Validation of Molecular Markers for New Stem Rust Resistance Genes in U.S. Hard Winter Wheat

Amy N. Bernardo, Robert L. Bowden, Matthew N. Rouse, Maria S. Newcomb, David S. Marshall, and Guihua Bai★

ABSTRACT

Stem rust, caused by *Puccinia graminis* f. sp. *tritici* (Pgt), is one of the most serious diseases of wheat (*Triticum aestivum* L.) worldwide. The discovery of new Pgt races in Africa, Ug99 and its variants, brings a new threat to global wheat production. Pyramiding several stem rust resistance genes into adapted varieties as opposed to breeding varieties with a single resistance gene is considered a more effective method to combat new races, but the success of gene pyramiding depends on the availability of molecular markers tightly linked to resistance genes. Markers for Ug99-effective genes, *Sr2*, *Sr22*, *Sr26*, *Sr32*, *Sr35*, *Sr39*, and *Sr40*, were evaluated for usefulness in marker-assisted selection (MAS) of hard winter wheat (HWW) using 10 resistance gene donor lines, 17 recently released U.S. HWW varieties or breeding lines, and 20 advanced introgression lines. Markers *XcsIH81-BM* and *XcsIH81-AG* for *Sr22*, *Xsr26#43* and *XBE51879* for *Sr26*, *Xbarc55* for *Sr32*, *Xbarc51* for *Sr35*, *Xrwg27* for *Sr39*, *Xsr39#22r* for *Sr40*, and *csSr2*-derived single nucleotide polymorphism (SNP) marker for *Sr2* are diagnostic for the set of HWW accessions evaluated in this study. These markers should be useful in marker-assisted pyramiding of stem rust resistance genes to develop HWW cultivars with multiple gene resistance against Ug99 races.

A.N. Bernardo, Dep. of Plant Pathology, Kansas State Univ., 4024 Throckmorton Hall, Manhattan, KS 66506; G. Bai and R.L. Bowden, Hard Winter Wheat Genetics Research Unit, USDA-ARS, 4008 Throckmorton Hall, Manhattan, KS 66506; M.N. Rouse and M.S. Newcomb, Cereal Disease Laboratory, USDA-ARS, 1551 Lindig Street, St. Paul, MN 55108; D.S. Marshall, USDA-ARS, Plant Science Research Unit, 3411 Gardner Hall, North Carolina State University, Raleigh, NC 27695.
★Corresponding author (guihua.bai@usda.ars.gov; gbai@ksu.edu).

Abbreviations: CAPS, cleaved amplified polymorphic site; HWW, hard winter wheat; IT, infection type; MAS, marker-assisted selection; MR, moderately resistant; MS, moderately susceptible; PCR, polymerase chain reaction; Pgt, *Puccinia graminis* f. sp. *tritici*; R, resistant; RWG, Red River Wheat Germplasm; S, susceptible; SNP, single nucleotide polymorphism; SSR, simple sequence repeat.

WHEAT STEM RUST, caused by *Puccinia graminis* f. sp. *tritici* (Pgt), is a devastating fungal disease of wheat. This pathogen infects leaves, stems, and glumes, and reduces the supply of water and nutrients to the developing kernels, which results in shriveled grain (Roelfs et al., 1992; Schumann and Leonard, 2000). Yield loss due to stem rust was estimated at 20 to 50% in severe epidemics (Zadoks, 1963; Rees, 1972; Joshi and Palmer, 1973; Leonard,

2001). Wheat stem rust was a problem in the United States until the 1950s when barberry (*Berberis vulgaris* L.), its alternate host, was eradicated. Stem rust–resistant varieties were effectively deployed (Singh et al., 2006), and earlier-maturing wheat varieties reached advanced development stages before temperatures became warm enough for rapid stem rust increase (Marshall, 1989). For half a century, losses due to stem rust in the United States have been minimal (Leonard and Szabo, 2005); however, the recent discovery in Africa of Ug99, a virulent strain of the stem rust pathogen, brings a new threat to global wheat production (Singh et al., 2011).

Ug99, also known as race TTKSK, was first characterized from Uganda in 1999 (Pretorius et al., 2000). Ug99 caused severe infections in wheat known to have the stem rust resistance gene *Sr31*. *Sr31* was transferred from rye (*Secale cereale* L.) to common wheat and had been effective for more than 30 yr; Ug99 is the first race identified to be virulent to this widely deployed resistance gene (Pretorius et al., 2000). From Uganda, this stem rust race has migrated to Kenya, Ethiopia, Sudan, and Yemen and more recently to Iran (Singh et al., 2006; FAO, 2008). The race PTKST, with virulence to *Sr24* and *Sr31* and belonging to the Ug99 lineage, was detected in South Africa in 2009 (Pretorius et al., 2010). New variants of Ug99 have been identified with virulence to extensively used resistance genes *Sr24* and *Sr36* (Jin and Singh, 2006; Singh et al., 2011). Because *Sr24* and *Sr36* were among the most important sources of resistance to stem rust in North American winter wheat (Jin and Singh, 2006), the great majority of U.S. winter wheat varieties are now genetically vulnerable to the Ug99 group of isolates.

Screening of worldwide wheat accessions has identified several stem rust resistance genes that remain effective against Ug99, including *Sr2*, *Sr22*, *Sr26*, *Sr32*, *Sr35*, *Sr39*, and *Sr40* (Pretorius et al., 2000; Singh et al., 2011). These resistance genes were transferred to wheat from cultivated emmer (*Triticum turgidum* L. subsp. *dicoccon* (Schrank) Thell.] and other wild relatives. Because the pathogen has demonstrated an ability to adapt to different resistance genes by gaining virulence, deployment of single new resistance genes is unlikely to be durable. A more effective method to combat Ug99 races would be to stack several new resistance genes into each new adapted variety (Leonard and Szabo, 2005; Mago et al., 2011b). However, the success of gene pyramiding cannot rely on the availability of isolates of Ug99 and other new stem rust races that can differentiate the set of resistance genes to be stacked. It is not feasible to send all breeding materials to African stem rust nurseries or screen under containment in the United States.

Molecular markers can predict the presence of a specific gene with very high probability without the need for disease evaluation and thus aid the transfer of several resistance genes into adapted materials to pyramid several genes in one plant. Markers linked to resistance genes *Sr2*, *Sr22*, *Sr26*, *Sr32*, *Sr35*, *Sr39*, and *Sr40* have been reported (Hayden et al., 2004; Khan et al., 2005; Mago et al., 2005, 2009, 2011a; Babiker, 2009; Dundas et al., 2007; McNeil et al., 2008; Yu et al., 2009; Liu et al., 2010; Wu et al., 2009; Niu et al., 2011; Periyannan et al., 2011), but most of these markers were identified using a specific biparental mapping population, and levels of polymorphism for these markers may vary with parents. Genetic distances between markers and the resistance genes are also different among the genes. In addition, many of these markers were developed based on agarose gels, and subtle differences in amplicon size between accessions may be difficult to distinguish. This study aimed to (i) validate the DNA markers for *Sr2*, *Sr22*, *Sr26*, *Sr32*, *Sr35*, *Sr39*, and *Sr40* in selected U.S. hard winter wheat (HWW) varieties and breeding lines with different genetic backgrounds using a high-throughput genotyping system and (ii) evaluate the usefulness of the markers for those genes in marker-assisted selection (MAS). This information will aid wheat breeders in selecting markers for use in MAS and gene pyramiding to enhance durability of stem rust resistance.

MATERIALS AND METHODS

Plant Materials

The wheat materials used in this study were 10 stem rust resistant donor lines (positive controls), including Sr22Tb (*Sr22*), WA-1 (*Sr26*), CnsSr32As (*Sr32*), Mq(2)5★G2919K (*Sr35*), P8810-B5B3A2A2 (*Sr39*), RL6088 (*Sr40*), CS-Hope DS 3B (*Sr2*), Hope (CI 8178) (*Sr2*), 17 recently released HWW varieties or breeding lines, and 20 advanced stem rust resistance gene introgression breeding lines (Table 1). All resistance gene donors have been confirmed for rust resistance (Dundas et al., 2007; Jin et al., 2007; Yu et al., 2010) and all recently released cultivars do not contain any new alien resistance genes listed in this study according to their release documents. The cultivar 2174 was reported to be negative for *Sr2* (Mago et al., 2011a), and Thunder CL was reported to be positive for *Sr2* (Haley et al., 2009), but the status of the others is unknown.

Stem Rust Evaluation

Greenhouse evaluation of selected accessions for seedling host response against Pgt race TTKSK (Table 1) was conducted at the USDA Cereal Disease Laboratory in St. Paul, MN. Protocols for inoculum preparation, inoculation, incubation, and disease rating were as described by Jin and Singh (2006). Seedlings with infection type (IT) 0, ;, 1, 2, or combinations thereof were considered resistant and those with an IT of 3 and/or 4 were classified as susceptible. Adult plant resistance was evaluated on 14 Oct. 2011 on the same set of materials in Njoro, Kenya, following the method described by Njau et al. (2010). Disease severity was assessed using the modified Cobb Scale (Peterson et al., 1948), and infection response was rated as resistant (R), moderately resistant (MR), moderately susceptible (MS), or susceptible (S) as described by Roelfs et al. (1992).

Table 1. Wheat accessions, host responses to stem rust *Puccinia graminis* f. sp. *tritici* race TTKSK, and marker results.

Selection	Pedigree	Greenhouse[†]	Field[‡]	Marker results[§]
CS-Hope DS 3B	Hope(2B)/*Chinese-Spring	3+		*Sr2* (Control)
Hope	Yaroslav-emmer/Marquis	3+		*Sr2* (Control)
Sr22Tb	Steinwedel*2//Spelmar*2/*Triticum monococcum* subsp. *aegilopoides* G-21	2–		*Sr22* (Control)
U5615-98-120-2	2174/Sr22Tb	22–		*Sr22* (Control)
U5616-20-154-7	Lakin/Sr22Tb	2–		*Sr22* (Control)
WA-1	Eagle/Chinese Spring Ph1ph1b/*6 Angas			*Sr26* (Control)
CnsSr32As	*Aegilops speltoides* and Chinese Spring	1+[¶]		*Sr32* (Control)
Mq(2)5*G2919K	Marquis*5/G2919K	;[¶]		*Sr35* (Control)
P8810-B5B3A2A2	HY366/RL5711//2*HY366/3/3*HY366-BL31.RL5711	2		*Sr39* (Control)
RL6088	RL6071*7/PGR6195	1[¶]		*Sr40* (Control)
2174	IL-71-5662/PL-145//PIONEER-2165	3+		None
Armour	B1551-WH/KS94U326	2		None
Art	Jagger/W94-244-132	4		None
Aspen	TAM302/B1551W			None
Billings	Erythrospermum-2755-91/Odisseya(N-566)//OK-94-P-597	2		None
Cedar	TAM302/B1551W			None
CJ	W-99-188-S-1/BC-950814-1-1			None
CO01W172	96HW100-5/96HW114	3+		None
Duster	W0405/NE78488//W7469C/TX81V6187	3+		None
Everest	HBK1064-3/Betty 'S'//VBF0589-1/IL89-6483 (Pioneer9021L//Roland/IL77-2656)			None
Fuller	Unknown	4		None
Hitch	G53/3/Abilene/G1113//Karl92/4/Jagger/5/KS89180B			None
KS05HW14-1	KS98HW452(KS91H153/KS93HW255)/CO960293//KS920709B-5-2(T67/X84W063-9-45//K92)	4	50S	None
Lakin	Arlin/KS89H130	3+		None
PostRock	Ogallala/KSU94U261//Jagger	4 and 3+		None
Thunder CL	KS01-5539/CO99W165	3+		*Sr2*
Tiger	KS98HW518(93HW91/93HW255)//KS98H245(IKE/TA2460//*3T200)/Trego			None
U5924-10-1	Fuller*2//Sr22Tb/2*2174	2	TrR	*Sr22*
U5924-10-6	Fuller*2//Sr22Tb/2*2174	22–	5R	*Sr22*
U5926-2-8	Duster*2//CnsSr32As/2*2174	3+	45MSS	Non-*Sr32*
U5926-3-4	Duster*2//CnsSr32As/2*2174	3+	30MSS	Non-*Sr32*
U5928-1-5	Fuller*2//CnsSr32As/2*2174	3+	55S	Non-*Sr32*
U5930-11-3	Duster*2//Mq(2)5*G2919K(Sr35)/2*2174	0 and 3+	5R and 30MSS	*Sr35*[#]
U5930-13-5	Duster*2//Mq(2)5*G2919K(Sr35)/2*2174	0	5R	*Sr35*
U5931-3-1	PostRock*2//Mq(2)5*G2919K(Sr35)/2*2174	0	TrR	*Sr35*
U5932-2-4	Fuller*2//Mq(2)5*G2919K(Sr35)/2*2174	0 and 3+	25MSS	*Sr35*[#]
U5935-2-3	PostRock*2//P8810-B5B3A2A2 (Sr39+Lr35)/2*2174	2	TrR	*Sr39*
U5937-4-2	Duster*2//P8810-B5B3A2A2 (Sr39+Lr35)/2*2174	3+ and 2–	10R and 35MSS	*Sr39*[#]
U5938-10-5	Fuller*2//P8810-B5B3A2A2 (Sr39+Lr35)/2*2174	2 and 3+	5R and 40S	*Sr39*[#]
U5941-1-6	Fuller*2//RL6088 (Sr40)/2*2174	3+	60S	Non-*Sr40*
U5942-10-1	PostRock*2//RL6088 (Sr40)/2*2174	2 and 3+	30S and 15MR	*Sr40*
U5947-1-3	Duster/3/2174//RL6088 (Sr40)/2*2174	2	5RMR	*Sr40*
U5948-11-1	2174*2//Sr22Tb/2*2174	12–	–	*Sr22*
U5950-11-2	KS05HW14*2/3/CnsSr32As/Lakin//KS05HW14	2 and 3+	5RMR and 40S	*Sr32*[#]
U5951-5-2	KS05HW14*2/3/Mq(2)5*G2919K(Sr35)/Lakin//KS05HW14	0	TrR	*Sr35*
U5952-5-4	KS05HW14*2/3/Mq(2)5*G2919K(Sr35)/Lakin//KS05HW14	3+	0 and 35S	*Sr35*[#]
U5954-1-5	KS05HW14*2/3/P8810-B5B3A2A2 (Sr39+Lr35)/Lakin//KS05HW14	3+ and 2	0 and 40S	*Sr39*[#]

[†]Seedling disease rating based on the scale by Stakman et al. (1962), wherein seedlings with low infection type (<3) were considered resistant to race TTKSK and those with a high infection type (≥3) were classified as susceptible.

[‡]Adult plant field rating of disease severity assessed using the modified Cobb scale (Peterson et al., 1948) and infection response (Roelfs et al., 1992) rated as trace to resistant (TrR), resistant (R), resistant to moderately resistant (RMR), moderately resistant (MR), moderately susceptible (MS), moderately susceptible to susceptible (MSS), or susceptible (S); S check had 80S rating.

[§]Results for markers *XcsSr2-SNP* (Sr2), *XcsIH81-BM* and *XcsIH81-AG* (Sr22), *Xsr26#43* and *XBE51879* (Sr26), *Xbarc55* (Sr32), *Xbarc51* (Sr35), *Xrwg27* (Sr39), and *Xsr39#22r* (Sr40). Other stem rust resistance genes could be present.

[¶]Data from Jin et al. (2007).

[#]Heterozygous for the stem rust resistance gene based on marker data.

Table 2. List of primers tested.

Stem rust gene	Primer name	Forward primer[†]	Reverse primer	Reference
Sr22	Wmc633	ACACCAGCGGGGATATTTGTTAC	GTGCACAAGACATGAGGTGGATT	Olson et al., 2010
Sr22	csIH81-BM	TTCCATAAGTTCCTACAGTAC	TAGACAAACAAGATTTAGCAC	Periyannan et al., 2011
Sr22	csIH81-AG	CTACCTCTGTCAATTTGAAC	GAAAAATGACTGTGATCGC	Periyannan et al., 2011
Sr22	Cfa2123	CGGTCTTTGTTTGCTCTAAACC	ACCGGCCATCTATGATGAAG	Khan et al., 2005
Sr26	Sr26#43	AATCGTCCACATTGGCTTCT	CGCAACAAAATCATGCACTA	Mago et al., 2005
Sr26	BE518379	AGCCGCGAAATCTACTTTGA	TTAAACGGACAGAGCACACG	Liu et al., 2010
Sr32	Stm773	AAACGCCCCAACCACCTCTCTC	ATGGTTTGTTGTGTTGTGTGTAGG	Dundas et al., 2007
Sr32	Barc55	GCGGTCAACACACTCCACTCCTCTCT	CGCTGCTCCCATTGCTCGCCGTTA	Yu et al., 2009
Sr35	Cfa2076	CGAAAAACCATGATCGACAG	ACCTGTCCAGCTAGCCTCCA	Babiker et al., 2009
Sr35	Barc51	CGCATGAGCAAACAAGCCAACAACT	CGGCCACAGCATCGGTTCTCCAAA	Yu et al., 2009
Sr39	Sr39#50s	CCAATGAGGAGATCAAAACAACC	TAGCAAGGACCAAGCAATCTTG	Mago et al., 2009
Sr39	Sr39#22r	AGAGAAGATAAGCAGTAAACATG	TGCTGTCATGAGAGGAACTCTG	Mago et al., 2009
Sr39	Rwg27	GCCTTGGTGGATTTTGTGAT	GCGCTTTCAGTACAGGGTTC	Niu et al., 2011
Sr39	Rwg28	AGAGCCTGGGACTGTTGCTA	CAATGGCACTCTTCAAAGCA	Niu et al., 2011
Sr39	Rwg29	CGGCTATTGCTCAAAGAAGG	TGTTTCTGTCAGAGGCAACG	Niu et al., 2011
Sr39 Sr40	Wmc474	ATGCTATTAAACTAGCATGTGTCG	AGTGGAAACATCATTCCTGGTA	G. Bai and P. St. Amand, personal communication, 2012; Wu et al., 2009
Sr40	Wmc344	ATTTCAGTCTAATTAGCGTTGG	AACAAAGAACATAATTAACCCC	Wu et al., 2009
Sr40	Wmc661	CCACCATGGTGCTAATAGTGTC	AGCTCGTAACGTAATGCAACTG	Wu et al., 2009
Sr40	Wmc477	CGTCGAAAACCGTACACTCTCC	GCGAAACAGAATAGCCCTGATG	Wu et al., 2009
Sr40	Gwm374	ATAGTGTGTTGCATGCTGTGTG	TCTAATTAGCGTTGGCTGCC	Wu et al., 2009
Sr40	Gwm319	GGTTGCTGTACAAGTGTTCACG	CGGGTGCTGTGTGTAATGAC	Wu et al., 2009
Sr2	3B042G11	ACAAACACACCGCAAAAAG	TGTCATTGGTGCCTCAGC	McNeil et al., 2008
Sr2	3B028F08	ACGAACAAGGGGAAGACG	TTTCGGTAGTTGGGGATGC	McNeil et al., 2008
Sr2	Stm559TGAG	AAGGCGAATCAAACGGAATA	TGTGTGTGTGTGTGAGAGAGAG	Hayden et al., 2004
Sr2	csSr2	CAAGGGGTTGCTAGGATTGGAAAAC	AGATAACTCTTATGATCTTACATTTTTCTG	Mago et al., 2011a
	csSr2-SNP[‡]	AAGCTCTAATTTCTTTGGAATC		

[†]M13 tail-ACGACGTTGTAAAACGAC added to 5′-end of all forward primers except csSr2.

[‡]SNP, single nucleotide polymorphism.

Marker Analysis

Genomic DNA was extracted from leaf tissue (bulk of 3–5 seedlings per line) following a cetyltrimethylammonium bromide protocol (Yu et al., 2008). One single nucleotide polymorphism (SNP) marker for *Sr2* and 24 simple sequence repeat (SSR) markers were evaluated (Table 2). A 10 μL polymerase chain reaction (PCR) mix contained 1x NH_4 buffer (Bioline), 2.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphates, 50 nM forward M13-tailed primer, 50 nM M13-dye-labeled primer, 100 nM reverse primer, 100 ng DNA, and 1 unit *Taq* polymerase (Promega). Polymerase chain reaction was performed in a DNA Engine thermal cycler (Bio-Rad) using a touchdown program described by Sun et al. (2009). The thermal cycling conditions for SSR primer *Wmc633* consisted of an initial denaturation at 95°C for 5 min followed by 40 cycles of 96°C for 30 s, 48°C for 1 min, and 72°C for 1 min with a final extension step of 5 min at 72°C. *Xsr39#22r* and *Xrwgs* markers were run based on conditions described by Mago et al. (2009) and Niu et al. (2011), respectively. For the *Sr2* SNP marker, SNaPshot (Life Technologies) analysis was done following the protocol described by Bernardo et al. (2012). *Sr2* PCR was done at 58°C annealing temperature and single base extension at 56°C. Polymerase chain reaction products were mixed with Hi-Di formamide and GeneScan 120, 500, or 1200 Liz size standard (Applied Biosystems), depending on the expected fragment sizes of PCR. Electrophoresis was done on a 3730

DNA Analyzer (Applied Biosystems), and amplification products were scored using GeneMarker software (Soft Genetics, 2010). All band or peak sizes mentioned herein include the M13 tail added to each forward SSR primer during primer synthesis and one of the following dyes incorporated during the PCR reaction: 6-FAM, VIC, PET, or NED (Applied Biosystems).

RESULTS

Markers for *Sr22*

Sr22Tb is the donor of *Sr22* and together with accessions U5615-98-120-2 and U5616-20-154-7 were used as positive controls for *Sr22*. Sr22Tb contains a *Triticum monococcum* L. subsp. *aegilopoides* (Link) Thell. (syn. *Triticum boeoticum* Boiss.) fragment that carries the *Sr22* gene in chromosome 7AL (The, 1973). Four markers linked to *Sr22*, *Xcfa2123*, *Xwmc633*, *XcsIH81-BM*, and *XcsIH81-AG* (Khan et al., 2005; Olson et al., 2010; Periyannan et al., 2011), were tested. Marker *XcsIH81-BM* amplified a 257 bp amplicon from the 7AL segment of *T. monococcum* subsp. *aegilopoides* in the controls and three other resistant accessions (Table 1; Supplemental Table S1) whereas *XcsIH81-AG* amplified a 385 bp amplicon from the 7AL corresponding segment of *T. aestivum*, which is present in all susceptible accessions but not in the resistant accessions (null allele), suggesting

that this marker is diagnostic for *Sr22*. These two markers can be used together as codominant markers for *Sr22*. For marker *Xcfa2123*, the expected fragment size linked to *Sr22* is 254 bp, and it was present in all five resistant accessions except U5615–98–120–2 and one (U5941–1–6) of the 41 *Sr22* S accessions. This marker also did not amplify (null allele) in 18 accessions without *Sr22*. Marker *Xwmc633* amplified a 135 bp fragment in the *Sr22* controls and all other *Sr22*-carrying accessions and a 178 bp fragment in non–*Sr22*-carrying accessions. Three non–*Sr22* genotypes also amplified the 135 bp fragment observed in *Sr22* positive accessions in addition to the 178 bp fragment, but the peak height of the 135 bp fragment was at least seven times smaller than that of the 178 bp band (data not shown).

Markers for *Sr26*

The *Sr26* resistance gene was introgressed from *Thinopyrum ponticum* (Podp.) Barkworth & D. R. Dewey to chromosome 6AL of wheat accession WA-1 (Dundas et al., 2007). Two markers were evaluated for *Sr26* (Mago et al., 2005; Liu et al., 2010). Marker *Xsr26#43* amplified a 233 bp fragment from WA-1, and an amplicon did not appear (null allele) in the other accessions tested. In contrast, the S marker *XBE51879* amplified a 328 bp fragment from all non–*Sr26* accessions and a null allele in the positive control. Thus, a combination of these two dominant markers can be used as a codominant diagnostic marker for *Sr26*.

Markers for *Sr32*

CnsSr32As (which is probably the same line as C77.19 produced by E.R. Sears, USDA at University of Missouri, Columbia, MO, deceased) carries *Sr32* on a translocation, which is a relatively large alien fragment in wheat, from *Aegilops speltoides* Tausch to chromosome 2B (McIntosh et al., 1995). Two markers have been developed for this gene: *Xstm773* and *Xbarc55* (Dundas et al., 2007; Yu et al., 2009). *Xstm773* amplified a 209 bp fragment in CnsSr32As, U5950–11–2, and CS-Hope DS 3B. CS-Hope DS 3B is an *Sr2* resistant Chinese Spring accession with a 3B substitution from Hope; it does not carry any *A. speltoides* chromatin for *Sr32* resistance. Because CnsSr32As and CS-Hope DS 3B are both Chinese Spring derivatives, the amplification of the 209 bp fragment in both accessions implies that *Xstm773* is actually tagging Chinese Spring and not the alien fragment from *A. speltoides*. The codominant marker *Xbarc55* amplified a 128 bp band in the positive control and two bands (128 and 141 bp) in U5950–11–2, an accession that seems to be segregating for *Sr32* based on stem rust evaluation using Ug99 (Table 1). Three other accessions that were initially thought to have *Sr32* (U5926–2–8, U5926–3–4, and U5928–1–5) were negative for both *Sr32* markers and susceptible to TTKSK. The most common *Xbarc55* band sizes observed in non–*Sr32* accessions were 141 and 149 bp.

Markers for *Sr35*

The *Sr35* resistance gene from *Triticum monococcum* L. was transferred to chromosome 3AL of Mq(2)5★G2919K (Zhang et al., 2010). Two markers, *Xbarc51* and *Xcfa2076*, were reported to link to the gene (Babiker et al., 2009; Yu et al., 2009). *Xbarc51* is a codominant marker and homozygous (236 bp band) in the positive control, U5930–13–5, U5931–3–1, and U5951–5–2 and heterozygous in other accessions carrying *Sr35* (U5930–11–3, U5932–2–4, and U5952–5–4). In addition to the 236 bp band, a 327 bp band was amplified in the latter accessions; the 327 bp band was the most common band observed in non–*Sr35* genotypes including recipient parents 'Duster' (PI 644016), 'Fuller' (PI 653521), and KS05HW14-1. Other band sizes observed in genotypes lacking *Sr35* were 245, 249, and 325 bp. Accessions U5930–13–5, U5931–3–1, and U5951–5–2 were resistant to Ug99 based on stem rust phenotypes whereas the disease rating for accessions U5930–11–3, U5932–2–4, and U5952–5–4 ranged from R to MS-to-S and appears to be heterogeneous and segregating for *Sr35* (Table 1). Marker *Xcfa2076* amplified a 207 bp fragment in the donor parent and all other accessions with *Sr35* except U5952–5–4. *Xcfa2076* allele sizes observed in S accessions were 88, 143, 150, 155, 160, 211, and 213 bp. Moreover, no amplification (null allele) was observed in eight non–*Sr35* samples, including 'Cedar', 'CJ', Sr22Tb, and P8810–B5B3A2A2.

Markers for *Sr39*

P8810–B5B3A2A2, a chromosome 2B recombinant containing an *A. speltoides* fragment (Friebe et al., 1996; Knox et al., 2000), is the donor for *Sr39*. Three markers were developed for this gene: *Xsr39#50s*, *Xsr39#22r*, and *Xwmc474* (Mago et al., 2009; P. St. Amand, personal communication, 2011). Markers *Xsr39#50s* and *Xsr39#22r* are dominant for *Sr39* susceptibility and resistance, respectively. The expected band size is 259 bp for *Xsr39#50s* and 818 bp for *Xsr39#22r*. CnsSr32As (*Sr32*) and RL6088 (*Sr40*) exhibited the same banding pattern as P8810–B5B3A2A2 for both markers and is therefore a false positive for *Sr39*. *Xwmc474* amplified a 171 bp band in all five accessions known to have *Sr39* (P8810–B5B3A2A2, U5935–2–3, U5937–4–2, U5938–10–5, and U5954–1–5) based on stem rust evaluations (Table 1) and a smaller fragment (133–158 bp) in non–*Sr39* accessions. In addition, this marker also generated a 156 or 158 bp amplicon in three *Sr39*-carrying accessions (U5937–4–2, U5938–10–5, and U5954–1–5), which suggests *Sr39* heterozygosity. These genotyping results are consistent with stem rust phenotypic data where U5935–2–3 was clearly resistant whereas U5937–4–2, U5938–10–5, and U5954–1–5 appear to be segregating (R to S disease rating) for *Sr39*.

Three new markers, *Xrwgs27*, *Xrwgs28*, and *Xrwgs29*, were recently developed for *Sr39* using Red River Wheat Germplasm (RWG) accessions that carry a reduced-size
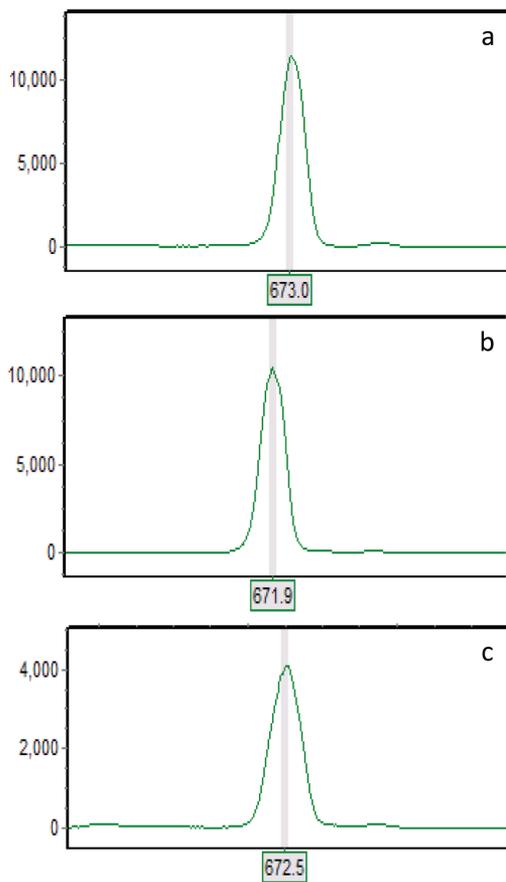
Figure 1. Electropherograms showing the amplification product of *Sr39* marker *Xrwgs27* in (a) P8810-B5B3A2A2, (b) CnsSr32As, and (c) equal ratio of P8810-B5B3A2A2 and CnsSr32As DNA. The *x*-axis shows the fragment size in base pairs (including an 18 bp VIC-dye-labeled M13 primer tail), and the *y*-axis represents peak signal intensity in relative fluorescence units.

Sr39 alien fragment in a wheat background (Niu et al., 2011). *Xrwgs27* is a codominant marker and was able to differentiate *Sr39*-resistant accessions (673 bp) from genotypes lacking *Sr39* (426, 665, 666, 672, 677, and/or 683 bp) and heterozygotes (673 bp plus another band). P8810-B5B3A2A2 and U5935-2-3 were resistant homozygotes based on *Xrwgs27* marker data whereas U5937-4-2, U5938-10-5, and U5954-1-5 were heterozygous; the *Xrwgs27* marker data support the TTKSK bioassay results. The 673 bp amplicon in *Sr39*-resistant accessions is only 1 bp bigger than that of CnsSr32As, a Chinese Spring accession with *Sr32* in the same wheat chromosome 2B (Fig. 1). Rwgs28 primers amplified three to four fragments with the 433 bp band tagging the susceptible allele and 458 bp band for the resistance allele; however, two accessions (CnsSr32As and RL6088) without the *Sr39* gene also amplified the same banding pattern as the *Sr39* donor P8810-B5B3A2A2. *Xrwgs29* did not show polymorphism between accessions with or without *Sr39* tested in this study. In addition, *Xwmc474* developed for the larger alien fragment generated a 145 bp amplicon in the RWG accessions with a shortened *Sr39* alien segment instead of a 171 bp band amplified in the accessions with a larger alien fragment, which suggests that this marker is located in an *A. speltoides* translocation that is now absent in the RWG accessions. This 145 bp band is similar in size to that generated in the Chinese Spring derivatives CS-Hope DS 3B and WA-1; the RWG accessions have Chinese Spring background (Niu et al., 2011).

Markers for *Sr40*

Sr40 in RL6088 is located in chromosome 2BS and originated from *Triticum timopheevii* (Zhuk.) Zhuk. subsp. *armeniacum* (Jakubz.) Slageren (Dyck, 1992). RL6088 still contains a large alien segment, so linkage drag is expected. Six markers linked to *Sr40* (Wu et al., 2009) were tested. *Xgwm319* amplified a 195 bp band not only in the donor line RL6088 but also in many non–*Sr40*-carrying genotypes such as Fuller, 'Lakin' (PI 617032), 'PostRock', KS05HW14-1, 'Thunder CL', and Duster. Similarly, for marker *Xwmc344*, many accessions were false positives for *Sr40*, including PostRock, WA-1, and *Sr2* donor lines. The 154 bp fragment amplified by *Xwmc474* in RL6088 was also observed in the non–*Sr40* accessions PostRock, 'Art', 'Aspen', and Thunder CL. *Xgwm374* generated a 232 bp fragment in RL6088, U5942-10-1, U5947-1-3, and '2174' (GSTR 12101). Because U5942-10-1 and U5947-1-3 have RL6088 and 2174 in their pedigrees, we cannot ascertain whether these accessions have *Sr40* resistance based on this marker. *Wmc477* primers amplified one to three bands. The expected band associated with the resistance allele of *Sr40* is 182 bp. Mq(2)5★G2919K and *Sr22*-carrying accessions Sr22Tb, U5924-10-1, and U5924-10-6 were false positive for this marker. In addition, scoring for this band is sometimes complicated by stutter bands in other fragments of bigger size. The last marker tested was *Xwmc661*, and it generated one to four bands in all tested accessions. A 190 bp band was amplified in RL6088, but this band was absent in two other *Sr40* accessions (U5942-10-1 and U5947-1-3).

Interestingly, *Xsr39#22r*, a dominant marker developed for *Sr39*, showed good association with *Sr40*. This marker generated an 820 bp band (Fig. 2) in *Sr40*-carrying RL6088, U5942-10-1, and U5947-1-3 and an 818 bp band or no band (null allele) in others including U5941-1-6, a non–*Sr40* accession based on TTKSK bioassay. U5941-1-6 consistently showed the Fuller (recipient parent) alleles and not the expected *Sr40* resistance alleles in all markers tested, so this line does not have *Sr40* based on genotypic and phenotypic data.

Markers for *Sr2*

Unlike the other previously described resistance genes in this study, *Sr2* is a non-race-specific adult plant resistance gene (Spielmeyer et al., 2003). *Sr2* originated from *T. turgidum* subsp. *dicoccum* and was transferred to wheat chromosome 3BS (McFadden, 1930). *Sr2* alone is not enough to protect wheat
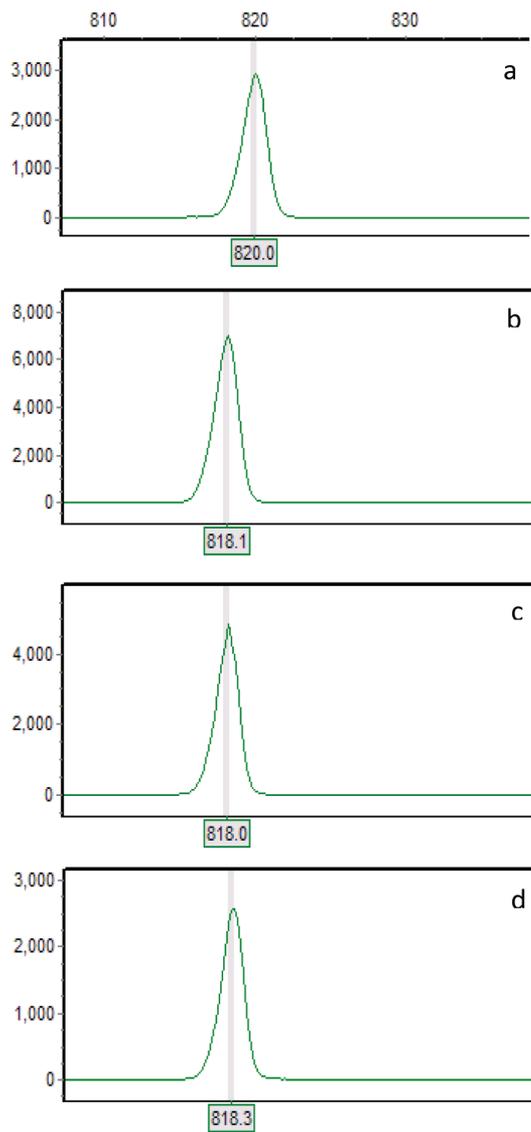
Figure 2. Electropherograms showing the amplification product of marker *Xsr39#22r* in (a) RL6088, (b) P8810-B5B3A2A2, (c) Red River Wheat Germplasm 1 (RWG1), and (d) CnsSr32As. The *x*-axis shows the fragment size in base pairs (including an 18 bp VIC-dye-labeled M13 primer tail), and the *y*-axis represents peak signal intensity in relative fluorescence units. The 820 bp fragment in RL6088 is associated with *Sr40* resistance.
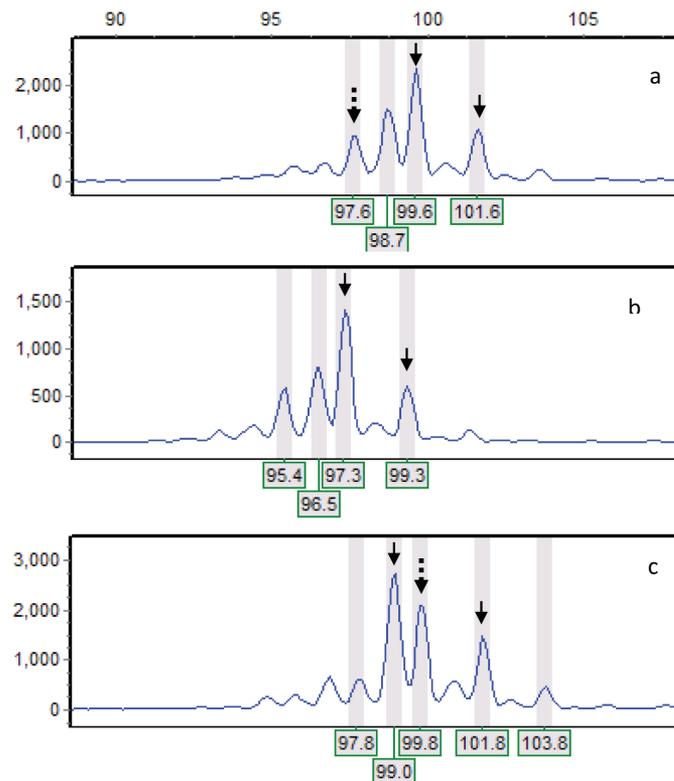

Figure 3. Electropherograms showing the amplification products of marker *Xstm559TGAG* in *Sr2* resistant control CS-Hope DS 3B (a), *Sr2* susceptible Billings, PI 646843 (b), and 2174 (c). The *x*-axis shows the fragment sizes in base pairs (including an 18 bp FAM-dye-labeled M13 primer tail), and the *y*-axis represents peak signal intensity in relative fluorescence units. The solid arrows indicate the two major peaks in each panel. The broken arrow in (a) points to a stutter peak in CS-Hope DS 3B that is similar in size to the 97.3 bp susceptibility peak in Billings; the one in (c) indicates a plus-A peak that is similar in size to the 99.6 bp peak in the resistant control. The presence of stutter and plus-A peaks complicates data scoring.

from losses due to stem rust, but it enhances resistance when combined with other stem rust resistance genes (Spielmeyer et al., 2003, Singh et al., 2011). Four markers, *XcsSr2*, *X3B042G11*, *X3B028F08*, and *Xstm559TGAG*, were reported for the gene (Hayden et al., 2004; McNeil et al., 2008; Mago et al., 2011a). Two wheat accessions, 'Hope' and CS-Hope DS 3B, are known to carry *Sr2* and were used as positive controls. The expected *X3B042G11* fragment size in *Sr2* positive accessions is 172 bp, but non–*Sr2* accessions have amplicons larger than 172 bp. The dominant marker *X3B028F08* amplified a 260 bp fragment in non–*Sr2* accessions and a null allele in those with *Sr2*. *X3B042G11* and *X3B028F08* genotyped 18 and 32 accessions as *Sr2* false positive, respectively, including

2174, Aspen, Cedar, Duster, RL6088, and 'Tiger'. Marker *Xstm559TGAG* amplified several alleles and was difficult to score. The expected fragment sizes for this marker were 98 and 100 bp for non–*Sr2* accessions and 100 and 102 bp for *Sr2* positive accessions; however, the amplification of stutter bands inherent in SSR markers and the tendency of *Taq* polymerase to add an additional nucleotide (usually A) at the 3′-end of PCR products (plus-A peaks) complicated data scoring (Fig. 3). Non–*Sr2* accessions Aspen, 'Hitch' (PI 655954), P8810-B5B3A2A2, Tiger, and U5947-1–3 showed the same banding pattern as the *Sr2* positive controls and are therefore false positives. Marker *XcsSr2* is a cleaved amplified polymorphic site (CAPS) marker (Mago et al., 2011a) that we converted into a SNP single base extension assay. Both positive controls and Thunder CL showed the expected 'A' allele for *Sr2* whereas 32 non–*Sr2* accessions showed the 'G' allele, which indicates the absence of *Sr2*. The other 12 non–*Sr2* accessions did not have an amplification product; among them were Art, CJ, 'Everest', PostRock, Tiger, Sr22Tb, WA-1, and CnsSr32As.

DISCUSSION

Markers for Ug99–effective resistance genes *Sr2*, *Sr22*, *Sr26*, *Sr32*, *Sr35*, *Sr39*, and *Sr40* were evaluated for usefulness in MAS of HWW using wheat resistance gene donors, recently released U.S. HWW varieties, and advanced elite breeding lines. Markers that worked well across a wide range of accessions include *XcsIH81-BM* and *XcsIH81-AG* for *Sr22*, *Xsr26#43* and *XBE51879* for *Sr26*, *Xbarc55* for *Sr32*, *Xbarc51* for *Sr35*, *Xrwg27* for *Sr39*, *Xsr39#22r* for *Sr40*, and *XcsSr2-SNP* for *Sr2*. These markers showed a high level of polymorphism between the resistant accession or accessions and a wide range of U.S. HWW breeding materials without the target genes and therefore are diagnostic markers for these genes.

In this study, some accessions had a mixed rating of R to MS-to-S to TTKSK in both greenhouse and field conditions and were clearly segregating based on Ug99 stem rust evaluations. Codominant markers were able to detect these accessions as heterozygous. *Xbarc51*, *Xbarc55*, and *Xrwg27* were good codominant markers for *Sr35*, *Sr32*, and *Sr39*, respectively. *XcsIH81-BM* coupled with *XcsIH81-AG* also can be used as codominant markers for *Sr22* as well as markers *Xsr26#43* and *XBE51879* for *Sr26*. These results mean that these codominant markers will be useful in screening for the presence of heterozygotes during early breeding generations. In contrast, the marker associated with *Sr40* (*Xsr39#22r*) is dominant and cannot distinguish heterozygotes from homozygotes. These results will help breeders to select parents for crosses, use the best markers to predict stem rust resistance genes in germplasm lines, and stack several resistance genes in a single cultivar to improve durability of stem rust resistance in HWW.

The resistance genes *Sr32*, *Sr39*, and *Sr40* are all located in chromosome 2B, and each is probably in a nonrecombining linkage block. *Sr40* is a translocation from the G genome of *T. timopheevii* subsp. *armeniacum* whereas *Sr39* and *Sr32* were derived from the S genome of *A. speltoides*. The B and G genomes of polyploid wheat are closely related to the S genome of *A. speltoides* (Dvorak and Zhang, 1990; Talbert et al., 1991), and the presence of a large alien translocation segment in *Sr40* may explain why *Xsr39#22r* can be used to select for *Sr40*. The target PCR product of *Xsr39#22r* is 820 bp for *Sr40*, which can be visualized easily in agarose gels if the samples being genotyped do not have *Sr39*; otherwise, the 818 bp band for *Sr39* cannot be distinguished from the 820 bp fragment for *Sr40* without a high-resolution system that can differentiate DNA fragments with a 2 bp difference. Likewise, for *Xrwg27* the amplified product in the *Sr39* donor is only 1 bp larger than that of the *Sr32* donor (CnsSr32As); therefore, a high-resolution fragment analysis system and careful data scoring are necessary for the separation of *Sr39* from *Sr32* when *Xrwg27* is used.

A CAPS marker for *Sr2* was successfully converted into a SNP marker. This marker was the best marker for *Sr2*

based on our results. The disadvantage of this marker is that it behaves as a dominant marker in cases where some other resistance gene donors or some recently released HWW varieties are used as elite parents because this marker showed a null allele instead of a 'G' allele in these accessions. However, all accessions with the null allele in this study were *Sr2* susceptible, which is consistent with the report by Mago et al. (2011a), which found that most Australian lines also had the null allele. This result suggests that non-*Sr2* accessions can have either the 'G' or null allele for this SNP marker; therefore, this marker is suitable for predicting *Sr2*.

Because most of the markers tested in this study were developed using a specific mapping population, not all of the markers worked well across the HWW accessions tested. Several markers showed polymorphism between the resistant accessions and most but not all of the susceptible accessions. These markers will remain useful for a limited number of breeding populations if they are polymorphic between the parents. For example, *Xgwm374* may be used as an alternate marker for *Sr40* and will give reliable results as long as line 2174 is not in the pedigree of any parent because it is monomorphic between the *Sr40* donor, RL6088, and 2174. However, polymorphism was detected between RL6088 and other non–*Sr40* accessions tested in this study. Similarly, markers *Xsr39#50*, *Xsr39#22r*, and *Xrwg28* for *Sr39* are also polymorphic between the *Sr39* donor and other non–*Sr39* accessions except two accessions with other resistance genes in 2B (CnSSr32As and RL6088).

Although several markers were reported as tightly linked to target resistance genes in a specific population in previous studies, they were not diagnostic when in different backgrounds. Those markers gave false positives in different accessions without target resistance genes and are therefore not recommended for detecting the presence of target resistance genes and MAS. Those markers include *X3B042G11*, *X3B028F08*, and *Xstm559TGAG* for *Sr2*, *Xcfa2123* for *Sr22*, *Xstm773* for *Sr32*, *Xrwg29* for *Sr39*, and *Xwmc344*, *Xwmc477*, *Xgwm319*, and *Xwmc661* for *Sr40*.

In summary, closely linked markers to all stem rust resistance genes studied (*Sr2*, *Sr22*, *Sr26*, *Sr32*, *Sr35*, *Sr39*, and *Sr40*) were identified and validated. Validated markers for the seven genes worked well across the U.S. HWW accessions used in this study. These markers should be useful in stacking different resistance genes to develop wheat cultivars with durable stem rust resistance against Ug99 and its variants and in MAS of those resistance genes in HWW breeding materials. Marker-assisted analysis of these resistance genes is important to U.S. breeders because they cannot directly evaluate resistance to Ug99 and associated foreign races in their breeding. Using molecular markers in pyramiding two to three genes of *Sr24*, *Sr26*, *Sr31*, and *SrR* has been reported (Mago et al., 2011b). *Sr22*, *Sr26*, and *Sr35* confer resistance to Ug99 and other important races (Singh et al., 2011), and breeding efforts to pyramid these genes are already underway.

Supplemental Information Available

Supplemental material is included with this manuscript.

Supplemental Table S1. Applicon sizes (bp) of markers for stem rust resistance genes in different hard winter wheat varieties.

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