W	T
10	187	T	W	T	W	W	T
11	7	T	W	T	W	–	T
12	2	T	W	T	W	W	–
13	1	–	T	T	W	W	T
14	5	T	W	T	W	T	T
15	2	T	W	T	T	W	T

[a] Marker haplotypes: W, homozygous for wheat CS alleles; T, *Thinopyrum ponticum* alleles and wheat alleles coexist; –, missing data

Table 3 Primer sequences and melting temperature (Tm) of the STS marker *Xrwgs30* and the SSR *Xcfa2040* linked to *Sr43* on *Th. ponticum* chromosome segments in two new wheat lines

Primer name	Primer sequences	Tm (°C)[a]	EST accession/contig or reference
Xrwgs30	CTCTTGGTGCCACACTCTGA	60	BE443432, BQ161328, NSFT03P2_Contig14171[b]
	TCAGTTCCCTCCCATTCATC	60	
Xcfa2040	TCAAATGATTTCAGGTAACCACTA	60	Sourdille et al. (2001)
	TTCCTGATCCCACCAAACAT	60	

[a] Melting temperature at the condition of 50 mM Na^+

[b] Wheat ESTs (BE443432 and BQ161328) and contig (NSFT03P2_Contig14171) mapped to deletion bin 7DL3-0.82-1.00 (http://wheat.pw.usda.gov/cgi-bin/graingenes/report.cgi?class=sequence;query=n*;name=NSFT03P2_Contig14171)

develop molecular markers closely linked to *Sr43* in these two new lines, we designed primers based on the sequences of the wheat ESTs mapped to deletion bin 7DL3-0.82-1.00. A total of 26 STS primer pairs were tested for polymorphisms among Thatcher, CS, KS10-2, and BC_2F_2 (CS// CS *ph1bph1b**2/KS10-2) resistant and susceptible bulks. Eleven primer pairs detected polymorphisms among the lines with two of them behaving as co-dominant STS markers. The other nine were dominant markers with the null alleles in the *Th. ponticum* segments. The two co-dominant STS markers were derived from the EST accessions BE443432 and BQ161328 that were assembled in the same EST contig (NSFT03P2_Contig14171, Table 3). Thus, these two primer pairs amplified the same locus and functioned as a single STS marker designated *Xrwgs30* (Table 3). This co-dominant STS marker amplified a 1,078-bp fragment in KS10-2 and amplified two fragments in sizes of approximately 872 and 1,500 bp in Thatcher and CS (Fig. 3a). The SSR marker *Xcfa2040* amplified a 233-bp fragment in KS10-2, a 261-bp fragment in Thatcher, and a 260-bp fragment in CS (Fig. 3a and Online Resource Fig. S1).

Similar to *Sr43*, stem rust resistance gene *Sr25* is also located on the distal *Th. ponticum* segment of the translocated chromosome T7DS·7DL-Ae#1 (Friebe et al. 1994). To test if the two genes are same, three wheat lines (KS10-2, RWG33 and RWG34) carrying *Sr43* and wheat line LcSr25Ars and cultivar Wheatear having *Sr25* were genotyped with *Xcfa2040*, *Xrwgs30*, and two *Sr25*-digonostic PCR markers *Gb* and *BF145935* (Ayala-Navarrete et al. 2007; Liu et al. 2010; Yu et al. 2010b). Marker analysis showed that these markers generated different banding profiles from the wheat lines carrying *Sr25* and *Sr43*, respectively (Fig. 3b), indicating that *Sr25* and *Sr43* are different genes from *Th. ponticum*. Most interestingly, the marker *Xrwgs30* for *Sr43* generated a unique band in size of approximately 600 bp from the two wheat lines carrying *Sr25*, while the *BF145935* marker for *Sr25* amplified two unique bands in sizes of approximately 200 and 205 bp from the three lines carrying *Sr43* (Fig. 3b). Thus, *Xrwgs30* and *BF145935* are also potential markers for detecting *Sr25* and *Sr43*, respectively.

Both RWG33 and RWG34 exhibited similar disease reactions to TTKSK and eight local stem rust races as KS10-2 (Table 4; Fig. 3c), but they were morphologically closer to CS than KS10-2 (Fig. 3d). In addition, RWG33, RWG34, and KS10-2 showed temperature-sensitive reactions to the local stem rust races, similar to line ISr6-Ra which has the temperature-sensitive stem rust resistance gene *Sr6*. At 21 °C, ISr6-Ra (*Sr6*) was susceptible to races RHFSC and QTHJC and highly resistant to all other six races; RWG33, RWG34, and KS10-2 were highly resistant to all the local races except for QFCSC. KS10-2 was moderately resistant to QFCSC, but RWG33 and RWG34 were susceptible to this race. Because Thatcher was also moderately resistant to QFCSC, the resistance to this race in KS10-2 is likely controlled by an *Sr* gene from Thatcher, which was eliminated during the development of RWG33 and RWG34. At 26 °C, ISr6-Ra and RWG33 were susceptible to all eight local races, while KS10-2 and RWG34 had decreased level of resistance (ITs 23 and 23;) to RHFSC and MCCFC and were susceptible to the other six races, indicating that *Sr43* is a temperature-sensitive *Sr* gene like *Sr6*.

The two *Sr43*-linked markers, *Xrwgs30* and *Xcfa2040*, were validated with 32 common wheat lines and cultivars (Online Resource Table 1S and Fig. S2). Two alleles, i.e., either 258-bp or 260-bp fragments, were detected at the *Xcfa2040* locus in all 32 wheat lines and cultivars (Online Resource Table 1S and Fig. S2). At the *Xrwgs30* locus, only one allele (i.e., 1,500-bp fragment) was detected in 32 wheat lines and cultivars. The *Th. ponticum* alleles at these two marker loci in KS10-2 and the two new wheat lines, RWG33 and RWG34, were different from those in all the common wheat cultivars tested. The two markers, therefore, can be used for marker-assisted selection of *Sr43*.

Wheat kernel and flour color characteristics are listed in Table 5. The KS10-2 flour sample showed lower brightness than CS, RWG33, and RWG34, while CS flour had higher redness than KS10-2, RWG33, and RWG34. Flour

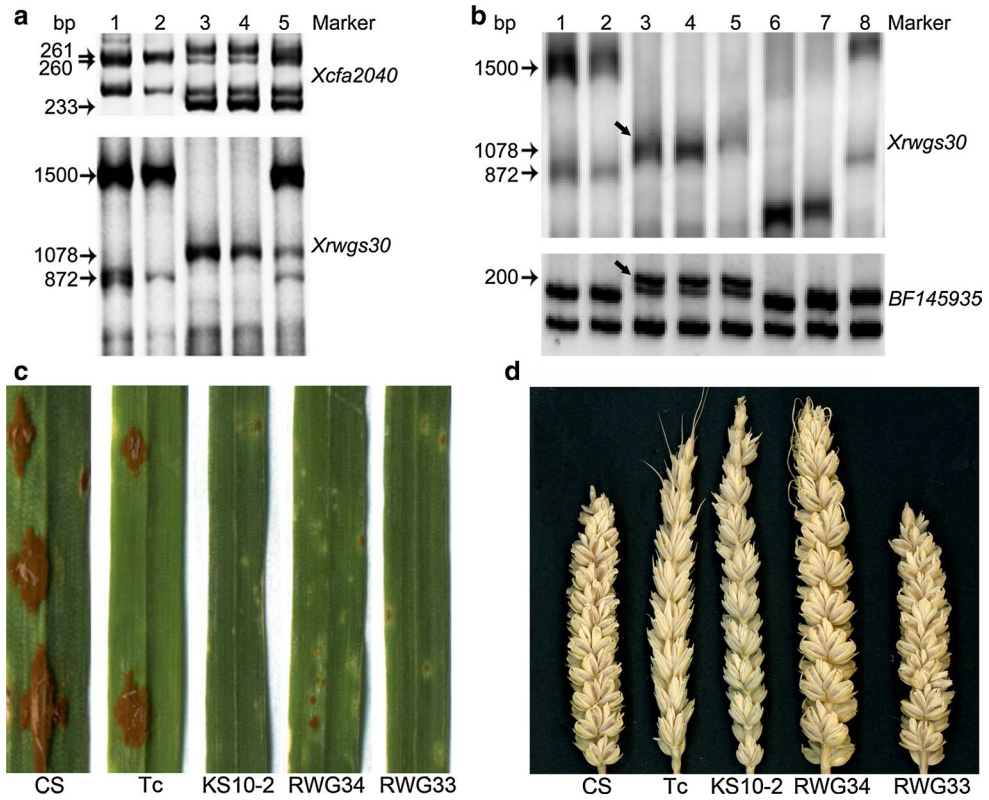

Fig. 3 Characterization of two new wheat lines (RWG33 and RWG34) carrying *Sr43* on a small segment of *Th. ponticum* chromatin. **a** Gel images of the PCR products of two co-dominant markers (*Xrwgs30 and Xcfa2040*) run on an 8 % non-denaturing polyacrylamide gel. The numbers at the top of the gels are lane numbers: 1, Thatcher (Tc); 2, Chinese Spring (CS); 3, KS10-2; 4, RWG34; 5, heterozygous BC_2F_2 plants for RWG34. The numbers on the left side represent the size of the fragment in base pair (bp). **b** Gel images of the PCR products of co-dominant marker *Xrwgs30* and dominant marker *BF145935* run on an 8 % non-denaturing polyacrylamide gel. The numbers at the top of the gels are lane numbers: 1, Tc; 2, CS; 3, KS10-2; 4, RWG33; 5, RWG34; 6, LcSr25Ars; 7, Wheatear; 8, CS. The numbers on the *left side* represent the size of the fragment in base pair (bp). **c** Photograph of stem rust reactions to race TMLKC. **d** Photograph of spike morphology

Table 4 Infection types produced by two new wheat lines with *Th. ponticum* chromosome segment carrying *Sr43* and parental lines to TTKSK and eight local races of *Puccinia graminis* f. sp. *tritici* (*Pgt*) at two temperature condition (21 and 26 °C)

Lines	TTKSK[a]	TPMKC		TMLKC		TPPKC		RHFSC		QTHJC		MCCFC		QCCJB		QFCSC	
	21[b]	21	26	21	26	21	26	21	26	21	26	21	26	21	26	21	26
Thatcher	–[c]	43	43	432	43	43	32	34	32	32	32	;2	23	–	321	23	34
CS	43	43	4	43	4	43	43	43	34	43	43	43	43	43	43	34	4
KS10-2	;	;	32	;1	342	;1–	32	;1–	23	12;	32	;	23	;	321	231	34
RWG33	–	;1–	43	;1	43	;	324	;1–	324	213	43	;1-	34	;1–	324	43	43
RWG34	;1	;	43	;1–	32	;	324	;1–	23;	;12	32	;1	23	;	32;	34	43
ISr6-Ra	–	;12	43	;1–	34	;1–	4	43	43	43	43	;1–	4	;1–	4	;1–	4

Infection types follow Stakman (1962) where 0, ; , 1, 2, or combinations were considered low infection types, and 3–4 were considered high infection types

[a] The *Pgt* races were designated based on the North American stem rust nomenclature system (Roelfs and Martens 1988) expanded to five differential sets (Jin et al. 2008)

[b] 21 and 26 refer to 21 and 26 °C, respectively

[c] – refers to missing data

Table 5 Wheat kernel characteristics, flour ash, protein content, and color parameters of the parental lines and two new *Sr43* carrying lines with *Th. ponticum* chromosome segments

Lines	Single kernel characteristics			Flour ash (%)	Flour protein (%)	Flour color			YPC (Abs_{440nm})
	Hardness index	Weight (mg)	Diameter (mm)			Brightness (L*)	Redness (a*)	Yellowness (b*)	
KS10-2	78.3a	28.1b	2.52b	0.46a	17.6a	88.8c	−3.6c	16.4a	0.30a
CS	44.0b	34.3a	2.78a	0.38a	14.7a	92.4a	−1.7a	6.3d	0.06c
Thatcher	55.0b	36.5a	2.88a	0.36a	14.6a	89.6bc	−1.8a	7.9c	0.06c
RWG33	57.0ab	29.4b	2.59b	0.37a	15.9a	91.4ab	−3.4bc	11.9b	0.21b
RWG34	40.2b	35.7a	2.70ab	0.38a	13.7a	91.5ab	−3.3b	12.0b	0.21b
LSD (0.05)	22.3	4.6	0.19	0.16	4.1	1.6	0.3	1.5	0.04

Values within a column followed by different letters are significantly different at $P < 0.05$

YPC yellow pigment concentration and, $Abs_{440\ nm}$ absorbance at 440 nm

yellowness and YPC values of RWG33 and RWG34 were higher than those of CS and Thatcher, indicating that the shortened *Th. ponticum* segments in both RWG33 and RWG34 probably still carry the *Y* gene for flour yellowness. However, RWG33 and RWG34 showed lower flour yellowness than KS10-2, suggesting that expression of the gene related to flour yellowness might be partially suppressed in the new wheat lines due to changes in genetic background, or the additional gene controlling yellow pigment content (Zhang and Dubcovsky 2008) may have been eliminated in the short translocations.

Discussion

Stem rust resistance genes transferred from alien genomes of wild Triticeae species are useful resources in the effort to contain the stem rust Ug99 threat to world wheat production. In addition to previously deployed alien *Sr* genes such as *Sr24*, *Sr36*, and $Sr1R^{Amigo}$ (Jin and Singh 2006; Olson et al. 2010), several other Ug99-effective *Sr* genes such as *Sr25* (Liu et al. 2010), *Sr26* (Dundas et al. 2007), *Sr32* (Mago et al. 2013), *Sr39* (Kerber and Dyck 1990; Mago et al. 2009; Niu et al. 2011), *Sr40* (Dundas et al. 2007), *Sr47* (Faris et al. 2008; Klindworth et al. 2012), and *Sr50* (Anugrahwati et al. 2008) have recently been made available to wheat breeding programs. Three new alien *Sr* genes, including *Sr51* from *Aegilops searsii* Feldman and Kislev ex Hammer (Liu et al. 2011a), *Sr52* from *Dasypyrum villosum* (L.) Candargy (Qi et al. 2011), and *Sr53* from *Ae. geniculata* Roth (Liu et al. 2011b), were recently transferred into the wheat genome. More recently, another major Ug99-effective gene *Sr44* from *Th. intermedium* (Host) Barkworth and D.R. Dewey was transferred into wheat as a compensating wheat–*Th. intermedium* Robertsonian translocation (Liu et al. 2013). All these studies represent major efforts in the utilization of alien species-derived *Sr* genes for managing Ug99.

In the present study, we attempted to make *Sr43* usable in wheat breeding by minimizing alien chromatin associated with *Sr43* using an improved *ph1b* mutant-mediated chromosome engineering procedure (Niu et al. 2011). Two introgression lines (RWG33 and RWG34) were developed to contain *Sr43* on a shortened *Th. ponticum* segment in the T7DS·7DL-$7el_2$L-7DL translocated chromosome. The alien segments in these two lines were shortened by approximately 80 % compared to the original stock KS10-2. In addition, two markers (*Xcfa2040* and *Xrwgs30*) closely linked to *Sr43* were identified and developed, respectively. This is the first report of substantial reduction of alien segments carrying *Sr43* and the development of *Sr43*-linked molecular markers.

Kim et al. (1993) designated the wheat translocation line KS10-2 as $7el_2$S·$7el_2$-7DL following RFLP analysis. After screening with the SSR markers located on 7D, we found that all the polymorphic markers between wheat (CS and Thatcher) and translocation lines (KS10-2 and KS24-1) were located on 7DL, and KS10-2 and KS24-1 had the same genotype at these SSR marker loci. This indicated that the long arm of the group 7 chromosomes in KS10-2 and KS24-1 were derived from *Th. ponticum*. The GISH results further proved that the translocated chromosome in KS10-2 had a distal segment of 7DS and the whole long arm and part of the short arm derived from *Th. ponticum*. Therefore, the translocated chromosome in KS10-2 should be designated 7DS-$7el_2$S·$7el_2$L. The shortened *Th. ponticum* segments carrying *Sr43* in the two new wheat lines (RWG33 and RWG34) were physically located in the subterminal region of 7DL. Because the stem rust resistance gene *Sr25* is also located on the distal *Th. ponticum* segment of the translocated chromosome T7DS·7DL-7Ae#lL in wheat lines Agatha and Agatha-28 (Friebe et al. 1994), the possibility that *Sr25* and *Sr43* are homoeo-allelic should be considered. Several previous studies demonstrated that *Sr25* and *Sr43* were derived from two different

Th. ponticum 7E chromosomes, which were identified as $7el_1$ and $7el_2$, respectively (Dvořák 1975; Kim et al. 1993; Zhang et al. 2011). The two chromosomes exhibited massive divergence as revealed by chromosome pairing and marker analysis (Dvořák 1975; Zhang et al. 2011). Stem rust tests showed that *Sr43* and *Sr25* exhibited different reactions to race TTKSK; in this study, *Sr43* conditioned an IT; (fleck) or ;1 while *Sr25* conditions an IT 2 or 2^+ (Jin et al. 2007b). Thus, the differing origin, marker analysis data and stem rust tests all suggested that *Sr25* and *Sr43* were different genes from *Th. ponticum*.

Some stem rust resistance genes, such as *Sr6*, are temperature sensitive (Knott and Anderson 1956). The evaluation of the two shortened translocation lines RWG33 and RWG34 and their parents with eight local races under two different temperatures (21 and 26 °C) showed that *Sr43* was also a temperature-sensitive *Sr* gene. Among the three lines carrying *Sr43*, RWG33 was susceptible to all eight local races and KS10-2 and RWG34 were susceptible to six races and moderately resistant to two races (RHFSC and MCCFC) at 26 °C. The different reactions of RWG33 from KS10-2 and RWG34 to the two races might reflect the difference in gene interaction or genetic backgrounds in these lines. Because Thatcher was resistant to MCCFC at both 21 and 26 °C (Table 4), it should carry a temperature-insensitive *Sr* gene to the race. The evaluation data suggested that KS10-2 and RWG34 carry the *Sr* gene from Thatcher, but RWG33 likely lost the gene. Thatcher was susceptible to RHFSC at both temperature conditions (Table 4). The IT 23 to RHFSC at 26 °C in these two lines might result from experimental error or indicate that *Sr43* was not completely ineffective to the race at high temperature. The three lines carrying *Sr43* have not been tested with Ug99 lineage races under different temperatures. If *Sr43* is also temperature sensitive to Ug99 lineage races, it might limit the efficacy of this gene in wheat breeding programs, especially for those targeting wheat-growing regions with hot weather conditions. This limitation might be overcome by pyramiding *Sr43* with other major *Sr* genes.

The preliminary quality analysis showed that similar to the original translocation line KS10-2, the two new wheat lines (RWG33 and RWG34) retained the *Y* gene. However, RWG33 and RWG34 showed significantly less flour yellowness than KS10-2, suggesting that expression of the gene(s) related to flour yellowness might be partially suppressed in the new wheat lines. Tsilo et al. (2011) reported that a QTL for flour yellowness was closely linked to the kernel hardness locus on 5DS. The 5D QTL reported by Tsilo et al. (2011) might affect kernel hardness as well as flour yellowness and result in less flour yellowness and kernel texture for RWG33 and RWG34 than KS10-2 in this research. Zhang and Dubcovsky (2008) indicated that the *Phytoene synthase 1* (*PSY-1*) gene located on the distal regions of 7AL and 7BL in wheat has been proposed as a candidate gene controlling grain yellow pigment content. *Th. ponticum* ortholog *PSY-E1* is linked to differences in grain yellow pigment content. Zhang and Dubcovsky (2008) hypothesized that *PSY-1* and at least one additional gene in the distal region of the long arm of homoeologous group 7 are associated with variation in grain yellow pigment content. Thus, it is possible that the *Th. ponticum* segment carrying the additional gene controlling yellow pigment content may have been eliminated in the new short translocations.

RWG33 and RWG34 flours were not desirable for making bread due to low kernel hardness and high flour yellowness, while they appeared to be good germplasm to develop wheat for the production of oriental noodle flour for which high flour brightness, yellowness, and low ash content is desirable. The quality analysis performed in this study was mainly used to determine flour yellowness of the two new wheat lines. The data for other quality parameters such as single kernel characteristics, flour protein content, and flour ash are preliminary. A comprehensive field trial with replications and multiple environments is necessary for determining the effects of the shortened *Th. ponticum* chromosome segments carrying *Sr43* on end-use quality as well as yield potential.

The two new wheat lines and associated molecular markers identified in this study should be useful for targeted separation of *Sr43* from the yellow flour gene *Y* using marker-assisted chromosome engineering. In our ongoing research, we are developing a large backcross population of *ph1b*-induced homoeologous recombinants using RWG34 as the donor of *Sr43*. Separation of *Sr43* from *Y* is expected by selection for homoeologous recombinants with further shortened *Th. ponticum* chromosome segments. The ease of this separation will depend on how tightly *Sr43* and *Y* are linked. In $7el_1$, deletion mapping has determined that *Sr25* and *Y* are very tightly linked (Prins et al. 1996; Groenewald et al. 2005), so that no recombinants of *Sr25* and *Y* have been recovered. If *Sr25* and *Sr43* are allelic, then a very tight linkage of *Sr43* with *Y* probably exists.

Because *Th. ponticum* and its derived wheat lines (e.g., wheat–*Th. ponticum* amphiploids and chromosome addition, substitution and translocation lines) are rich sources of genes for stem rust resistance (Xu et al. 2009; Turner et al. 2013), the molecular markers linked to the *Th. ponticum* chromosomes reported in this study are valuable tools for characterizing and genotyping the *Th. ponticum* collections and their derived wheat lines with stem rust resistance.

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Ethical standards The experiments were performed in compliance with the current laws of the USA.

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