RESEARCH

Genetic Variation for Markers Linked to Stem Rust Resistance Genes in Pakistani Wheat Varieties

Mahwish Ejaz, Muhammad Iqbal,* Armghan Shahzad, Atiq-ur-Rehman, Iftikhar Ahmed, and Ghulam M. Ali

ABSTRACT

Stem rust, caused by *Puccinia graminis* Pers. f. sp. *tritici* Eriks. and E. Henn., is one of the major diseases of wheat throughout the world. New *P. graminis* f. sp. *tritici* races including Ug99 (strain TTKS) and its variants, as well as local stem rust races, pose a serious threat to wheat (*Triticum aestivum* L.) production in Pakistan. Identifying resistance genes effective against the prevalent races and incorporating these genes into adapted wheat varieties can contribute to stem rust control. In this study, 117 Pakistani wheat varieties were screened with 18 DNA markers to detect the presence of stem rust resistance genes *Sr2*, *Sr6*, *Sr22*, *Sr24*, *Sr25*, *Sr26*, *Sr31*, and *Sr38*. Stem rust resistance genes *Sr22*, *Sr24*, *Sr25*, and *Sr26* were absent from all varieties, whereas *Sr2*, *Sr6*, *Sr31*, and *Sr38* were present at various frequencies. The highest frequency was observed for *Sr2* (9–79% by different markers), followed by *Sr31* (35%), *Sr6* (11%), and *Sr38* (9%). These results indicated that Pakistani varieties are being protected by very few resistance genes and lack resistance genes potentially effective against new stem rust races. Therefore, there is a need to incorporate stem rust resistance genes *Sr22*, *Sr24*, *Sr25*, and *Sr26* into Pakistani wheat varieties. Different markers used for adult plant resistance gene *Sr2* indicated different frequencies of this gene in the tested varieties. More reliable and efficient markers need to be developed for marker-assisted selection of this and other genes.

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Abbreviations: BAC, bacterial artificial chromosome; CAPS, cleaved amplified polymorphic sequences; CTAB, cetyltrimethylammonium bromide; MAS, marker-assisted selection; PCR, polymerase chain reaction; *Pgt*, *Puccinia graminis* Pers. f. sp. *tritici* Eriks. and E. Henn.; SCAR, sequence characterized amplified region; *Sr*, stem rust resistance; SSR, simple sequence repeat; STS, sequence tagged site.

Bread or common wheat (*Triticum aestivum* L.) is affected by three species of rusts, that is, leaf rust, stripe rust, and stem rust. Leaf rust is caused by *Puccinia triticina* Eriks., stripe rust by *Puccinia striiformis* West. f. sp. *tritici* Eriks., and stem rust by *Puccinia graminis* Pers. f. sp. *tritici* Eriks. and E. Henn. (*Pgt*) (Bariana et al., 2007). Under favorable conditions, yield losses due to leaf rust can be up to 30% (Roelfs et al., 1992). Yield losses due to yellow rust ranging from 10 to 70% have been reported (Chen, 2005). Stem rust epidemics have resulted in as much as 50% yield losses in recent years (Beard et al., 2006), whereas yield losses due to Ug99 can be as high as 90% (Beard et al., 2006).

Ug99 is the most devastating race of *Puccinia graminis* f. sp. *tritici* and is a major threat to wheat production. It first appeared in Uganda in 1999 and now has spread throughout East Africa, Yemen, Sudan, and Iran. Its spread has now been predicted toward North Africa, Middle East, Asia, and beyond, raising serious concerns of major epidemics that could destroy wheat crops in various areas (Singh et al., 2008). Two variant strains of Ug99, TTKST and TTSSK,

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were detected in Kenya in 2006 and 2007, indicating the evolution of Ug99. In 2007, there was a severe epidemic in some regions of Kenya by TTKST, and half of the global wheat germplasm that was resistant to Ug99 appeared to be susceptible to this variant (Singh et al., 2008). Ug99 has not, so far, been detected in Pakistan but the migration pattern suggests that it may spread to Pakistan through Iran. Moreover, some local stem rust races have been evolving to greater virulence in Sindh and southern Punjab provinces. Consequently, there is a need to develop effective strategies to mitigate the present and future challenges posed by *Pgt* in Pakistan to ensure food security.

To date, >50 stem rust resistance genes have been reported in wheat and its wild relatives (McIntosh et al., 2008). Most of these genes are specific to pathogen race except *Sr2*, which is race-nonspecific and provides durable resistance (McIntosh et al., 1995; Singh et al., 2006). *Sr2* confers slow rusting, which may not substantially reduce yield losses under severe epidemics (Singh et al., 2006). Therefore, deployment of *Sr2* with other rust resistance minor genes, commonly called *Sr2*-complex, can provide resistance against most of the stem rust races, including Ug99 (Singh et al., 2006).

Screening of breeding material for disease resistance genes using conventional approaches requires time, as some genes express only at later stages of plant growth. Another drawback of the conventional approach is that disease inoculum has to be applied on plants, which is dangerous in regions where a particular pathogenic race is not present.

Gene-for-gene specificity between host resistance genes and different avirulence genes in the pathogen can be employed for postulation of resistance genes in the host plant. However, this method is best suited for seedling resistance genes because the interaction between resistance genes and stage of development of plant at which these genes express can obscure the gene postulation (Kolmer, 1996). These problems can be overcome by using DNA-based markers to identify the resistance genes that may be present (McCartney et al., 2005).

Molecular markers provide an efficient way to address problems faced in conventional breeding methods. Rust resistance genes can be tagged with tightly linked DNA markers and selection based on these markers improves the efficiency of breeding programs (Todorovska et al., 2009). With the advent of marker-assisted selection (MAS), gene pyramiding, in which genes identified in different genotypes are deployed into a single cultivar that contains desired alleles at more than one locus, has become efficient (Joshi and Nayak, 2010).

Several DNA markers linked to various stem rust resistance genes in wheat have been identified and developed. The genes include *Sr2* (Spielmeyer et al., 2003; Hayden et al., 2004), *Sr1R^{Amigo}* (Olson et al., 2010), *Sr6* (Tsilo et al., 2009), *Sr9a* (Tsilo et al., 2007), *Sr24* (Mago et al., 2005; Olson et al., 2010), *Sr25* (Liu et al., 2010), *Sr26* (Mago et al., 2005; Liu et al., 2010), *Sr31* (Das et al., 2006), *Sr35* (Zhang et al., 2010), *Sr36* (Tsilo et al., 2008), *Sr38* (Helguera et al., 2003), *Sr39* (Gold et al., 1999), *Sr40* (Shuangye et al., 2009), *SrCad* (Hiebert et al., 2011), *SrWeb* (Hiebert et al., 2010), *Sr51* (Liu et al., 2011b), *Sr52* (Qi et al., 2011), and *Sr53* (Liu et al., 2011a).

There is limited information on the presence/absence of major stem rust resistance genes in Pakistani-adapted spring wheat. The objective of this study was to detect major stem rust resistance genes in Pakistani-adapted spring wheat varieties, using DNA markers to assist future rust resistance breeding.

MATERIALS AND METHODS

Plant Material and Genomic DNA Extraction

Seeds of 117 Pakistani wheat varieties along with positive and negative controls for stem rust resistance genes were obtained from Wheat Program and Crop Diseases Research Program, National Agricultural Research Centre, Islamabad, Pakistan. Eight to 10 seeds of each genotype were sown in pots in a greenhouse, and leaf tissue from three to four plants of each genotype were harvested after 2 to 4 wk of growth for genomic DNA extraction.

DNA was extracted from fresh leaf tissue using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) with some modification. About 200 mg of fresh leaf tissues were ground vigorously in 2 to 3 mL of prewarmed 2% (w/v) CTAB (with 1% [v/v] mercaptoethanol) using an autoclaved mortar and pestle. About 750 μL of emulsified sample in a 1.5-mL Eppendorf tube were incubated at 65°C for 30 min in a water bath. Following incubation, 750 μL of chloroform:isoamyl alcohol (24:1) was added to each tube; the tube was inverted four to five times to mix the contents and then centrifuged at 9726 × *g* for 10 min. About 500 to 600 μL of supernatant were transferred to a new Eppendorf tube to which 0.8 volume of ice-chilled 2-propanol was added and incubated at 4°C for 10 min. DNA was precipitated by centrifuging the tubes at 14,006 × *g* for 10 min; the supernatant was discarded. DNA pellets were washed with 70% (v/v) ethanol with centrifugation at 14,006 × *g* for 10 min at room temperature. Ethanol was removed from the tubes, and DNA pellets were air-dried and resuspended in 50 μL of Tris-EDTA buffer. RNA was removed by adding 1 μL of RNase A (10 mg mL^{-1}) and incubating for 30 min at 37°C. DNA was quantified by gel electrophoresis of the DNA samples along with two known DNA standards. Quantity of DNA in the samples was estimated by comparing their band strengths with those of standards. Quantified DNA samples were diluted to working concentration of about 25 ng μL^{-1}.

Polymerase Chain Reaction Analysis

The 20 μL of polymerase chain reaction (PCR) mixture used contained 1× PCR buffer (75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01% Tween [v/v] 20), 2.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 10 pmol each of the forward and reverse primers except for primers CSH81-BM, CSH81-AG, which were used in 2:1 ratio, 1 unit of *Taq* DNA Polymerase (Fermentas, Life Sciences) and 1 μL of DNA template (25 ng μL^{-1}). Eighteen simple sequence repeat/

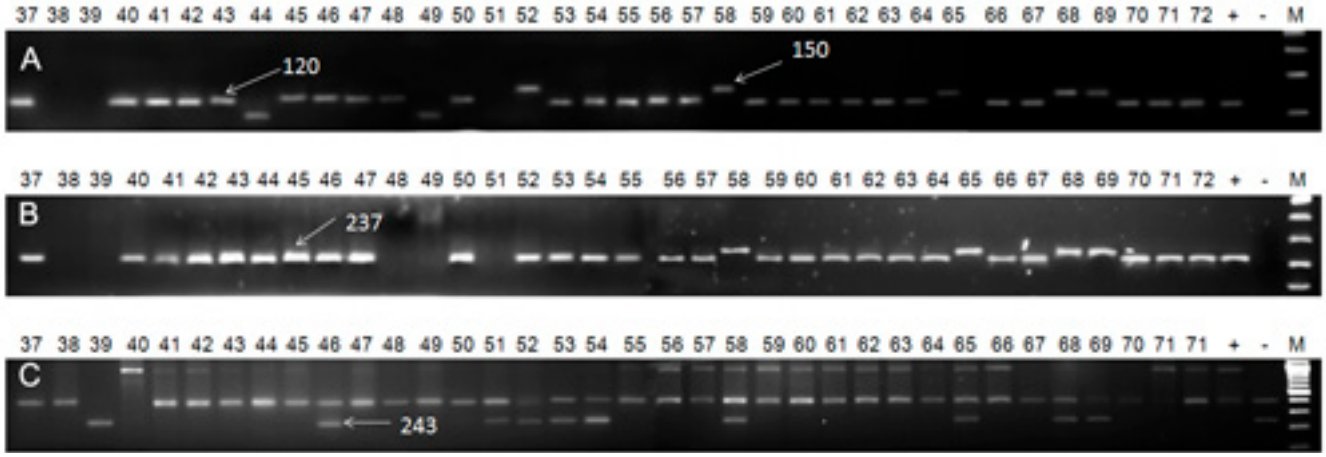

Figure 1. Polymerase chain reaction amplification of (A) *Xgwm533* locus in Pakistani wheat on 3% high-resolution agarose, (B) marker stm559tgag for *Sr2* (2% high-resolution agarose), and (C) marker X3B028F08 for *Sr2* on 1.5% agarose. M = 100 bp; + means positive ('Pavon'), whereas – means negative control 'Morocco'.

sequence tagged site/cleaved amplified polymorphic sequences/sequence characterized amplified region (SSR/STS/CAPS/SCAR) primer pairs were used to determine the presence/absence of the stem rust resistance genes.

Amplification reaction was performed in an automated Thermal Cycler (Applied Biosystems Veriti 96-well). Optimized PCR profiles of all primer pairs are given in supplementary table. Amplified products were electrophoresed on different concentrations of normal and high-resolution agarose gel (depending on product size of each primer) stained with ethidium bromide. Gels were photographed using a Gel Documentation system (UVIpro Platinum, Uvitec, Cambridge, UK) and bands were scored to indicate the presence/absence of stem rust resistance genes.

For CAPS marker, 25 μL of PCR Master Mix was prepared and 10 μL of PCR product was electrophoresed on 1.5% agarose gel. The remaining 15 μL PCR product of samples showing 337-bp bands were digested with *PagI* (*Bsp*HI) 10 U μL^{-1} (Fermentas, Life Sciences). Restriction digestion was done by adding 0.5 μL of restriction enzyme, 2 μL of 10× buffer O, and 2.5 μL of nuclease-free water in each reaction tube, followed by incubating at 37°C for 1 h.

RESULTS

Eighteen SSRs/STSs markers were used to determine presence/absence of stem rust resistance genes in 117 Pakistani wheat varieties. DNA markers *Xgwm533*, X3B042G11, X3B061C22, X3B028F08, *csSr2*, *stm559tgag*, *Xcfd#43*, CSH81-BM, CSH81-AG, Sr24#12, Gb, Sr26#43, BF518379, iag95, SCSS30.2, SCSS26.1, and VENTRIUP-LN2 gave reproducible results, whereas *Xgwm47*, SCAR39-F2/R3, and *Xwmc453* failed to amplify fragments previously reported diagnostic for *Sr6*, *Sr9a*, and *Sr39*.

Six markers, *Xgwm533*, X3B042G11, X3B061C22, X3B028F08, *csSr2*, and *stm559tgag*, were used to detect adult plant stem rust resistance gene *Sr2* derived from 'Hope' (Hare and McIntosh, 1979). Microsatellite dominant marker *Xgwm533* produced a 120-bp fragment associated with the presence of *Sr2*, in positive control 'Pavon' and 92 varieties, whereas 11 varieties resulted in 130- and 150-bp fragments that are not diagnostic for the presence of the *Sr2* gene (Fig. 1 and Table 1). Thirteen varieties and negative control 'Morocco' did not result in amplification of any fragment for *Xgwm533*.

Sequence tagged microsatellite marker *stm559tgag* was assayed for *Sr2* with new forward primer stm559n (Pretorius et al., 2012). This marker produced a 237-bp fragment known to be associated with stem rust resistance gene *Sr2* in 92 varieties and positive control (Fig. 1). Twenty-five varieties were negative for *stm559tgag*, among which 10 produced fragments >237 bp in size and 15 varieties showed no amplification (Table 1). Four varieties, 'Pirsabak-08', 'Bahawalpur-2000', 'KT-2000', and 'Zardana' showed presence of the *Sr2* gene based on marker *Xgwm533*, but absence of this gene based on marker *stm559tgag*. Similarly 'Barani-70', 'Hashim-08', 'Fsd-85', 'MH-97', 'Khushal', and 'NIA-Sunehri' showed presence of the *Sr2* gene with marker *stm559*, whereas absence with *Xgwm533*.

The three bacterial artificial chromosome (BAC)–derived SSR markers, X3B042G11, X3B061C22, and X3B028F08, have been reported more closely linked with *Sr2* than *Xgwm533* (McNeil et al., 2008). Marker X3B042G11 produced two bands (180 and 200 bp) in the negative control and 'Hashim08', whereas positive control 'Pavon' as well as 'NIFA-Bathor', 'NIFA-Barsat', 'BARS-2009', 'Gomal-08', and 'Ghaznavi' yielded only a 180-bp fragment (data not shown). Marker X3B061C22 also was tested on these same varieties. This marker produced two fragments (180 and 200 bp) in negative control 'Morocco' as well as 'NIFA-Barsat', 'BARS-2009', and 'Ghaznavi'. Positive control for *Sr2* 'Pavon' as well as 'Gomal-08' and 'Hashim08' produced a 200-bp fragment, whereas no band was found in 'NIFA-Bathor' (data not shown). These markers were not further tested on remaining varieties. Marker X3B028F08 produced a 243-bp fragment in negative control 'Morocco' and 34

Table 1. Allelic variation at the marker loci linked with stem rust resistance genes in Pakistani wheat varieties.

		Sr2			Sr6	Sr31			Sr38
No.†	Variety	Xgwm533	Stm559tgag	X3B028F08	Xcfd43	iag95	SCSS26.1‡	SCSS30.2	Ventriup-LN2
1	Mexipak-65	+	+	–	–	–	+	–	–
2	Barani-70	–	+	–	+	–	+	–	–
3	Chenab-70	–	–	–	–	–	+	–	–
4	Yecora-70	+	+	+	–	–	+	–	–
5	Blue silver-71	–	–	–	–	–	+	–	–
6	Lyallpur-73	+	+	+	–	–	+	–	–
7	Sandal-73	+	+	+	–	–	+	–	–
8	Punjab-76	+	+	+	–	–	+	–	–
9	Pavon-76	+	+	+	–	+	–	+	–
10	Zarghoon-76	+	+	+	–	–	+	–	–
11	Lu26–77	+	+	+	–	–	+	–	–
12	Pak-81	+	+	+	±	+	–	+	–
13	Punjab-81	+	+	+	+	–	+	–	–
14	Sarhad-82	+	+	+	–	+	+	+	–
15	Barani-83	+	–	–	±	–	+	–	–
16	Fsd-83	+	–	–	+	–	+	–	–
17	Kohinoor-83	+	+	+	–	+	–	+	–
18	Tandojam-83	+	+	+	–	+	–	+	–
19	Chakwal-86	+	+	+	–	–	+	–	–
20	Sarsabz-86	+	+	+	–	–	+	–	–
21	Khyber-87	+	+	+	–	+	–	+	–
22	Shalimar-88	+	+	+	+	–	+	–	–
23	Zardana-89	+	+	+	–	–	+	—	–
24	Pasban-90	+	+	+	–	+	–	+	–
25	Rohtas-90	+	+	–	–	+	–	+	–
26	Soghat-90	+	+	+	–	–	+	–	–
27	Inqalab-91	+	+	+	–	–	+	–	–
28	Pirsabak-91	+	+	+	–	–	+	–	–
29	Bakhtawar-92	+	+	–	–	+	–	–	–
30	Sariab-92	+	+	+	–	–	+	–	–
31	Kaghan-93	+	+	+	–	+	–	+	–
32	Potowar-93	+	+	+	–	–	+	–	–
33	Parwaz-94	+	+	+	+	–	+	–	–
34	Kiran-95	+	+	+	–	–	+	–	–
35	Kohsar-95	+	+	+	–	–	+	–	–
36	Shahkar-95	+	+	+	–	+	+	+	+
37	Noshehra-96	+	+	+	–	–	–	–	–
38	Suleman-96	–	–	+	–	–	+	–	+
39	Tatara-96	–	–	–	–	+	–	–	+
40	B.Pur-97	+	+	+	–	+	–	+	–
41	Chakwal-97	+	+	+	–	–	+	–	–
42	F.Sarhad-97	+	+	+	–	–	+	–	+
43	Kohistan-97	+	+	+	–	+	+	+	–
44	MH-97	–	+	+	–	–	+	–	+
45	Margalla-99	+	+	+	–	–	+	–	–
46	Zarlashta-99	+	+	–	–	+	–	+	–
47	Auqab-2000	+	+	+	–	–	+	–	–
48	B.Pur-2000	+	–	+	–	+	+	+	–
49	Chenab-2000	–	–	–	±	+	–	+	–
50	Haider-2000	+	+	–	–	–	+	–	–
51	Iqbal-2000	–	–	–	+	+	–	+	–
52	Marvi-2000	–	–	–	+	–	+	–	–
53	Saleem-2000	+	+	–	–	+	–	+	–
54	Marwat-2001	+	+	–	–	–	+	–	–
55	Wafaq-2001	+	+	+	–	+	–	+	–
56	Aufaq-2002	+	+	+	+	–	+	–	–
57	Bhakkar-02	+	+	+	–	–	+	–	–
58	GA-2002	–	–	–	–	–	+	–	+
59	Moomal-2002	+	+	+	–	–	+	–	+

(cont'd)

Table 1. Continued.

		Sr2			Sr6	Sr31			Sr38
No.†	Variety	Xgwm533	Stm559tgag	X3B028F08	Xcfd43	iag95	SCSS26.1‡	SCSS30.2	Ventriup-LN2
60	SH-2002	+	+	+	−	−	+	+	−
61	Manthar-2003	+	+	+	−	+	−	+	−
62	SH-2003	+	+	+	−	−	+	−	−
63	Imdad-05	+	+	+	−	−	+	−	−
64	Pirsabak-05	+	+	+	−	+	−	+	−
65	Rashkoh-05	−	−	−	−	−	+	−	−
66	Fareed-06	+	+	+	−	−	+	−	−
67	Khirman-06	+	+	+	−	+	−	+	−
68	Sassui-06	−	−	−	−	+	−	+	−
69	Sehar-2006	−	−	−	−	−	+	−	−
70	Shafaq-2006	+	+	+	−	−	+	−	−
71	SKD1-2006	+	+	+	+	−	+	−	−
72	Fsd-2008	+	+	+	+	−	+	−	−
73	Lasani-2008	+	+	+	−	−	+	−	−
74	Mairaj-2008	+	+	+	−	−	+	−	−
75	Pirsabak-08	+	−	+	−	+	−	+	−
76	Chakwal-50	−	−	+	−	+	−	+	−
77	NARC-09	+	+	+	−	−	+	−	−
78	Local White	−	−	+	−	−	+	−	−
79	SA-42	−	−	−	−	−	+	−	−
80	Sonalika	+	+	+	+	−	+	−	−
81	Khushal	−	+	−	−	+	+	+	−
82	SA-72	+	+	−	−	−	+	+	−
83	WL-711	+	+	+	+	−	+	−	−
84	SAAR	+	+	+	−	−	+	−	−
85	Panjnad-1	+	+	+	−	+	+	+	−
86	Bhittai	+	+	+	−	+	−	+	−
87	Aas-2009	+	+	+	−	+	−	+	−
88	AARI-2010	−	−	+	−	+	−	+	−
89	NIFA-Bathor	+	+	−	−	+	−	−	−
90	NIFA-Barsat	+	+	+	−	−	+	−	−
91	BARS-2009	+	+	+	−	−	+	−	−
92	Gomal-08	−	−	+	−	+	−	+	−
93	Hashim-08	−	+	−	−	−	+	−	−
94	Ghaznavi	+	+	+	−	+	−	+	−
95	NIA-Amber	−	−	+	−	−	+	−	−
96	NIA-Sunehri	−	+	−	−	−	+	−	−
97	KT-2010	+	+	+	−	−	+	−	−
98	Sutlaj-86	+	+	+	−	−	+	−	−
99	Derawar-97	+	+	+	−	+	−	−	+
100	Daman-98	+	+	+	−	−	+	+	−
101	Dera-98	+	+	+	−	−	+	−	−
102	Nasir-2000	+	+	−	−	+	−	+	−
103	Raj	+	+	−	−	−	+	−	−
104	Zam-04	−	−	−	−	+	−	+	−
105	Mehran-89	+	+	+	−	−	−	+	−
106	Anmol-91	+	+	+	−	−	+	−	+
107	TD1	+	+	+	−	−	+	−	−
108	Pirsabak-04	+	+	+	−	+	−	+	−
109	Jauhar-78	+	+	−	−	−	+	−	−
110	Sindh-81	+	+	+	−	−	+	−	−
111	KT-2000	+	−	−	−	−	+	−	−
112	Fsd-85	−	+	−	−	+	−	+	−
113	Shaheen-94	+	+	+	−	−	−	+	−
114	Zardana	+	−	−	−	−	−	+	+
115	AS-2002	−	−	−	+	+	−	+	−
116	Takbeer	+	+	+	−	−	+	−	−
117	Watan	+	+	+	−	+	−	+	−

†Corresponds to the varieties number given in the figures.

‡The symbol + indicates the presence of marker allele but absence of *Sr31*.

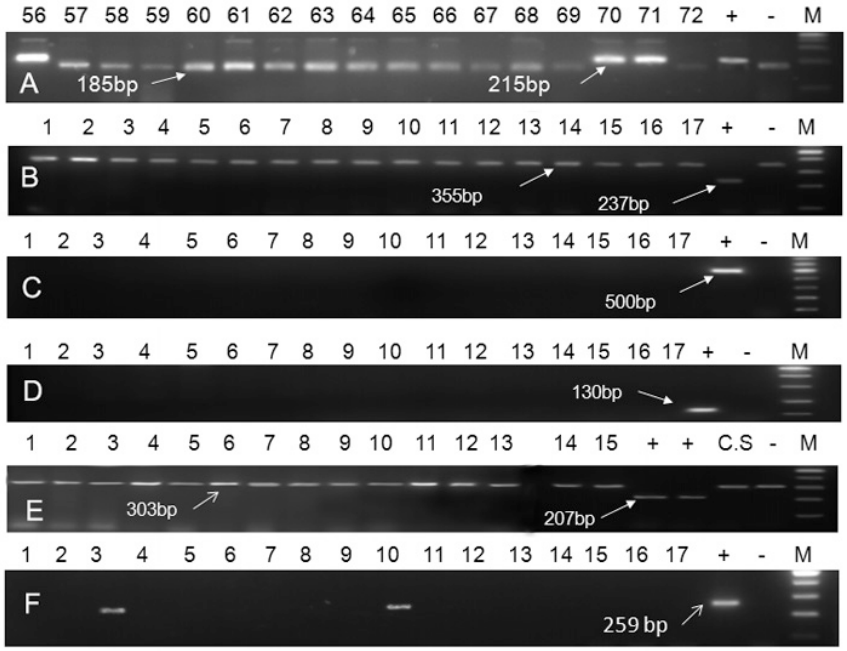

Figure 2. Polymerase chain reaction amplification of (A) *Xcfd#43* locus in Pakistani wheat for *Sr6* on 3% agarose; (B) markers CSH81-BM and CSH81-AG for *Sr22* gene on 2% agarose; (C) marker Sr24#12 for *Sr24* on 1.5% agarose; (D) marker Gb for *Sr25* on 2% agarose; (E) markers Sr26#23 and BE518379 for *Sr26* on 2% agarose; (F) marker VENTRIUP-LN2 for *Sr38* in Pakistani spring wheat on 2% agarose. M = 100 bp; + means positive control 'Chris','Sr22TB', 'LcSr24Ag', 'Superseri', and 'RL6081' for *Sr6*, *Sr22*, *Sr24*, *Sr25*, and *Sr38*, respectively, whereas – means negative control 'Morocco', and C.S means 'Chinese Spring'.

varieties, indicating the absence of *Sr2* (Fig. 1 and Table 1). This fragment has been reported diagnostic for the absence of *Sr2*. Eighty-three varieties and positive control 'Pavon' showed a smeared pattern (Fig. 1) as reported by McNeil et al. (2008), indicating the presence of *Sr2* gene.

A recently developed CAPS marker, *csSr2*, was also used to detect the presence of the *Sr2* gene. Marker *csSr2* produced no band in 15 varieties, whereas the remaining 102 varieties produced a 337-bp fragment (data not shown), suggesting the presence of the *csSr2* marker. After restriction digestion of the PCR products, 90 varieties produced 53-, 112-, and 225-bp fragments (data not shown), which showed loss of the restriction site. Ten varieties and positive control produced 53-, 112-, and 172-bp fragments; the 172-bp fragment was associated with *Bsp*HI restriction site. Our results did not match those of Mago et al. (2011) with respect to the presence of a 53-bp fragment in *Sr2*-positive varieties.

The highest number of *Sr2*-positive varieties was observed with marker *Xgwm533* and *stm559tgag*, followed by X3B028F08 and *csSr2*. Twelve varieties, namely 'Chenab-70', 'Tatara-96', 'Chenab-2000', 'Iqbal-2000', 'Marvi-2000', 'GA-2002', 'AS-2002', 'Rashkoh-05', 'Sassui-06', 'Sehar-2006', and 'Zam-04' were negative for *Sr2* based on all five markers used. Similarly, all markers indicated the presence of *Sr2* in 'Yecora-70', 'Lyallpur-73', 'Sandal-73', 'Pak-81', 'Pasban-90', 'Soghat-90', 'Potowar-93', 'Parwaz-94', and 'NIFA-Barsat'.

Two microsatellite markers, *Xwmc453* and *Xcfd43*, were used to detect the presence of the *Sr6* gene. Marker *Xwmc453* produced two monomorphic fragments (150 and 200 bp) in eight varieties including positive and negative controls (data not shown). Therefore, this marker was not further used to screen the remaining varieties. Marker *Xcfd43* produced a 215-bp fragment in the positive control as well as in 13 varieties including 'Barani-70', 'Punjab-81', 'Fsd-83', 'Shalimar-88', 'Parwaz-94', 'Iqbal-2000', 'Marvi-2000', 'Aufaq-2002', 'AS-2002', 'SKD1-2006', 'Fsd-2008', 'Sonalika', and 'WL-711', indicating the likely presence of *Sr6* in these varieties. One hundred varieties and the negative control 'Chinese Spring' showed 185-bp fragment, suggesting the absence of the *Sr6* gene (Fig. 2 and Table 1). Three varieties, 'Pak-81', 'Barani-83', and 'Chenab-2000', showed both positive and negative alleles of the *Sr6* gene.

Restriction fragment length polymorphism–converted STS markers CSIH81-BM and CSIH81-AG linked to *Sr22* (Periyannan et al., 2010) were used as codominant markers in multiplex PCR reaction. Positive control line 'Sr22TB' showed a band size of 237 bp, showing the presence of the *Sr22* gene. A DNA fragment of 355 bp was observed in the negative control 'Morocco' and all 117 varieties tested (Fig. 2), indicating the absence of the *Sr22* gene.

The dominant STS marker Sr24#12 was used to detect stem rust resistance gene *Sr24*. This marker amplified a 500-bp fragment only in the positive control 'LcSr24Ag' (Fig. 2). All other varieties, including negative control 'Morocco', did not yield any band associated with *Sr24* (Fig. 2), suggesting the likely absence of this gene.

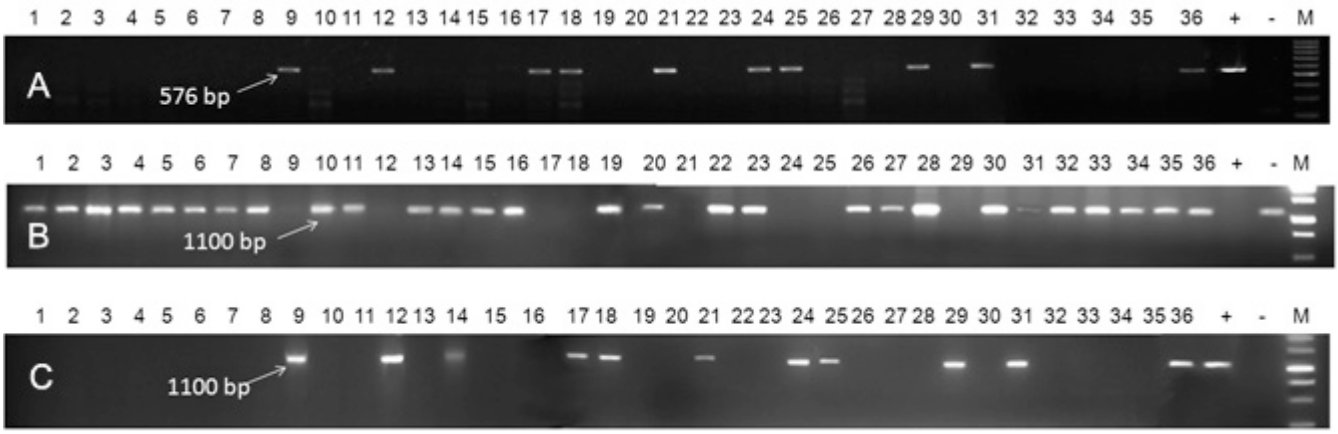

Figure 3. Polymerase chain reaction amplification pattern of (A) marker SCSS30.2 showing presence of *Sr31* (2% agarose; M = 100 bp); (B) marker SCSS26.1 for the absence of *Sr31* in Pakistani wheat (1.5% agarose; M = 1 kb); (C) fragments amplified by STS marker iag95 (1.5% agarose; M = 1 kb). + means positive 'Seri-82', whereas – means negative control 'Morocco'.

The presence/absence of *Sr25* was determined using marker Gb. This marker produced a 130-bp fragment only in the positive control 'Superseri'. All the tested Pakistani wheat varieties along with the negative control did not produce any fragment (Fig. 2), indicating the likely absence of this gene.

Two dominant markers, BE518379 and Sr26#43, were used in combination to detect the presence of the *Sr26* gene. These markers produced a 207-bp band only in positive control 'Eagle', whereas a 303-bp band (Fig. 2) was observed in all 117 varieties and the negative control. This suggested that none of the Pakistani wheat varieties tested in this study had *Sr26* gene.

Two SCAR markers, SCSS30.2 and SCSS26.1, and one STS marker, iag95, were used to detect *Sr31*. Marker SCSS30.2 resulted in a 576-bp fragment (Fig. 3 and Table 1) in 42 varieties and the positive control 'Seri-82', showing the presence of the *Sr31* gene. This fragment was absent from the remaining 75 varieties and negative control 'Morocco', indicating the absence of *Sr31* gene. Marker SCSS26.1 produced a 1100-bp fragment (Fig. 3 and Table 1) in 78 varieties, mostly those that did not produce a 576-bp fragment with marker SCSS30.2, showing absence of the *Sr31* gene. Thirty-nine varieties did not show the 1100-bp fragment for marker SCSS26.1, suggesting presence of *Sr31* in these varieties. Results from these two markers were complementary with few exceptions. Dominant marker iag95 amplified the expected 1100-bp band in 41 varieties and the positive control 'Seri-82', suggesting the presence of *Sr31* gene (Fig. 3 and Table 1). The remaining 77 varieties and 'Morocco' did not amplify the 1100-bp band, indicating the absence of *Sr31*.

Primer pairs VENTRIUP and LN2 were used to detect *Sr38* gene. Ten varieties, namely 'Anmol-91', 'Shahkar-95', 'Punjab-96', 'Fakhar-e-Sarhad-97', 'Derawar-97', 'MH-97', 'GA-2002', 'Moomal-2002', 'Zardana', and the positive control 'RL6081' showed a 259-bp fragment (Fig. 2 and Table 1), indicating the presence of *Sr38* gene. The remaining 107 varieties and 'Morocco' did not produce the 259-bp fragment, suggesting the absence of *Sr38*.

The SCAR primers Sr39F2/R3 (Gold et al., 1999) were used to detect *Sr39* gene. These primers failed to amplify the expected 900-bp fragment associated with the presence of *Sr39* gene. Instead, they produced three monomorphic bands of sizes ranging between 100 and 200 bp (data not shown) in all varieties and positive control 'RL6082'.

DISCUSSION

Global wheat production is threatened by the evolution of new races of *Pgt* in North Africa and their migration to other parts of the world. The new races have broken down the resistance of widely deployed stem rust resistance genes, especially *Sr31*. Development of resistant wheat varieties is one way of coping with this threat. The present study was conducted to determine the presence/absence of *Sr* genes in Pakistani-adapted spring wheat so as to facilitate future *Sr* gene pyramiding.

Stem rust resistance gene *Sr2* provides nonhypersensitive response at adult plant stage (McIntosh et al., 1995). We used six DNA markers to detect *Sr2* gene in Pakistani-adapted spring wheat. Microsatellite marker *Xgwm533* produced 120-bp fragment in 79% Pakistani wheat varieties, indicating the presence of *Sr2*. However, Spielmeyer et al. (2003) reported that some *Sr2* noncarriers also produced 120-bp fragment. To reliably detect *Sr2* gene, we used STS marker *stm559tgag* developed by Hayden et al. (2004) with the new forward primer referred to as *stm559n* (Pretorius et al., 2012), which showed the same frequency as *Xgwm533* for presence of *Sr2* gene with few exceptions. McNeil et al. (2008) found three BAC-derived markers, X3B042G11, X3B061C22, and X3B028F08, closer to *Sr2* gene than *Xgwm533*. These three markers produced polymorphic bands between positive and negative control in our study.

However, the *Sr2* gene-associated alleles of the first two markers were not similar to those reported by McNeil et al. (2008). Therefore, we did not apply these markers on all varieties. Our results for marker X3B028F08 were consistent with McNeil et al. (2008). Based on the results of this marker, 70% of Pakistani wheat varieties likely carry the *Sr2* gene. We suggest that this marker can be helpful in MAS for *Sr2*. The CAPS marker *csSr2* is diagnostic to detect single nucleotide polymorphism for *Bsp*HI restriction site (Mago et al., 2011). Our results of *csSr2* marker were 87% and 82% similar to that of *Xgwm533* and *stm559tgag*, respectively. However, after restriction with *BspH1*, only 9% of Pakistani varieties showed presence of the *Sr2* gene. This marker has been reported as more accurate for *Sr2* as compared to other markers reported previously. However, our results suggest that this marker probably underestimated the frequency of *Sr2* in Pakistani wheat germplasm. Moreover, CAPS markers require an additional step of restriction digestion, which makes them costly and time-consuming compared to STS markers. It is, therefore, recommended to use both *stm559tgag* and BAC-derived marker X3B028F08 for screening of wheat germplasm in Pakistan. As *Sr2* is a race-nonspecific adult plant resistance gene, efforts should be made toward the development of a gene-specific marker to assist future incorporation of this gene into wheat varieties.

We used two closely linked (1.1 and 1.5 cM, respectively) microsatellite markers, *Xwmc453* and *Xcfd43*, reported by Tsilo et al. (2009) to detect the presence of *Sr6*. The marker *Xwmc453* did not produce fragments associated with the presence/absence of *Sr6*, indicating that this marker is probably not diagnostic for *Sr6*. On the contrary, marker *Xcfd43* produced the expected fragments. Screening of Pakistani varieties with this marker showed that 11% of varieties likely have *Sr6*.

Stem rust resistance gene *Sr22* is effective against Ug99 and all other stem rust pathotypes, except races 316 and 317 from Israel (Periyannan et al., 2010). To date, this gene has only been incorporated in Australian commercial cultivar 'Schomburgk' (Singh, 1991; Khan et al., 2005). The limited use of this gene in cultivated wheat might be due to a yield penalty associated with this gene (Paull et al., 1994). The STS markers csIH81-BM and csIH81-AG are diagnostic to detect the presence/absence of *Sr22* (Periyannan et al., 2010). These markers showed absence of *Sr22* in Pakistani wheat varieties. It is, therefore, recommended to incorporate this gene into Pakistani wheat varieties to broaden their genetic base against *Pgt* races. Stem rust resistance gene *Sr24* confers resistance to stem rust race TTKS but not to its variants. Our results showed absence of this gene in Pakistani wheat varieties, so deployment of this gene in Pakistani cultivars should be encouraged. This will provide resistance to other prevalent *Pgt* races and may provide residual resistance to its variants as suggested by Knott (2008). Moreover, *Sr24* gene is also useful due to its linkage with *Lr24*. Klindworth et al. (2011) reported the occurrence of this gene in U.S. winter wheat, which can be used as source for the introgression of *Sr24*.

Stem rust resistance genes *Sr25* and *Sr26* are effective against variants of Ug99, TTKST and TTTSK (Singh et al., 2006; Jin et al., 2007). We used STS marker Gb (Prins et al., 2001) to detect *Sr25* gene. Our results showed absence of *Sr25* in Pakistani wheat varieties. This marker was also validated by Liu et al. (2010) and Njau et al. (2010). Liu et al. (2010) also tested a more accurate codominant marker BF145935 for *Sr25*, which showed 198- and 180-bp fragments in *Sr25*-positive varieties, and 202- and 180-bp bands in *Sr25* noncarriers. We preferred using Gb, as the 4-bp difference resulting from BF145935 was relatively difficult to resolve on agarose gel. This gene has been widely exploited in Australian and CIMMYT germplasm (Bariana et al., 2007). This gene needs to be incorporated into Pakistani wheat varieties so as to broaden their genetic base against the various *Pgt* races.

The STS markers Sr26#43 (Mago et al., 2005) and BE518379 (Liu et al., 2010) were used in combination to serve as a codominant marker. These markers showed absence of the *Sr26* gene in Pakistani wheat varieties. Similar to *Sr25*, *Sr26* is also effective against Ug99 and *Sr24*-virulent races. Use of this gene has been limited to Australia where 'Eagle' was the first cultivar possessing *Sr26* (Martin, 1971). The limited use of this gene might be due to a 9% yield penalty associated with this gene (The et al., 1988). This problem was later solved with the development of new lines having reduced alien segment (Dundas et al., 2007). Thus, this gene can easily be transferred through Australian germplasm into Pakistani wheat varieties for broadening the genetic base of future wheat varieties against *Pgt* races.

Before the emergence of Ug99, stem rust resistance was maintained mainly by *Sr31* in most of the countries around the world except Australia (Singh et al., 2008). We used STS marker iag95 (Mago et al., 2002) and SCAR markers $SCSS30.2_{576}$ and $SCSS26.1_{1100}$ (Das et al., 2006) to assay Pakistani wheat varieties for this gene; 35% of the varieties tested had the *Sr31* gene. Das et al. (2006) reported that $SCSS30.2_{576}$ and $SCSS26.1_{1100}$ were more reliable than previously developed STS markers. Our results of the three markers were 98% similar, suggesting that these markers are equally reliable for detection of *Sr31* gene. However, the two SCAR markers can be used as codominant markers in segregating generations to distinguish homozygous dominant from heterozygous carriers of *Sr31*. Due to the large difference in the annealing temperatures of the two SCAR markers, these cannot be used in a multiplex PCR. Marker iag95 also has been successfully validated on South African germplasm (Pretorius et al., 2012).

Most Pakistani wheat varieties are highly susceptible to Ug99 but are resistant to local stem rust races (Mirza et al., 2010a). Our results showed the presence of *Sr31* in these varieties, indicating that *Sr31* probably is effective against Pakistani stem rust races. Moreover, susceptible genes can still provide resistance along with effective genes, a phenomenon known as ghost or residual resistance (Knott, 2008). So other stem rust resistance genes need to be incorporated into these varieties. Varieties 'Kiran-95', 'Tandojam-83', and 'Sarsabz-86' were found susceptible to a local stem rust race (Khanzada, 2008) named RRTTF (Mirza et al., 2010b) present in southern Pakistan. Among these cultivars, 'Tandojam-83' showed presence of *Sr31*, whereas the other two showed absence of *Sr31*. However, our results do not provide evidence that local race(s) carry virulence for *Sr31*, so the local races need to be tested against all stem rust resistance genes to know their virulence/avirulence pattern.

Stem rust resistance gene *Sr38* confers resistance against stem rust race TPPKC (Klindworth et al., 2011) and is linked with *Yr17* and *Lr37*. This gene was found in very low frequency (9%) in the Pakistani wheat varieties tested. Due to its linkage with stripe and leaf rust resistance genes, this gene cluster should be incorporated in future Pakistani wheat varieties to increase its frequency and to confer multiple rust resistance. Gold et al. (1999) developed SCAR markers to detect *Sr39* gene in Canadian wheat. However, we failed to produce the amplicon diagnostic for *Sr39* gene in Pakistani-adapted spring wheat. Instead, we observed three monomorphic bands ranging from 100 to 200 bp in size. Hence, there is need for further testing of this marker and for development of a more reliable marker for *Sr39*. This gene has not been exploited extensively and there is no report of quality deterioration associated with *Sr39*/*Lr35* segment. Therefore, this gene should be introgressed into Pakistani wheats.

Our results showed that a majority of Pakistani wheat varieties are prone to infection by Ug99 and its variants, as most carry only the *Sr31* gene. Phenotypic screening data of Pakistani germplasm in Kenya from 2005 to 2010 (Anonymous, 2010) also suggested that a majority of varieties are susceptible to Ug99. Therefore, it is important to broaden the genetic base of stem rust resistance in future wheat varieties by pyramiding multiple stem rust resistance genes, especially those effective against Ug99 and its variants. Marker-assisted selection can greatly facilitate the transfer of these needed *Sr* genes; the markers and their frequencies described in this study provide a basis on which to develop MAS protocols.

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