Sources of Stem Rust Resistance in Wheat-Alien Introgression Lines

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Abstract

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Stem rust is one of the most devastating diseases of wheat. Widely virulent races of the pathogen in the Ug99 lineage (e.g., TTKSK) are threatening wheat production worldwide; therefore, there is an urgent need to enhance the diversity of resistance genes in the crop. The objectives of this study were to identify new sources of resistance in wheat-alien introgression derivatives from *Secale cereale*, *Leymus mollis*, *L. racemosus*, and *Thinopyrum junceiforme*, postulate genes conferring the resistance, and verify the postulated genes by use of molecular markers. From seedling tests conducted in the greenhouse, the presence of seven known stem rust resistance genes (*Sr7b*, *Sr8a*, *Sr9d*, *Sr10*, *Sr31*, *Sr36*, and *SrSatu*) was postulated in the wheat-alien introgression lines. More lines possessed a high level of resistance in the field compared with the number of lines that were resistant at the seedling stage. Three 2R (2D) wheat-rye substitution lines (SLU210, SLU238, and SLU239) seemed likely to possess new genes for resistance to stem rust based on their resistance pattern to 13 different stem rust races but the genes responsible could not be identified. Wheat-rye, wheat-*L. racemosus*, and wheat-*L. mollis* substitutions or translocations with single and multiple interchanges of chromosomes, in particular of the B and D chromosomes of wheat, were verified by a combination of genomic in situ hybridization and molecular markers. Thus, the present study identified novel resistance genes originating from different alien introgressions into the wheat genome of the evaluated lines. Such genes may prove useful in enhancing the diversity of stem rust resistance in wheat against widely virulent pathogen races such as those in the Ug99 lineage.

Stem rust or black rust of wheat, caused by *Puccinia graminis* f. sp. *tritici* Erikss. & Henning, is one of the most devastating diseases of wheat worldwide, causing yield losses of 80 to 100% in susceptible cultivars (Singh et al. 2011). The new, widely virulent group of pathogen races in the Ug99 lineage (as exemplified by race TTKSK, also called isolate Ug99) originating from East Africa in the late 1990s has caused great concern for wheat food security. Members of this race group are virulent to the widely deployed resistance genes *Sr24*, *Sr31*, and *Sr36* (Jin et al. 2008, 2009; Pretorius et al. 2000). The resistance genes *Sr9h*, *Sr13*, *SrTmp*, and *Sr1RS*$^{\mathrm{Amigo}}$, found effective against race TTKSK, have been overcome by other pathogen races originating in Yemen and Africa such as TRTTF, TTKSF+, and TKTTF (Olivera et al. 2012b, 2015; Rouse et al. 2014a). Moreover, new races TTKTK and TTKTT with additional virulence to important resistance genes were recently detected (Patpour et al. 2016) and have now spread across large wheat-growing regions (Patpour et al. in press). Race TKTTF (not a member of the Ug99 lineage) recently caused severe epidemics in Ethiopia, resulting in significant economic losses (Olivera et al. 2015). Race TKTTF has spread widely and is now found in more than 10 countries, including Sweden, Denmark, and Germany (http://rusttracker.cimmyt.org/?page_id=6811; M. S. Hovmøller, personal communication). About 35 of the 73 described *Sr* resistance genes were derived from cultivated bread wheat but only a few are effective against races in the Ug99 lineage (Singh et al. 2015). Due to the constant evolution and mutation of *P. graminis* f. sp. *tritici* races, many resistance genes are rendered ineffective within a relatively short period of time.

Therefore, it is necessary to use different genetic stocks as an effective disease management strategy to broaden the genetic base of stem rust resistance in wheat when suitable resistance genes are not available in the A, B, and D genomes of wheat (Kole 2011; Mujeeb-Kazi et al. 2013). The tertiary gene pool has proven particularly useful for improving the stem rust resistance of wheat. Examples of important resistance genes include *Sr27*, *Sr31*, *Sr1RS*$^{\mathrm{Amigo}}$, and *Sr50*, originating from wheat-rye introgressions (Mago et al. 2004, 2005b; Marais and Marais 1994; The et al. 1991); *Sr24*, *Sr25*, *Sr26*, and *Sr43*, originating from wheat-*Thinopyrum ponticum* introgressions; *Sr44* originating from a wheat-*Thinopyrum intermedium* introgression; and *Sr52* originating from a wheat-*Dasypyrum villosum* introgression (Liu et al. 2010; Mago et al. 2005a; Niu et al. 2014; Qi et al. 2011). To better understand the functionality of stem rust resistance genes, *Sr33* from *Aegilops tauschii* (Periyannan et al. 2013) and *Sr35* from *Triticum monococcum* (Saintenac et al. 2013) were cloned and found to encode nucleotide-binding site leucine-rich repeat proteins. Although a number of stem rust resistance genes have been identified in different genetic stocks, deleterious linkage drag associations might contribute to these genes not being deployed (Singh et al. 2015).

Wild relatives are important sources of genes that can be used for bread wheat (*T. aestivum* L., 2n = 6x = 42, AABBDD) improvement to address both abiotic and biotic stresses. Rye (*Secale cereale*,

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2n = 14), Russian wheatgrass (*Thinopyrum junceiforme*, 2n = 28), Dune grass (*Leymus mollis*, 2n = 28), and Mammoth wild rye (*L. racemosus* 2n = 28) are members of the tertiary genepool of wheat and contain useful genes for crop improvement (Merker 1984; Merker and Lantai 1997). The introgression of genes from *S. cereale*, *T. junceiforme*, *L. mollis*, and *L. racemosus* that are highly effective against African stem rust races offers great promise for enhancing the resistance of wheat. A large number of wheat-alien introgression lines were developed in the 1980s and 2000s by the late Professor Arnulf Merker at the Swedish University of Agricultural Sciences (SLU). These wheat-alien introgression lines have mainly been tested for resistance to powdery mildew, leaf rust, and the Russian wheat aphid (Andersson et al. 2015; Ellneskog-staam and Merker 2002; Forsström and Merker 2001). No studies have been advanced on these introgression lines for resistance to stem rust. Therefore, the aims of this study were to (i) evaluate and identify possible new resistance sources through seedling and adult plant phenotyping of wheat lines with alien introgressions from *S. cereale*, *T. junceiforme*, *L. mollis*, and *L. racemosus* with different races of stem rust and (ii) validate and confirm postulated *Sr* genes with available molecular markers.

Materials and Methods

Plant materials. The plant materials used in this study included 185 winter and 94 spring wheat-alien introgression lines. These lines were developed by crossing and backcrossing strategies during 1980 to 2000 by the late Professor Arnulf Merker at SLU (Forsström and Merker 2001; Merker 1984; Merker and Lantai 1997). The wheat-alien introgression lines used in this study are maintained at the Plant Breeding Department at SLU. These lines contain rye chromosomes 1R, 2R, 3R, 4R, 5R, and 6R in the form of single and multiple disomic substitutions. Lines with wheat-rye translocations such as 1DL.1RS, 1BL.1RS, 2RL.2BS, 3DL.3RS, and 5AL.5RS and lines with multiple combinations of rye chromosome substitutions such as 1R+2R, 1R+3R, 1R+6R, 5R+4R+7R, and 1R+6R+4R+7R were also present. The investigated materials also included wheat lines with introgressed chromatin from *L. racemosus, L. mollis*, and *T. junceiforme* (Table 1).

Seedling resistance tests. The seedling resistance tests of the 279 wheat-alien introgression lines were conducted at several institutions. For the highly virulent African stem rust races, the phenotyping was done in a biosafety level-3 containment facility at the University of Minnesota in St. Paul. Other stem rust tests were conducted at the United States Department of Agriculture–Agricultural Research Service (USDA-ARS) Cereal Disease Laboratory, St. Paul, MN; University of the Free State, Bloemfontein, South Africa; and the Regional Cereal Rust Research Center (RCRRC), located at the Aegean Agricultural Research Institute, International Center for Agricultural Research in the Dry Areas (ICARDA) in Izmir, Turkey (Table 2). In South Africa, lines carrying the 3R chromosome and lines with potentially sources of resistance—that is, 22 wheat-alien introgression lines, three wheat cultivars ('Sonett', 'Prins', and 'Drabant'), and triticale genotypes ('Coorong' with *Sr27*, 'Kiewiet' with *SrKw*, and 'Satu' with *SrSatu*)—were tested at the seedling stage. In the United States, stored urediniospores were removed from a –80°C freezer, immediately heat shocked for 10 min, and then placed in a rehydration chamber (at approximately 80% relative humidity) for 2 to 4 h (Rouse et al. 2011). The spores were then suspended in a lightweight mineral oil (Soltrol 170; Chevron Phillips Chemical Company LP) and inoculated onto 8- to 10-day-old seedlings at the first leaf stage. Inoculated plants were kept in a dark moist chamber at 18 to 20°C with relative humidity near 100% for 16 h to induce infection by urediniospores. Then, plants were incubated in a greenhouse at 18 to 20°C with supplemental light provided by pressure sodium vapor lamps (400 W and photo flux) for 16 h per day. The rust phenotyping protocol used at the University of the Free State is similar to the one employed in the United States (Pretorius et al. 2012). At the RCRRC, a similar methodology was followed for phenotyping experiments with race TKTTF, except that fresh urediniospores collected from plants in the field were used and suspended in the lightweight mineral oil. Ten-day-old seedlings were inoculated according to Rouse et al. (2011). Seedling infection types were recorded 14 days after incubation using a 0-to-4 scale, where 0; = immune to very resistant, 1 = resistant, 2 = moderately resistant, 3 = moderately susceptible, 3+ = susceptible, and 4 = very susceptible (Jin et al. 2007; Stakman et al. 1962).

Assessment of field response to stem rust. In total, 182 winter and 65 spring wheat-alien introgression lines were evaluated for their stem rust response in the field at the Kenyan Agricultural and Livestock Research Organization (KALRO) in Njoro in 2011 and the RCRRC in 2014. Additionally, 74 spring wheat-alien introgression lines were evaluated in the field at the University of Minnesota in St. Paul in 2014. The limitation of lines tested in St. Paul was due to limited seed stocks of the winter lines, making it not possible to evaluate the entire collection in the field at St. Paul. Seed stocks of the wheat-*L. racemosus* and wheat-*T. junceiforme* lines, in particular, were extremely limited. Winter wheat-alien introgression lines were vernalized for 6 weeks at +4°C before transplanting in the field in KALRO. To provide sufficient rust infection in the nurseries, mixtures of susceptible wheat cultivars were used as spreader rows surrounding and between the plots at all locations. In Njoro, the spreader rows were inoculated at the booting and heading stages with races TTKSK+TTKST by the needle-injection method (i.e., injecting urediniospores directly into the stems of spreader plants) and also

Table 1. Wheat-alien introgression lines and respective parents evaluated in this study

Cross, pedigree	Plant habit	Number of lines	Type
Triticale[a]	Spring and winter	5	× *Triticosecale*
Wheat[a]	Spring and winter	8	*Triticum aestivum* and *T. carthlicum*
Sv 876012 × H	Winter	37	Wheat-rye introgressions
Sv 876032 × H × K	Winter	54	Wheat-rye introgressions
Sv 856003 × H	Winter	6	Wheat-rye introgressions
Sub 1R+2R	Winter	42	Wheat-rye introgressions
Malysh	Winter	6	Wheat-rye introgressions
Starke × Otello	Winter	7	Wheat-rye introgressions
Uno × Holme	Winter	8	Wheat-rye introgressions
Triticale VT828041	Spring	6	Wheat-rye introgressions
Triticale Drira	Spring	23	Wheat-rye introgressions
Triticale Beagle	Spring	12	Wheat-rye introgressions
Triticale VT83 615	Spring	2	Wheat-rye introgressions
Triticale VT83 591	Spring	4	Wheat-rye introgressions
Triticale VT 82 8039	Spring	5	Wheat-rye introgressions
3R BB14 (CIMMYT 1974)	Spring	4	Wheat-rye introgressions
Leymus spp.	Spring and winter	34	Wheat-*Leymus* spp. introgressions
Thinopyrum junceiforme	Spring	16	Wheat-*T. junceiforme* introgressions
Total	…	279	…

[a] Parental cultivars and lines.

by direct foliar inoculation of the urediniospore-oil suspension on spreader plants under mist-irrigated conditions (Njau et al. 2013). At Izmir, the spreader rows were inoculated three times with race TKTTF by dusting with a mixture of fresh urediniospores and talcum powder under mist-irrigated conditions. Spreader rows at the St. Paul nursery were inoculated with race MCCFC at the early tillering and heading stage using the needle-injection and direct urediniospore-oil foliar application methods (Olivera et al. 2012a). In all locations, the adult plant response to stem rust was assessed between growth stages 50 and 90 based on the Zadoks scale (Zadoks et al. 1974) when the susceptible control reached maximum (approximately 90%) severity. Disease severity was assessed using the modified Cobb scale (Peterson et al. 1948) and adult plant infection responses were rated according to Roelfs et al. (1992). In addition, the presence of the pseudo black chaff (PBC) phenotype in some of the spring wheat-alien introgression lines were assessed on a 0-to-4 scale (Juliana et al. 2015) in Njoro and Izmir.

Molecular marker analysis. Genomic DNA was isolated from 10-day-old leaf tissue of the wheat-alien introgression lines according to the methods of Edwards et al. (1991), with some slight modifications. Initially, 40 mapped wheat simple sequence repeat (SSR) markers for the B and D chromosomes (Röder et al. 1998; Somers et al. 2004); 30 rye-specific SSR and expressed sequence tagged (EST)-derived SSR markers for the 1R, 2R, and 3R chromosomes (Hackauf and Wehling 2002; Khlestkina et al. 2004; Martis et al. 2013; Saal and Wricke 1999); and 12 *Leymus* EST-SSR markers for the wheat-*L. racemosus* introgressions (Kaur et al. 2008; Larson et al. 2012) were assayed. The diagnostic markers *XcsSr2* for *Sr2* (Mago et al. 2011), SSR *Xscm* and STS *Xiag95* for *Sr31* (Mago et al. 2002; Saal and Wricke 1999), SSR *Xstm773* for *Sr36* (Tsilo et al. 2008), and *VENTRIUP-LN2* for *Sr38* (Helguera et al. 2003) were also assayed. The polymerase chain reaction (PCR) master mix for all markers consisted of 2 μl of 25 ng of genomic DNA template, 0.6 μl of a 10 μM mixture of forward and reverse primers, 0.075 μl (0.5 U) of Taq polymerase, 1.75 μl of 10× Ex Taq Buffer (10 mM Tris-HCI, 50 mM KCl, and 1.5 mM $MgCl_2$, pH 8.3), 1.75 μl of a 2.5 mM mixture of dNTP, and 9 μl of double-distilled H_2O, bringing the total reaction volume to 15 μl. The *XcsSr2* marker was digested by adding 5 μl of 1.5 ml of 10× NEB Buffer 4 and 0.5 μl of *Bsp*HI (10 U/ml, New England BioLabs) and incubated at 37°C for 1 h (Mago et al. 2011). PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining under UV light. The Kompetitive Allele-Specific PCR (KASP) markers for *Sr2* (*wMAS000005*) and *Sr36* (*wMAS000015*) (http://maswheat.ucdavis.edu/protocols/StemRust/index.htm) were analyzed using the Applied Biosystems StepOne Plus Real-Time PCR System. Each KASP PCR consisted of 50 ng of DNA template, 5 μl of 2× KASP buffer, and 1.4 μl of primer mixture. Thermal cycling conditions were 94°C for 15 min, followed by 10 cycles of touchdown PCR (94°C for 20 s and 65 to 57°C for 60 s, dropping 0.8°C per cycle), followed by 36 cycles of regular PCR (94°C for 20 s and 57°C for 60 s), followed by fluorescence reading at 20°C. Both thermal cycling and fluorescence reading were performed on the ABI Step One Plus Real-Time PCR system.

Genomic in situ hybridization. Genomic in situ hybridization (GISH) analysis was performed to detect alien chromatin in the resistant lines. Root tips of 2- to 3-day-old plants were treated for 24 h in ice-cold water and then fixed in a 3:1 mixture of ethanol and glacial acetic acid. Metaphase spreads were prepared according to the methods of Mirzaghaderi et al. (2014). Genomic DNA from *S. cereale*, *L. mollis*, and *L. racemosus* was used as a probe and labeled with fluorescein 12-dUTP green or Texas Red 12-dUTP by nick translation. GISH was performed as described by Schwarzacher et al. (1992). Denaturation of DNA was conducted at 80°C for 4 min, followed by hybridization at 80°C for 2 min, with subsequent incubation of samples in a moist chamber at 37°C overnight. Slides were washed in a water bath in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 20 min in darkness and, thereafter, were dehydrated in 70, 90, and 100% ethanol for 2 min. Finally, the slides were air dried at room temperature and counterstained with 4′,6-diamidino-2-phenylindole at 1 μg/ml in Vectashield (Vector Laboratories). Images were acquired with an epifluorescence microscope BX61 (Olympus) using a cooled CCD camera (Orca ER; Hamamatsu). Pictures were processed and merged by Adobe Photoshop (Adobe Systems Incorporated).

Results

Seedling resistance evaluation. Seedling resistance to races TTKSK, TTKST, TTTSK, TRTTF, TTTTF, TPMKC, QTHJC, RKQQC, TKTTF, and MCCFC was observed in a number of the wheat-alien introgression lines (Table 3). Individual plants were scored for their infection type and the number of resistant, susceptible, and heterogeneous (mix of susceptible and resistant) plants within lines was recorded (Table 3). Based on the virulence profile of the different races of *P. graminis* f. sp. *tritici*, some of the wheat-alien introgression lines were postulated to carry *Sr7b*, *Sr8a*, *Sr9d*, *Sr10*, *Sr31*, *Sr36,* and *SrSatu* (Supplementary Table S1). For some of the lines, resistance genes could not be postulated because their reactions did not correspond to the avirulence or virulence profile of the races tested. The resistance genes in such lines were designated as unknown. Eleven winter wheat-rye introgression lines with the 2R (2B) substitution were found to be resistant (infection type 22+) to races TTKSK and TTTSK although, in most cases, the lines were heterogeneous in their reaction.

The 3R chromosome is known to carry the *Sr27* and *SrSatu* resistance genes, which are effective against races TTKSK, TTKST, TTTSK, TRTTF, TTTTF, RKQQC, QTHJC, TPMKC, TKTTF, and MCCFC. To further resolve the identified resistance, all of the 14 3R (3D) substitution, 1R+3R (1D+3D) multiple substitution, and 3RS.3DL translocation lines were tested with the South African stem rust races BPGSC, BPGSC+*SrKiewiet*, and BPGSC+*SrSatu*. In addition, eight wheat-alien introgression lines and three wheat cultivars were tested for resistance to South African stem rust

Table 2. Origin and virulence phenotype of *Puccinia graminis* f. sp. *tritici* races used in this study

			Virulence profile[a]																			
Race	**Isolate**	**Origin**	***5***	***21***	***9e***	***7b***	***11***	***6***	***8a***	***9g***	***36***	***9b***	***30***	***17***	***9a***	***9d***	***10***	***Tmp***	***24***	***31***	***38***	***McN***
TTKSK	04KEN156/04	Kenya	*5*	*21*	*9e*	*7b*	*11*	*6*	*8a*	*9g*	–	*9b*	*30*	*17*	*9a*	*9d*	*10*	–	–	*31*	*38*	*McN*
TTKST	06KEN19-V-3	Kenya	*5*	*21*	*9e*	*7b*	*11*	*6*	*8a*	*9g*	–	*9b*	*30*	*17*	*9a*	*9d*	*10*	–	*24*	*31*	*38*	*McN*
TTTSK	07KEN24-4	Kenya	*5*	*21*	*9e*	*7b*	*11*	*6*	*8a*	*9g*	*36*	*9b*	*30*	–	*9a*	*9d*	*10*	*Tmp*	–	*31*	*38*	*McN*
TRTTF	06YEM34-1	Yemen	*5*	*21*	*9e*	*7b*	*11*	*6*	–	*9g*	*36*	*9b*	*30*	*17*	*9a*	*9d*	*10*	*Tmp*	–	–	*38*	*McN*
TTTTF	01MN84A-1-2	United States	*5*	*21*	*9e*	*7b*	*11*	*6*	*8a*	*9g*	*36*	*9b*	*30*	*17*	*9a*	*9d*	*10*	*Tmp*	–	–	*38*	*McN*
RKQQC	99KS76A-1	United States	*5*	*21*	–	*7b*	–	*6*	*8a*	*9g*	*36*	*9b*	–	–	*9a*	*9d*	–	–	–	–	–	*McN*
QTHJC	75ND717C	United States	*5*	*21*	–	–	*11*	*6*	*8a*	*9g*	–	*9b*	–	*17*	–	*9d*	*10*	–	–	–	–	*McN*
TPMKC	74MN1409	United States	*5*	*21*	*9e*	*7b*	*11*	–	*8a*	*9g*	*36*	–	–	*17*	–	*9d*	*10*	*Tmp*	–	–	–	*McN*
MCCFC	59KS19	United States	*5*	–	–	*7b*	–	–	–	*9g*	–	–	–	*17*	–	–	*10*	*Tmp*	–	–	–	*McN*
TKTTF	…	Turkey	*5*	*21*	*9e*	*7b*	–	*6*	*8a*	*9g*	*36*	*9b*	*30*	*17*	*9a*	*9d*	*10*	–	–	–	–	*McN*
BPGSC	UVPgt53	South Africa	–	–	–	–	*11*	–	*8a*	*9g*	–	*9b*	–	–	*9a*	*9d*	*10*	–	–	–	–	*McN*
BPGSC+*SrKiewiet*	UVPgt56	South Africa	–	–	–	–	*11*	–	*8a*	*9g*	–	*9b*	–	–	*9a*	*9d*	*10*	–	–	–	–	*McN*
BPGSC+*SrSatu*	UVPgt57	South Africa	–	–	–	–	*11*	–	*8a*	*9g*	–	*9b*	–	–	*9a*	*9d*	*10*	–	–	–	–	*McN*

[a] Symbol: – indicates avirulence.

races. Gene postulation results suggested the presence of *SrSatu* in four of the lines (SLU216, SLU217, SLU218, and SLU220) and *SrSatu* plus an additional gene in line SLU219. In summary, the seedling resistances found in the present materials with 1R (1D), 2R (2D), 3R (3D), 1R+6R (1D+6D), wheat-*T. junceiforme*, and wheat-*L. mollis* substitutions and 1BL.1RS and 5AL.5RS translocations showed the presence of *Sr7b*, *Sr8a*, *Sr9d*, *Sr10*, *Sr31*, *Sr36*, and *SrSatu* as well as uncharacterized or potentially new *Sr* genes. All details of the seedling infection types of wheat-alien introgressions are presented in Supplementary Table S1.

Field response to stem rust. In the field experiments carried out in Njoro, Izmir, and St. Paul, disease severity was sufficient to easily differentiate resistant and susceptible lines. Overall, 67 (27.1%) of the lines in Njoro, 23 (9.3%) of the lines in Izmir, and 6 (8%) of the lines in St. Paul exhibited markedly reduced rust severities (0 to 30%) compared with susceptible controls (approximately 90%). Additionally, 22 lines (8.9%) in Njoro, 90 lines (36.4%) in Izmir, and 2 lines (3%) in St. Paul displayed rust severities of 5 to 40% with MR-MS or MS-S infection types. Thus, these lines were susceptible at the seedling stage, yet they showed resistance in the field at the three diverse sites, suggesting that these lines have adult plant resistance (APR; Table 4; Fig. 1). All stages (seedling) of stem rust resistance response in 30 (12.1%) of the lines in Njoro, 39 (15.8%) of the lines in Izmir, and 28 (37.8%) of the lines in St. Paul with severities 0 to 30% (R-MR response) and 10 (4.1%) of the lines in both Njoro and Izmir with 5 to 40% severities (MR-MS and MS-MSS response) were observed in the field experiments (Table 4). Many of the lines—118 (44.8%) in Njoro, 85 (34.1%) in Izmir, and 38 (51.3%) in St. Paul—displayed susceptible responses at the seedling and adult stages (Table 4). The spring and winter wheat parental cultivars at all locations exhibited susceptible disease responses from 50S to 80MS-S. The PBC pigmentation score was 1 to 2 in 17 spring lines (Supplementary Table S2). A major gene governing the PBC trait is linked to the slow-rusting resistance gene *Sr2* (McFadden 1930) and was assayed in the field at Njoro and Izmir as well as with the *XcsSr2* and *wMAS000005* markers. However, none of these lines with the PBC phenotype was associated with gene *Sr2*. Also, the presence of *Sr2* could not be confirmed by use of markers *XcsSr2* or *wMAS000005*, which are specific for the gene. Hope and CS-Hope DS 3B (donors of *Sr2*) accessions were used as positive controls

Table 3. Number of wheat-alien introgression lines exhibiting resistant and susceptible reactions to *Puccinia graminis* f. sp. *tritici* races[a]

	Number of lines										
Infection type	**1 Rep. TTKSK**	**2 Rep. TTKSK**	**TTKST**	**TTTSK**	**TRTTF**	**TTTTF**	**TPMKC**	**QTHJC**	**RKQQC**	**TKTTF**	**MCCFC**
Resistant											
;0	30	31	38	22	20	17	14	32	35	18	32
;1	6	5	7	4	4	4	1	5	8	17	7
11+	…	…	7	…	8	1	4	11	9	9	6
2	6	7	1	13	4	3	2	4	2	8	…
2+	7	6	4	1	3	3	3	4	2	1	3
Subtotal	49	49	57	40	39	28	24	56	56	53	48
Susceptible											
3	17	16	3	2	2	3	3	2	1	3	…
3+	178	138	113	225	174	67	54	66	67	215	68
4	33	74	87	7	38	168	182	126	129	6	162
Subtotal	228	228	203	234	214	238	239	194	197	224	230
Heterogeneous	2	2	19	5	26	13	16	28	25	2	1
Total lines	279	279	279	279	279	279	279	279	279	279	279

[a] Infection types observed were based on a 0-to-4 scale (Stakman et al. 1962). Lines with ;0 to 2+ types were considered resistant and lines with 3 to 4 types were considered susceptible. Heterogeneous type consisted of a mix of susceptible and resistance reactions in one line.

Table 4. Number of wheat-alien introgressions exhibiting adult plant resistance (APR) in Kenya, Turkey, and St. Paul[a]

		Number of lines[b]				
Severity	**Response[c]**	**Kenya**	**Turkey**	**St. Paul**	**Gene[d]**	**Note**
R-RMR						
0–20	R	47	17	4	Minor	APR resistant and seedling susceptible
	…	21	23	28	Major	Seedling and APR resistant
5–30	RMR	20	6	2	Minor	APR resistant and seedling susceptible
	…	9	16	…	Major	Seedling and APR resistant
Total	…	97	62	34	…	…
MR-MS-MSS						
5–40	MR-MS	16	22	2	Minor	APR resistant and seedling susceptible
	…	10	5	…	Major	Seedling and APR resistant
5–30	MS-MSS	6	68	…	Minor	APR resistant and seedling susceptible
	…	…	5	…	Major	Seedling and APR resistant
Total	…	32	100	2	…	…
MSS-S						
40–70 and 50–100	MS-S and S	118	85	38	None	APR and seedling susceptible
Total	…	118	85	38	…	…

[a] APR was evaluated based on the Cobb Scale (Peterson et al. 1948) and host response to infection based on pustule type and size (Roelfs et al. 1992). Lines exhibiting severity scores ranging from 0 to 30 RMR may carry four or five APR genes (Singh et al. 2014). The lines exhibiting 5 to 40 MR-MS or 5 to 30 MS-MSS may carry two or three minor genes that implicate for slow rusting or partial resistance. Lines with 40 to 70 MSS and 50 to 100 S did not possess APR in the field. However, presence of APR genes in combination with for or five minor genes usually display high level of resistance (Singh et al. 2014).
[b] In St. Paul, only the spring materials were tested for APR.
[c] Host response.
[d] Gene effect.

for the *XcsSr2* and *wMAS000005* markers. The expected fragment size of 172 bp was found in the *Sr2* donors (Hope and CS-Hope DS 3B) for the *XcsSr2* marker whereas, for the wheat-alien introgression lines, the fragment size was about 240 bp. Using the *wMAS000005* marker, Hope and CS-Hope DS 3B gave a signal in the VIC (fluorescent dye) assay, while the wheat-alien introgression lines gave a signal in the FAM (fluorescent dye) assay.

Molecular marker validation. To validate the presence of the postulated resistance genes, a number of markers were used. Five lines with 1R (1D) and two lines with 2R (2D) wheat-rye substitutions were postulated to carry *Sr36*. In addition, three lines with wheat-*L. racemosus* substitutions and translocation were postulated to carry *Sr36* and validated with the KASP *wMAS0000015* and SSR *Xstm773* (155 bp) markers. By applying the *wMAS0000015* marker, lines with *Sr36* were detected by the VIC signal, and those without the gene were detected by the FAM signal. Two lines were evaluated with the *Xscm9* (220 bp) and Iag95 (1,100 bp) markers for the presence of the *Sr31* gene located on the 1BL.1RS wheat-rye translocation. The resistance spectra of some lines were phenotypically similar to other lines known to carry the *Sr38* gene. In these lines, the *VENTRIUP-LN2* marker was used for verification but the resistance gene was not detected. Details of the molecular marker validation in wheat-alien introgression lines are shown in Supplementary Table S2.

GISH analysis. The chromosome constitution of selected wheat-alien introgression lines was characterized by GISH at mitotic metaphase. In most of the lines, 42 chromosomes were present but, in some lines, signals for the presence of one, two, and five alien chromosome pairs were indicated. In general, one or two pairs of chromosomes from *S. cereal* or *L. racemosus* or five pairs of chromosomes from *L. mollis* were revealed (Fig. 2). In addition to GISH, a number of selected polymorphic wheat (30 markers), rye (14 markers), and *Leymus* SSR (5 markers) markers were used (Table 5; Supplementary Tables S3, S4, S5, and S6). Specific molecular markers for *L. mollis* and *T. junceiforme* were not applied, due to a lack of availability of such markers. In most cases, the D genome chromosomes of wheat were replaced. Thus, in most wheat-rye translocations and substitutions, the rye chromosomes 1R, 2R, and 3R replaced the 1B and 1D, 2B and 2D, and 3D wheat chromosomes, respectively. For the *L. racemosus* introgressions, two lines were identified as having substitutions and one line as possessing a translocation in the 6D wheat chromosome. All details of GISH and molecular marker analyses are presented in Supplementary Tables S3, S4, S5, and S6.

Discussion

The seedling and adult stage screening of wheat-alien introgression lines followed by GISH and molecular marker analyses clearly showed the presence of new stem rust resistance genes in the material studied. In summary, three of the 2R (2D) (SLU210, SLU238, and SLU239) substitution lines were resistant to all evaluated stem rust races at both the seedling and adult plant stages. Moreover, seven of the *T. junceiforme* (SLU251, SLU252, SLU253, SLU255, SLU256, SLU274, and SLU275) and one of the 3R (3D) (SLU214) wheat-alien introgressions were also identified as potential sources of new stem rust resistance genes because these lines were highly resistant to race TTKSK and TTTSK. Previous studies have documented several stem rust resistance genes (i.e., *Sr27*, *SrSatu*, *Sr31*, *Sr50*, and *Sr1RS*$^{\text{Amigo}}$) derived from *S. cereale* (Mago et al. 2004, 2005b; Marais and Marais 1994; The et al. 1991). Moreover, of the approximately 70 *Sr* genes recently cataloged to be effective against *P. graminis* f. sp. *tritici* races, including those in the Ug99 lineage, most are derived from alien species (Mujeeb-Kazi et al. 2013; Pumphrey et al. 2012; Singh et al. 2015). A number of the stem rust resistance genes derived from wheat-alien species have been transferred to wheat through wide hybridization (Dundas et al. 2007; Gill et al. 2006; Mujeeb-Kazi et al. 2013; Xu et al. 2009). In recent efforts to identify additional genes for resistance to TTKSK and other stem rust races, a large and diverse collection of wheat-alien germplasm was evaluated at both seedling and adult stages, indicating that the alien species are extremely rich sources of genes for resistance (Evanega et al. 2014; Mujeeb-Kazi et al. 2013). Wheat-alien introgressions have also been used as a method to transfer other important traits into wheat; for example, *S. cereale* has served as a source of resistance to all rusts and powdery mildew (*Sr31*/*Yr9*/*Lr26*/*Pm8*) (Friebe et al. 1996), resistance to cereal aphid and Hessian fly (Crespo-Herrera et al. 2013; Hysing et al. 2007), and significant genetic diversity for yield potential, drought and salinity tolerance, micronutrient content, and further additive genetic variation for wheat improvement (Mujeeb-Kazi et al. 2013; Peake et al. 2011). Because no stem rust resistance genes have previously been described from *S. cereale* chromosome 2R or from *T. junceiforme*, these genetic resources possess new stem rust resistance genes.

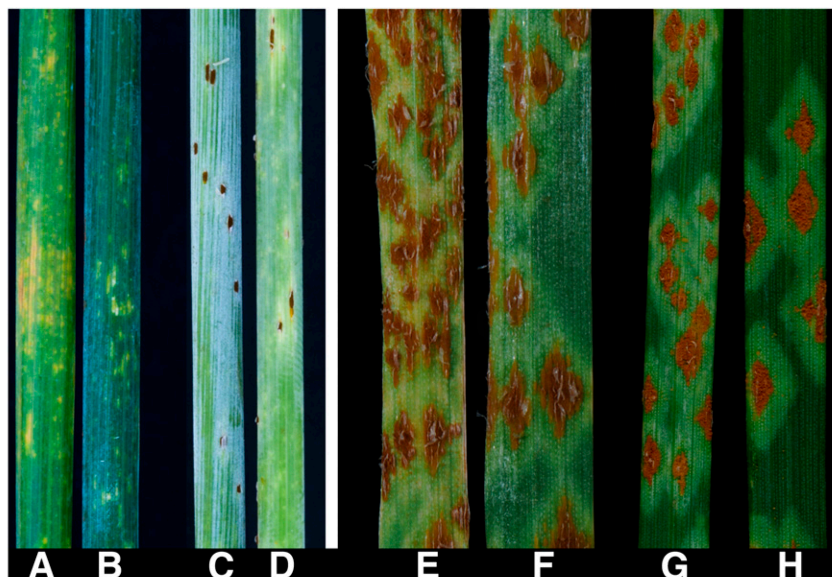

Fig. 1. Adult plant stem rust responses of **A,** SLU107; **B,** SLU157; **C,** SLU221; and **D,** SLU117 in the field in Kenya and seedling infection types of **E,** SLU107; **F,** SLU157; **G,** SLU221; and **H,** SLU117 of wheat-alien introgression lines to race TTKSK. Adult plant severity scoring was based on the modified Cobb scale (Peterson et al. 1948) and the host response to infection was assessed according to Roelfs et al. (1992). Seedling infection reactions to race TTKSK were assessed using a 0-to-4 scale (Stakman et al. 1962).

A number of the lines evaluated in this study showed the likely presence of previously reported stem rust resistance genes. For example, the presence of *Sr31* was inferred in two 1BL.1RS wheat-rye translocation lines (SLU168 and SLU173) based on the molecular markers *Xscm9* and *Xiag95* (Mago et al. 2002; Saal and Wricke 1999). *Sr31* has been deployed widely and provided stable resistance against stem rust races for over 30 years in commercial wheat cultivars (Singh et al. 2008). Furthermore, *Sr36* has been a key resistance gene in winter and spring wheats worldwide (Singh et al. 2011). The use of the molecular marker *Xstm773* (Tsilo et al. 2008) and *wMAS000015* (http://maswheat.ucdavis.edu/protocols/Sr36/index.htm) indicated the presence of *Sr36* in 10 spring lines. However, both *Sr31* and *Sr36* are ineffective against the virulent race TTTSK (Jin et al. 2009; Pretorius et al. 2000). Thus, based on the susceptible reactions of seven lines (SLU251, SLU252, SLU253, SLU255, SLU256, SLU274, and SLU275) to races TTTSK, TPMKC, TTTTF, RKQCC, TRTTF, and TKTTF, it is likely that *Sr36* is present. However, subsequent DNA fingerprinting with markers *Xstm773* and *wMAS000015* indicated the absence of the *Sr36* gene. Also, based on the assays with the South African races of BPGSC, BPGSC+*SrKiewiet*, and BPGSC+*SrSatu*, four of the spring lines were highly resistant and, therefore, postulated to carry *SrSatu* or this gene together with an additional unknown resistance gene. *SrSatu* is the most commonly used stem rust resistance gene in triticale breeding and is widely distributed in CIMMYT triticale germplasm (Olivera et al. 2013; Zhang et al. 2010). Aside from the above-mentioned resistance genes, *Sr7b* was postulated in three spring lines, *Sr8a* in two winter lines, *Sr9d* in two winter lines, and *Sr10* in five spring and two winter lines. These four genes originated from *Triticum aestivum* and *T. turgidum* and are ineffective against races in the Ug99 lineage (Singh et al. 2011). To conclude, the majority of the wheat-alien introgression lines with unknown genes (138 lines) exhibited a high level of resistance to some of the tested stem rust races with a broad range of virulence but a total of 110 of these lines were highly susceptible to all races at the seedling stage. It has been proposed that wheat lines often exhibit seedling susceptibility to rust diseases but possess multiple minor genes that are effective only at the adult plant stage (Singh et al. 2014). In previous studies, the effects of the *Sr2* (*Yr30*/*Lr27*), *Sr55* (*Yr46*/*Lr67*), *Sr57* (*Yr18*/*Lr34*), and *Sr58* (*Yr29*/*Lr46*) loci have been characterized to carry pleiotropic APR genes (Singh et al. 2015). Moreover, *Sr12* and *Sr56* were found to act in concert in conferring APR (Bansal et al. 2014; Rouse et al. 2014b). To fully elucidate the genetic architecture of APR, molecular mapping studies are a highly effective approach (Yu et al. 2014). In total, three of the 2R (2D) wheat-rye substitution lines (from triticale line VT828041 and 'Beagle') showed a high level of resistance to all 13 *P. graminis* f. sp. *tritici* races (including South African races) used in this investigation. We found that the 1R (1D), 3R (3D), and 3DL.3RS wheat-rye substitutions and translocation lines (SLU183, SLU184, SLU185, SLU186, SLU189, SLU190, SLU213, SLU214, SLU215 SLU321, SLU232, and SLU233) from VT828041 and Beagle triticale were not resistant to all tested races. Further studies are needed to clarify the genetic basis of resistance observed in several lines. Interestingly, a relatively high proportion of the lines were heterogeneous in their reaction to the races TTKST, TRTTF, TTTTF, TPMKC, QTHJC, and RKQQC, while only a few lines were heterogeneous to races TTKSK, TTTSK, TKTTF, and MCCFC (Table 3). This could be due to the fact that, in most of

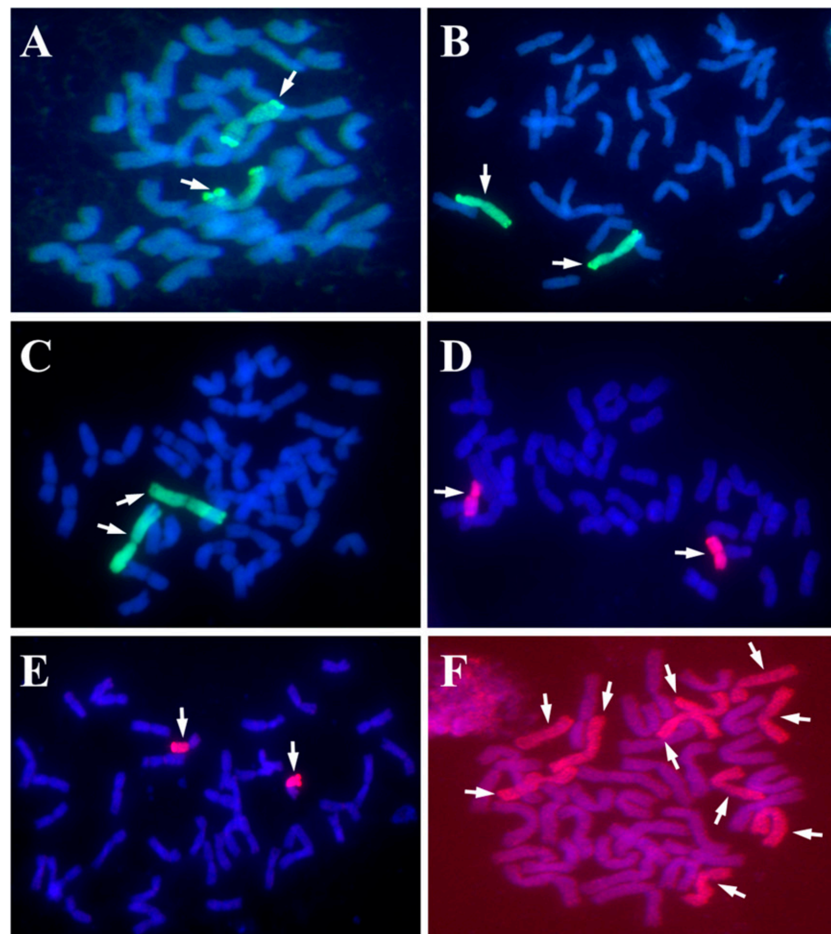

Fig. 2. Genomic in situ hybridization patterns of wheat-rye, wheat-*Leymus racemosus*, and wheat-*L. mollis* substitutions and translocation lines. **A,** SLU190, 1R (1D) substitution with 2n = 40+2; **B,** SLU238, 2R (2D) substitution with 2n = 40+2; **C,** SLU219, 3R (3D) substitution with 2n = 40+2; **D,** SLU235, wheat-*L. racemosus* substitution with 2n = 40+2; **E,** SLU237, wheat-*L. racemosus* translocation with 2n = 40+2; and **F,** SLU176, wheat-*L. mollis* substitution with 2n = 32+10. All chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (in blue) and rye-specific signals are shown in green. Chromosomes of *L. racemosus* and *L. mollis* chromosomes are shown in red. Scale bar = 10 μm.

the heterogeneous lines (SLU73 to SLU84), the 2R (2B) wheat-rye substitution was involved. Further tests with a wider array of races or genetic analysis are needed to define what genes are responsible for the seedling resistances observed among heterogeneous lines.

For a number of lines susceptible at the seedling stage, field testing indicated the presence of varying levels of APR. The resistance identified from seedling tests to races TTKSK, TKTTF, and MCCFC remained highly effective in the field experiments (Table 4). Lines exhibiting severity scores ranging from 0 to 20, 10 to 30, 5 to 30, and 5 to 40 (Table 4) might carry multiple minor-effect resistance genes. APR is known as a complex trait conferred by a number of quantitative trait loci (QTL). The combination of several of these minor-effect QTL can lead to APR or slow-rusting resistance (Singh et al. 2013b). APR is also often associated with additive and epistatic interactions (Singh et al. 2013a). Recent results on 1BL.1RS and 2RL.2BS double wheat-rye translocation lines have clearly shown that APR resistance against race TTKSK is likely due to the presence of multiple genes (Rahmatov et al. 2015). Furthermore, lines with high levels of stripe, leaf, and stem rust resistance have been reported to carry combinations of more than one APR gene, although analyses to understand the genetic background of such sources is necessary in for each case (Singh et al. 2014). Also, the genetic background for PBC has been associated with several QTL on chromosome arms 2DS, 3BS, 4AL, and 7DS (Singh et al. 2013a). Depending on the genotype and environment, *Sr2* is sometimes strongly associated with PBC (McFadden 1930). The genetic background effects with multiple genes and variations in the linkage relationships between PBC and *Sr2* can explain our results of markers *XcsSr2* and *wMAS000005* showing no association between PBC and *Sr2*. Likewise, the black glume color similar to PBC has also been mapped to chromosome 1A in a common wheat (Khlestkina et al. 2006). In general, several of the wheat-alien introgression lines in the present study, evaluated for field stem rust response at three locations, exhibited the possible presence of minor-effect resistance genes. Further studies are needed to characterize the genetic basis of resistance in these lines.

Table 5. Genomic in situ hybridization (GISH) and molecular marker results of wheat-alien introgression lines[a]

Germplasm[b]	GISH and molecular analysis			Introgression[c]	Chromosome number	Note	*Sr* genes[d]
	Rye	*Leymus*	Wheat				
SLU50	1R present	…	1D absent	Substitution	40+2 1R (1D)	1R replaced 1D	Unknown
SLU56	1R present	…	1D absent	Substitution	40+2 1R (1D)	1R replaced 1D	Unknown
SLU168	1RS present	…	1BS absent	Translocation	42 1BL.1RS	1RS present	*Sr31*
SLU173	1RS present	…	1BS absent	Translocation	42 1BL.1RS	1RS present	*Sr31*
SLU183	1R present	…	1D absent	Substitution	40+2 1R (1D)	1R replaced 1D	Unknown
SLU188	Rye absent	…	Wheat present	Normal wheat	42 AABBDD	Normal wheat	Unknown
SLU190	1R present	…	1D absent	Substitution	40+2 1R (1D)	1R replaced 1D	*Sr36*
SLU193	1R present	…	1D absent	Substitution	40+2 1R (1D)	1R replaced 1D	*Sr36*
SLU196	1R present	…	1D absent	Substitution	40+2 1R (1D)	1R replaced 1D	Unknown
SLU198	1R present	…	1D absent	Substitution	40+2 1R (1D)	1R replaced 1D	Unknown
SLU199	1R present	…	1D absent	Substitution	40+2 1R (1D)	1R replaced 1D	Unknown
SLU208	1R present	…	1DS absent	Translocation	42, 1RS.1DL	1RS replaced 1DS	Unknown
SLU234	1R present	…	1DS absent	Translocation	42, 1RS.1DL	1RS replaced 1DS	*Sr7b*
SLU73 to SLU83	2R present	…	2B absent	Substitution	40+2, 2R (2B)	2R replaced 2B	Unknown
SLU209	2R present	…	2D absent	Substitution	40+2, 2R (2D)	2R replaced 2D	*36*,+
SLU210	2R present	…	2D absent	Substitution	40+2, 2R (2D)	2R replaced 2D	*36*,+
SLU212	2R present	…	2D absent	Substitution	40+2, 2R (2D)	2R replaced 2D	Unknown
SLU223	2R present	…	2D absent	Substitution	40+2, 2R (2D)	2R replaced 2D	Unknown
SLU238	2R present	…	2D absent	Substitution	40+2, 2R (2D)	2R replaced 2D	Unknown
SLU239	2R present	…	2D absent	Substitution	40+2, 2R (2D)	2R replaced 2D	Unknown
SLU213	3R present	…	3D absent	Substitution	40+2, 3R (3D)	3R replaced 3D	Unknown
SLU214	3R present	…	3D absent	Substitution	40+2, 3R (3D)	3R replaced 3D	Unknown
SLU215	3R present	…	3D absent	Substitution	40+2, 3R (3D)	3R replaced 3D	Unknown
SLU216	3R present	…	3D absent	Substitution	40+2, 3R (3D)	3R replaced 3D	*SrSatu*
SLU217	3R present	…	3D absent	Substitution	40+2, 3R (3D)	3R replaced 3D	*SrSatu*
SLU218	3R present	…	3D absent	Substitution	40+2, 3R (3D)	3R replaced 3D	*SrSatu*
SLU219	3R present	…	3D absent	Substitution	40+2, 3R (3D)	3R replaced 3D	*SrSatu*,+
SLU220	3R present	…	3D absent	Substitution	40+2, 3R (3D)	3R replaced 3D	*SrSatu*
SLU221	3R present	…	3D absent	Substitution	40+2, 3R (3D)	3R replaced 3D	Unknown
SLU231	3R present	…	3DS absent	Translocation	3RS.3DL	3RS replaced 3DS	*Sr7b*
SLU232	3R present	…	3DS absent	Translocation	3RS.3DL	3RS replaced 3DS	*Sr7b*
SLU233	3R present	…	3DS absent	Translocation	3RS.3DL	3RS replaced 3DS	Unknown
SLU229	1R+6R present	…	1D+6D absent	Substitution	38+4, 1R+6R (1D+6R)	1R+6R replaced 1D+6D	Unknown
SLU230	1R+3R present	…	1D+3D absent	Substitution	38+4, 1R+3R (1D+3R)	1R+3R replaced 1D+3D	Unknown
SLU176	*Leymus mollis*	NsNsXmXm	…	Substitution	32+10	5 pairs of *L. mollis*	Unknown
SLU177	*L. mollis*	NsNsXmXm	…	Substitution	30+10	5 pairs of *L. mollis*	Unknown
SLU178	*L. mollis*	NsNsXmXm	…	Substitution	30+10	5 pairs of *L. mollis*	Unknown
SLU235	*L. racemousus*	NsXm	6D absent	Substitution	40+2 (6D)	2 pairs of *L. racemousus*	*Sr36*
SLU236	*L. racemousus*	NsXm	6D absent	Substitution	40+2 (6D)	2 pairs of *L. racemousus*	*Sr36*
SLU237	*L. racemousus*	NsXm	6D absent	Translocation	42 (6DL)	2 pairs of *L. racemousus*	*Sr36*

[a] In addition to GISH analysis, the wheat simple-sequence repeat (SSR) markers for 1D, 2B, 2D, 3D, and 6D (Röder et al. 1998; Somers et al. 2004); rye SSR and expressed sequence tag (EST)-derived SSR for 1R, 2R, and 3R (Khlestkina et al. 2004; Saal and Wricke 1999); and EST-SSR for *L. racemosus* (Kaur et al. 2008; Larson et al. 2012) were assayed for the presence and absence of wheat-alien introgression chromosomes. In most cases, the rye, *L. racemosus*, and *L. mollis* were substituted in the B and D chromosomes of wheat.

[b] Germplasm designation.

[c] Introgression level.

[d] Postulated *Sr* genes.

By using GISH in combination with molecular markers, we showed that lines with the 2R (2D) and 3R (3D) wheat-rye substitutions were resistant to all tested races, and the resistances were likely from rye. In the present study, the combination of GISH together with molecular markers for wheat, rye, and *L. racemosus* unequivocally validated the presence of alien chromatin introgressed into wheat (Table 5; Fig. 2). Both cytogenetic and molecular analyses supported the contention that most lines had the following chromosome composition: 2n = 40+2 1R (1D), 2n = 40+2 2R (2B), 2n = 40+2 2R (2D), 2n = 40+2 3R (3D), 2n = 42 1DL.1RS, and 2n = 42 3DL.3RS. Therefore, our study agrees with previous studies (Merker 1975, 1979, 1984) showing that introgressions of alien chromosomes were primarily in the B and D chromosomes of wheat. Wheat-alien addition, substitution, and translocation lines have been successfully used in wheat improvement. However, for stem rust resistance, translocation lines are preferred due to the smaller introgression of alien chromatin, less linkage drag, and regular meiotic behavior (Friebe et al. 1996; Liu et al. 2011; Niu et al. 2011, 2014; Tiwari et al. 2014). Transfer of stem rust resistance genes such as *Sr24* and *Sr26* (Mago et al. 2005a), *Sr39* (Niu et al. 2011), *Sr43* (Niu et al. 2014), and *Sr53* (Liu et al. 2011) has been successfully deployed by chromosome engineering such as radiation and *ph1b*-induced approaches. In conclusion, this study demonstrated that the wheat-alien introgression lines evaluated, particularly those from *S. cereale* and *Thinopyrum junceiforme* (effective only to Ug99 linage), had effective resistance against all stem rust races tested, and further genetic studies are needed to characterize these resistance sources.

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