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ORIGINAL PAPER

Fine mapping and characterization of *Sr21*, a temperature-sensitive diploid wheat resistance gene effective against the *Puccinia graminis* f. sp. *tritici* Ug99 race group

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Abstract

Key message **The diploid wheat stem rust resistance gene *Sr21* confers temperature-sensitive resistance to isolates of the Ug99 group and maps to the middle of the long arm of chromosome 2A^m.**

Abstract A race of *Puccinia graminis* f. sp. *tritici*, the causal pathogen of stem rust of wheat, known as Ug99, and its variants, are virulent to plants carrying stem rust resistance genes currently deployed in most wheat cultivars worldwide. Therefore, identification, mapping and deployment of effective resistance genes are critical to reduce this threat. Resistance gene *Sr21* identified in diploid wheat *T. monococcum* can be effective against races from the Ug99 race group, but both susceptible and partial resistant reactions have been reported in previous studies. To clarify this conflicting information we screened four monogenic lines with *Sr21* and four susceptible controls with 16 *Pgt* isolates including five isolates of the Ug99 race group under three different temperatures and three different photoperiods. We observed that, temperature influences the interaction between monogenic lines with *Sr21* and Ug99 race group isolates, and may be one source of previous inconsistencies. This result indicates that, although *Sr21* confers partial resistance against Ug99, its effectiveness can be modulated by environmental conditions and should not be deployed alone. Using two large diploid wheat-mapping populations (total 3,788 F_2 plants) we mapped *Sr21* approximately 50 cM from the centromere on the long arm of chromosome 2A^m within a 0.20 cM interval flanked by sequence-based markers *FD527726* and *EX594406*. The closely linked markers identified in this study will be useful to reduce the *T. monococcum* segments introgressed into common wheat, accelerate *Sr21* deployment in wheat breeding programs, and facilitate the map-based cloning of this gene.

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S. Chen, M. N. Rouse and W. Zhang contributed equally to this work.

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Introduction

Puccinia graminis f. sp. *tritici* (*Pgt*), the causal agent of wheat stem rust, has historically caused significant yield losses worldwide. Stem rust has been controlled over the past several decades by deploying resistance genes, eliminating the alternate host of *Pgt* in some areas, and monitoring the pathogen population for potentially dangerous races. The deployed resistance genes, including several

from wheat wild relatives, have provided adequate resistance for the last several decades.

Unfortunately, an isolate of *Pgt* was reported in Uganda in 1999 (Ug99) that is virulent to plants carrying the wheat resistance genes currently deployed in most of the large wheat growing regions of the world (Pretorius et al. 2000; Singh et al. 2006). This isolate was classified as race TTKSK according to the North American system for *Pgt* race nomenclature (Jin et al. 2007, 2008). The unique virulence combination of race TTKSK allows this race to overcome resistance in the majority of wheat cultivars in Asia (Singh et al. 2008), the United States (Jin and Singh 2006), and Canada (Fetch 2007) and is considered a threat to global wheat production. Subsequent monitoring of *Pgt* in Kenya identified race TTKSK variants with additional virulence to plants carrying resistance genes *Sr24*, *Sr36*, and *Sr9h* (Jin et al. 2008, 2009; Pretorius et al. 2012; Rouse et al. 2014). Genes *Sr24* and *Sr36* are relatively widespread in the southern United States (Jin and Singh 2006) increasing the threat of the Ug99 race group. Races from the Ug99 group subsequently spread to Yemen, Iran, and South Africa (Nazari et al. 2009; Pretorius et al. 2010).

The identification, mapping and deployment of resistance genes effective to the Ug99 race group are critical to reduce this new threat. Two stem rust resistance genes effective to race TTKSK have been recently cloned in diploid species *Aegilops tauschii* (D genome, *Sr33*, Periyannan et al. 2013) and *Triticum monococcum* (A^m genome, *Sr35*, Saintenac et al. 2013). However, additional genes are required to diversify the combinations of deployed resistance genes to minimize the risks associated with the deployment of limited sources of resistance.

Stem rust resistance gene *Sr21* was identified in diploid wheat *T. monococcum* and transferred to hexaploid wheat (*T. aestivum*) chromosome 2A (The 1973). The gene was mapped 2.4 cM distal to the centromere on the long arm of chromosome 2A in hexaploid wheat by using monosomic analyses and telocentric mapping (McIntosh and Bennett 1979). However, this genetic distance needs to be considered with caution because chromosomes of *T. monococcum* and *T. aestivum* are known to recombine poorly in the presence of the *Ph1b* gene in hexaploid wheat (Dubcovsky et al. 1995). The position of *Sr21* relative to mapped molecular markers was not known.

Conflictive or inconclusive results were reported regarding the effectiveness of *Sr21* resistance against race TTKSK (Jin et al. 2007). Accession 'Einkorn' (CI 2433) and other *T. monococcum* accessions were postulated to have *Sr21* based on their reduced infection in response to race TTKSK relative to susceptible controls (Rouse and Jin 2011b). Subsequent genetic analyses determined those *Sr21* gene postulations were accurate, confirming the reduced infection of race TTKSK isolate 04KEN156/04 to plants carrying *Sr21* (Rouse and Jin 2011a). Variable reactions of race TTKSK and related races to monogenic lines with *Sr21* were reported by different laboratories in the United States, Canada, and South Africa (Jin et al. 2007; Visser et al. 2011; Pretorius et al. 2010). Since the *Sr21* resistance gene is a part of the North American differential set, these variable reactions affect the five-letter designation of the Ug99 race group isolates. For example, isolates would be classified as race TTKSK if high infection is observed on plants carrying *Sr21*, and as race PTKSK if low infection is observed on plants carrying *Sr21*. In addition, it has previously been reported that the degree of resistance conferred by *Sr21* varies with ploidy level (McIntosh and Bennett 1979).

The first objective of this study was to identify the cause of the inconsistent reactions of Ug99 race group isolates observed in response to *Sr21*. We hypothesized that, temperature and photoperiod may affect the level of resistance of *Sr21* to the Ug99 race group. The second objective was to generate a precise map of *Sr21* to accelerate its deployment in breeding programs, assist in the reduction of the *T. monococcum* chromosome segment introgression in hexaploid wheat, and facilitate the cloning of *Sr21*. We hypothesized that the use of *T. monococcum* mapping populations will eliminate the distortions in the recombination rates known to occur between *T. monococcum* and *T. aestivum* chromosomes in hexaploid wheat (Dubcovsky et al. 1995).

Materials and methods

Lines used to test the effect of temperature and photoperiod

Eight wheat lines were used in this study for *Sr21* evaluation (Table 1). Chinese Spring (CSA), LMPG-6, W2691, and PI 272557 were used as susceptible checks. Stem rust susceptible line LMPG was used as recurrent parent (Knott 1990). LMPG-6 is a selection of LMPG available at the USDA-ARS Cereal Disease Laboratory. W2691 was highly susceptible to most races of the wheat stem rust pathogen and was frequently used to propagate this pathogen (Watson and Luig 1963; Rowell 1984). PI 272557 was characterized as an accession of *T. monococcum* ssp. *monococcum* with susceptibility to five races of *Pgt* (Rouse and Jin 2011b). CI 2433 (PI 10474) is the *T. monococcum* subsp. *monococcum* genetic stock for *Sr21*, and was referred to as 'Einkorn' in Stakman et al. (1962). Monogenic lines *T. monococcum* derivative, *T. monococcum* deriv./8*LMPG and W3586 (Table 1) were all proved to possess the same dominant gene *Sr21* derived from their diploid parents by using genetic and pathological tests (The 1973). Four to six plants of each line were inoculated with 16 isolates of *Pgt* corresponding to 14 races (Table 2).

Table 1 *Sr21* monogenic lines and susceptible controls used to test the effect of temperature and photoperiod

Line	Genome	Pedigree	Resistance gene	References
Chinese Spring	Hexaploid	–	None	–
T. monococcum derivative	Hexaploid	W1569/C68-123//Chinese Spring	*Sr21*	The (1973) Roelfs and McVey (1979)
LMPG-6	Hexaploid	Prelude/8*Marquis	None	Knott (1990)
T. monococcum deriv./8*LMPG	Hexaploid	*T. monoc.* deriv./8*LMPG	*Sr21*	Knott (1990)
W2691	Hexaploid	Little Club//Gabo/Charter	None	Watson and Luig (1963)
W3586	Hexaploid	*T. durum* W304/*T.monococcum* cv. Einkorn//unknown hexaploid	*Sr21*	The (1973)
PI 272557	Diploid	–	None	Rouse and Jin (2011b)
CI 2433	Diploid	–	*Sr21*	Stakman et al. (1962)

Table 2 Avirulence/virulence formulae for isolates of *Puccinia graminis* f. sp. *tritici* used in this study. *Sr21* is underlined

Isolate[a]	Race[b]	Origin	Avirulence	Virulence
04KEN156/04	TTKSK[a]	Kenya	24 36 Tmp	5 6 7b 8a 9a 9b 9d 9e 9g 10 11 17 30 31 38 McN
09KEN09-2	TTKSK	Kenya	24 36 Tmp	5 6 7b 8a 9a 9b 9d 9e 9g 10 11 17 30 31 38 McN
09TAN06-2	TTKSK	Tanzania	24 36 Tmp	5 6 7b 8a 9a 9b 9d 9e 9g 10 11 17 30 31 38 McN
06KEN19V3	TTKST	Kenya	36 Tmp	5 6 7b 8a 9a 9b 9d 9e 9g 10 11 17 24 30 31 38 McN
07KEN24-4	TTTSK	Kenya	24 Tmp	5 6 7b 8a 9a 9b 9d 9e 9g 10 11 17 30 31 36 38 McN
06YEM34-1	TRTTF	Yemen	8a 24 31	5 6 7b 9a 9b 9d 9e 9g 10 11 17 21 30 36 38 McN Tmp
01MN84A-1-2	TTTTF	USA	24 31	5 6 7b 8a 9a 9b 9d 9e 9g 10 11 17 21 30 36 38 McN Tmp
74MN1409	TPMKC	USA	6 9a 9b 24 30 31 38	5 7b 8a 9d 9e 9g 10 11 17 21 36 Tmp McN
06ND717C	QFCSC	USA	6 7b 9b 9e 11 24 30 31 36 38 Tmp	5 8a 9a 9d 9g 10 17 21 McN
99KS76A-1	RKQQC	USA	9e 10 11 17 24 30 31 38 Tmp	5 6 7b 8a 9a 9b 9d 9g 21 36 McN
75ND717C	QTHJC	USA	7b 9a 9e 24 30 31 36 38 Tmp	5 6 8a 9b 9d 9g 10 11 17 21 McN
77ND82A-1	RCRSC	USA	6 8a 9e 11 24 30 31 38 Tmp	5 7b 9a 9b 9d 9g 10 17 21 36 McN
07WA140-17-1	QCCLL	USA	6 7b 8a 9b 9d 9e 10 11 30 31 36 38 Tmp McN	5 9a 9g 17 21 24
75WA165-2A	QCCSM	USA	6 7b 8a 9b 9e 11 30 31 36 38 Tmp	5 9a 9d 9g 10 17 21 24 McN
09ID73-2	SCCSC	USA	6 7b 8a 9b 11 24 30 31 36 38 Tmp	5 9a 9d 9e 9g 10 17 21 McN
59KS19	MCCFC	USA	6 8a 9a 9b 9d 9e 11 21 24 30 31 36 38	5 7b 9g 10 17 Tmp McN

[a] Five isolates of the Ug99 race group were collected from three different locations. Isolates 04KEN156/04, 06KEN19V3, and 07KEN24-4 were derived from *Pgt* collections obtained at an inoculated stem rust screening nursery in Njoro, Kenya during 2004, 2006, and 2007, respectively. Isolate 06KEN19V3 was derived from a collection from *Sr24* isogenic line LcSr24Ag. Isolate 09KEN09-2 was derived from a *Pgt* collection from an unknown susceptible plant in a commercial field of wheat variety 'Kwale' in the Rift Valley province of Kenya in 2009. Isolate 09TAN06-2 was derived from a bulk *Pgt* collection from 10 wheat varieties from a fungicide trial in the Monduli district of Tanzania in 2009

[b] *Sr21* is not included in the formulae for Ug99 group isolates because of variation at different temperatures

Mapping populations

The two segregating populations used to map resistance gene *Sr21* in diploid wheat were derived from crosses between the susceptible *T. monococcum* ssp. *monococcum* PI 272557 described above and Ug99-resistant accessions DV92 and G3116. DV92 has a spring growth habit (Bullrich et al. 2002) and is postulated to carry both *Sr21* and *Sr35* (Zhang et al. 2010; Rouse and Jin 2011a). G3116 is a wild winter *T. monococcum* subsp. *aegilopoides* (Dubcovsky et al. 1996) that was postulated to contain resistance gene *Sr21* (Zhang et al. 2010).

To map *Sr21* without the interference of *Sr35* we used *Pgt* race MCCFC, which is virulent to plants carrying *Sr35* but shows low infection types on plants carrying *Sr21*. For the initial map we evaluated 204 F_2 plants from the cross of PI 272557 × DV92 and their corresponding $F_{2:3}$ families (~25 plants per family) with MCCFC. Evaluations were performed at 24 °C with a 16 h photoperiod. For the construction of the high-density map, we screened 3,584 F_2 individuals (734 plants in population PI 272557 × DV92 and 2,850 plants in population PI 272557 × G3116) with *Sr21*-flanking markers *gwm312* and *CJ653447*. Evaluations with MCCFC were performed only for the progeny

of plants carrying recombination events in the critical *Sr21* region (~25 plants per family).

Stem rust assays

Resistance evaluations were performed at the USDA-ARS Cereal Disease Laboratory. Four to six plants of each line were inoculated with 16 isolates of *Pgt* corresponding to 14 races (Table 2) in two different biological replicates: one in 2009 and one in 2011. Five isolates of the Ug99 race group were evaluated. In addition, we evaluated one isolate characterized as producing a low infection type on lines with *Sr21* (race MCCFC) and 10 isolates characterized as producing high infection on lines carrying *Sr21*. Of the five Ug99 race group isolates, three were evaluated in both years, and the other two were evaluated in only 1 year (09KEN09-2 and 09TAN06-2, in 2009 and 2011, respectively).

Procedures for inoculation were reported previously (Rouse et al. 2011). We studied the effect of temperature (16, 20 and 24 °C) and of photoperiod (10, 15 and 20 h photoperiod) on *Sr21* resistance to different *Pgt* races. The different temperatures were compared at the intermediate photoperiod (15 h, long-day photoperiod) and the different photoperiods were compared at the intermediate temperature (20 °C, effective *Sr21* resistance to Ug99 in preliminary experiments). After inoculation, plants were kept in one of five growth chamber environments with the following combinations of temperature and photoperiod: 16 °C/15 h, 20 °C/15 h, 24 °C/15 h, 20 °C/10 h and 20 °C/20 h. Infection types were scored 14 days after inoculation according to Stakman et al. (1962). For the genetic mapping of *Sr21*, plants with infection types ranging between ';1' and '2+' (similar to DV92, G3116, and Einkorn) were classified as resistant and those with infection types between '3−' to '4' (similar to PI 272557) were classified as susceptible. Based on previous studies, the repeatability of the infection type scores was estimated to be higher than 95 % (Rouse and Jin 2011b).

To allow statistical analyses of infection types, we converted the Stakman infection types to a 0–9 linear scale (Zhang et al. 2011). Briefly Stakman ITs 0, 1^-, 1, 1^+, 2^-, 2, 2^+, 3^-, 3, and 3^+ were coded as 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9, respectively. Stakman et al. ITs ';' and '4' were considered equal to '0' and '9', respectively, for the purpose of this linearized conversion. The effect of *Sr21* was calculated for each genetic background, isolate, and growth chamber treatment by subtracting the mean linearized infection type of *Sr21* monogenic lines from the mean linearized infection type of the corresponding susceptible check with similar genetic background. To test if the effect of *Sr21* differed among the isolates in the five treatments, the means of the *Sr21* effects across backgrounds were compared using Tukey's honest significant difference (HSD) test. Calculation of means and Tukey's HSD were performed in R (R Development Core Team 2011).

Molecular markers

Wheat genomic SSR markers previously mapped on chromosome 2AL and in the centromeric region of 2AS were screened for polymorphisms among the three parental lines (DV92, G3116 and PI 272557). The primer sequences of these SSR markers were obtained from GrainGenes database (http://wheat.pw.usda.gov). Polymorphic markers were then used to test both F_2 populations. Wheat genes orthologous to the *Brachypodium* and rice genes present in the region colinear with the wheat *Sr21* region were identified and PCR primers were developed. The PCR amplification products were then sequenced to identify sequence polymorphisms among the parents.

PCR were performed in Applied Biosystems® GeneAmp® PCR System 9700 (Applied Biosystems Inc., Foster City, CA) in volumes of 20 μL containing 1 unit *Taq* DNA polymerase, 2 μL 10× buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μL of each primer (10 μM), and 50–100 ng template DNA. The PCR conditions were as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 50–60 °C for 30 s (depending on primers), 72 °C for 1 m and a final extension for 7 m at 72 °C. PCR products were then separated in 6 % non-denaturing acrylamide gels or 1 % agarose gels and stained directly with ethidium bromide.

Linkage map construction

The genetic linkage map of *Sr21* was constructed using the software MapMaker 3.0b (Lander et al. 1987) at a LOD threshold of 3.0. The initial marker order was established using the COMPARE, ORDER and MAP commands and additional markers were then added using the TRY command. Finally, a genetic linkage map was drawn with the software MapDraw V2.1 (Liu and Meng 2003).

Results

Sr21 resistance at different temperatures and photoperiods

The infection types of the eight wheat lines in response to the 16 *Pgt* isolates in the five treatments are displayed in Supplementary Table S1. In the 16 °C treatment, race TTKSK produced high infection types on the *Sr21* lines except for CI 2433 where an intermediate infection type was observed (Fig. 1a). In the four other treatments, race TTKSK produced low infection types on the *Sr21* monogenic lines with occasional intermediate reactions observed on line *T. monococcum* derivative (Fig. 1b). Race MCCFC,

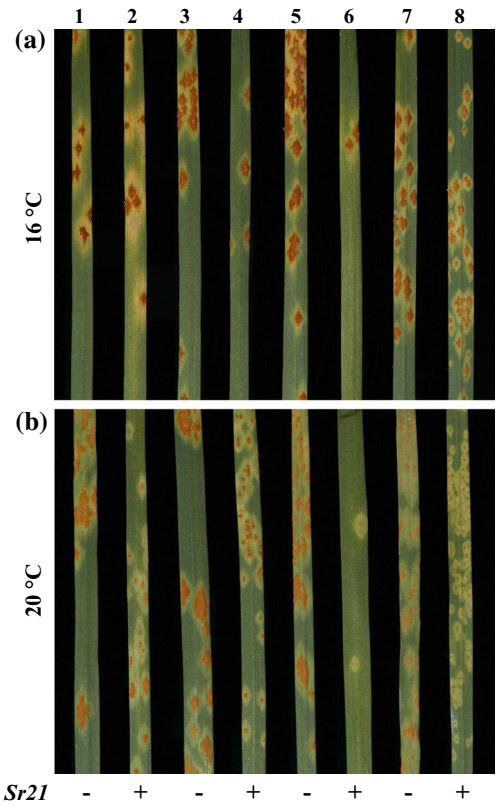


Fig. 1 Reaction to *Puccinia graminis* f. sp. *tritici* race TTKSK of **1** Chinese Spring; **2** *T. monococcum* Deriv.; **3** LMPG-6; **4** *T. monococcum* Deriv./8*LMPG; **5** W2691; **6** W3586; **7** PI 272557; and **8** CI 2433 at **a** 16 °C and **b** 20 °C, both with a 15 h photoperiod

was avirulent to plants carrying *Sr21* except for the 16 °C treatment, where obvious avirulence was present only on CI 2433. The ITs for race MCCFC in this treatment were intermediate on *T. monococcum* Deriv./8*LMPG and W3586 and high on *T. monococcum* Deriv. Overall, the disease reaction between race TTKSK and related races to the *Sr21* monogenic lines and susceptible controls resembled that of race MCCFC, which results in reduced infections in plants carrying *Sr21*.

Figure 2 shows in a quantitative way that resistance conferred by *Sr21* to the Ug99 race group was significantly higher than isolates previously characterized as virulent to plants carrying *Sr21* (Fig. 2b–e). The exception to this was the 16 °C treatment, where lines carrying *Sr21* were as susceptible to the Ug99 race group isolates as to the isolates know to be virulent to plants carrying *Sr21* (Fig. 2a). *Sr21* sometimes exhibited a stronger resistance to race MCCFC than to the Ug99 race group, particularly in the long and short photoperiod treatments. In the 20 °C, 15 h photoperiod treatment, *Sr21* exhibited significantly greater resistance to race TTTSK than to race TTKSK. In all other treatments there was no significant difference in the effectiveness of *Sr21* among isolates of the Ug99 race group. The *Pgt* isolates previously characterized as virulent to plants carrying *Sr21*, all conferred high reactions to the different *Sr21* monogenic plants in the different treatments with one exception: the disease response on CI 2433 was often intermediate with ITs such as '2+3' to *Sr21*-virulent isolates.

There were three inconsistencies between the two replications of the seedling assays. First, *Pgt* race QCCLL was avirulent on LMPG-6 and *T. monococcum* Deriv./8*LMPG in all treatments of the first replication, but not in the second replication. Second, race QCCSM was avirulent to LMPG-6 and *T. monoc.* Deriv./8*LMPG in the 16 °C treatment in the first replication, but not the second. Third, race QCCLL was avirulent on both W2691 and W3586 in the 24 °C treatment only in the first replication. These three inconsistencies account for only 2.5 % of the phenotypic data-points used in our analyses and did not affect the conclusions of the study. All of these departures from the expected high reactions are likely independent of *Sr21* since both the *Sr21* genetic stock and recurrent parent exhibited unexpected ITs.

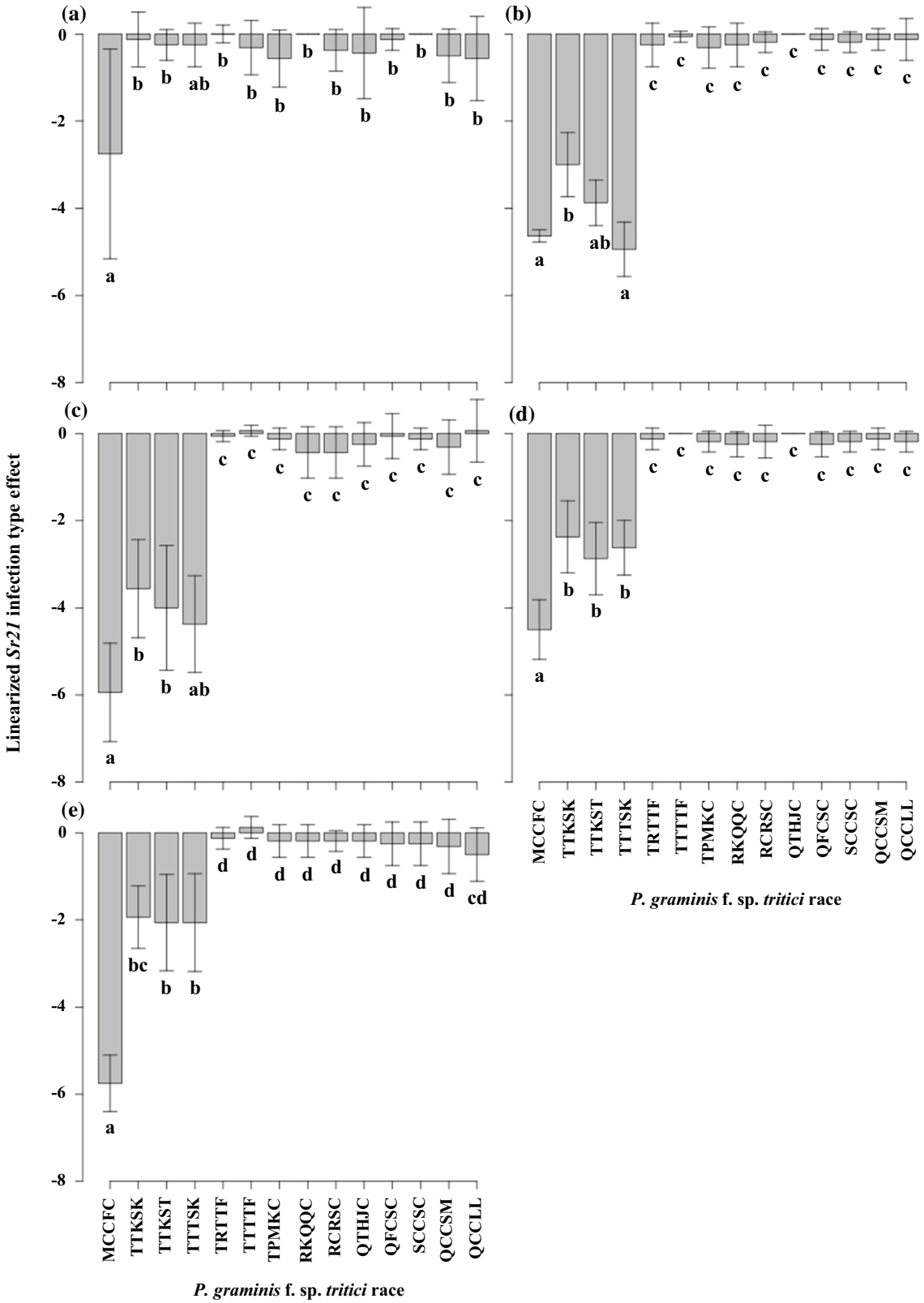
Microsatellite marker analyses and development of sequenced-based markers

Sr21 was initially mapped on the long arm of chromosome $2A^m$ using a subset of 204 F_2 individuals from population PI 272557 × DV92. Sixty SSR markers previously mapped on chromosome arm 2AL and near the centromere in the GrainGenes database were used to screen for polymorphisms. After testing the F_2 segregating population, six markers, *wmc453*, *gwm448*, *gwm356*, *gwm249*, *wmc407* and *gwm312*, were linked to *Sr21* or to each other (Fig. 3a; Table 3).

To increase the marker density in the *Sr21* region and to provide sequence-based markers for comparative genomics studies, we selected several EST sequences mapped to this region of chromosome 2AL for marker development. Four sequence-based markers, *BE406908*, *BF473744*, *CJ653447* and *BG313738* (Table 3), were mapped and shown to be linked to *Sr21* (Fig. 3a).

High-density genetic map

Screening of 734 F_2 plants from the PI 272557 × DV92 population and 2,850 F_2 plants from the PI 272557 × G3116 population yielded 203 plants with recombination events between *Sr21*-flanking markers *gwm312* and *CJ653447*. Four plants from PI 272557 × G3116 carrying recombination events in the targeted region (1.97 %) did not survive, so the phenotyping and genotyping was based on the progeny of 199 F_2 plants. The same proportion was used



to adjust the total number of gametes of this population to avoid distortions in the calculation of the genetic distances (from 5,700 to 5,588).

We then established the colinearity of this region with the orthologous region in the *Brachypodium* genome to generate additional markers. *CJ653447*, the

◀**Fig. 2** Reduction in linearized infection type (IT) produced by the *Sr21* lines to various isolates of *P. graminis* f. sp. *tritici* at **a** 16 °C, 15 h photoperiod; **b** 20 °C, 15 h photoperiod; **c** 24 °C, 15 h photoperiod; **d** 20 °C, 10 h photoperiod; and **e** 20 °C, 20 h photoperiod. Bars with different letters are significantly different among isolate means based on Tukey's Honest Significant Difference test. Values in the *Y* scale were calculated by subtracting linearized ITs (0, 1, 2, 3, 4, 5, 6, 7, 8, and 9) of the susceptible checks (Chinese Spring, LMPG-6, W2691, and PI 272557) from the linearized ITs of monogenic lines with *Sr21* (*T. monococcum* derivative, *T. monoc.* Deriv./8*LMPG, W3586, and CI 2433, respectively) averaged across genetic backgrounds. Values close to 0 represent no difference between monogenic lines for *Sr21* and susceptible checks and more negative values represent increasing resistance in the *Sr21* monogenic lines compared to the susceptible checks

closest sequence-based marker to *Sr21* (2.4 cM, Fig. 3a), was orthologous to gene *Bradi5g22430* on *Brachypodium* chromosome 5. Out of the 22 *Brachypodium* genes selected from this region, nine were successfully converted into additional wheat markers (Table 3) and mapped to the *Sr21* region. The wheat orthologs to *Bradi5g21960* (*HX077369*) and *Bradi5g22040* (*BJ305745*) were mapped in the PI 272557 × DV92 population proximal to the flanking marker *gwm312* outside of the candidate region for *Sr21* (Fig. 3b) and therefore, were not mapped in the PI 272557 × G3116 population (Fig. 3c). The other seven wheat orthologs of *Bradi5g22070* (*CK210684*), *Bradi5g22090* (*FD527726*), *Bradi5g22200* (*EX594406*), *Bradi5g22210* (*BJ233471*), *Bradi5g22230* (*EG377826*), *Bradi5g22280* (*CJ566493*) and *Bradi5g22340* (*HX186716*) were used to genotype the 199 lines with recombination events between *gwm312* and *CJ653447* and to construct a high density map of the *Sr21* region (Fig. 3c). Based on this map, *Sr21* was mapped 0.15 cM distal to *FD527726* and 0.05 cM proximal to *EX594406*.

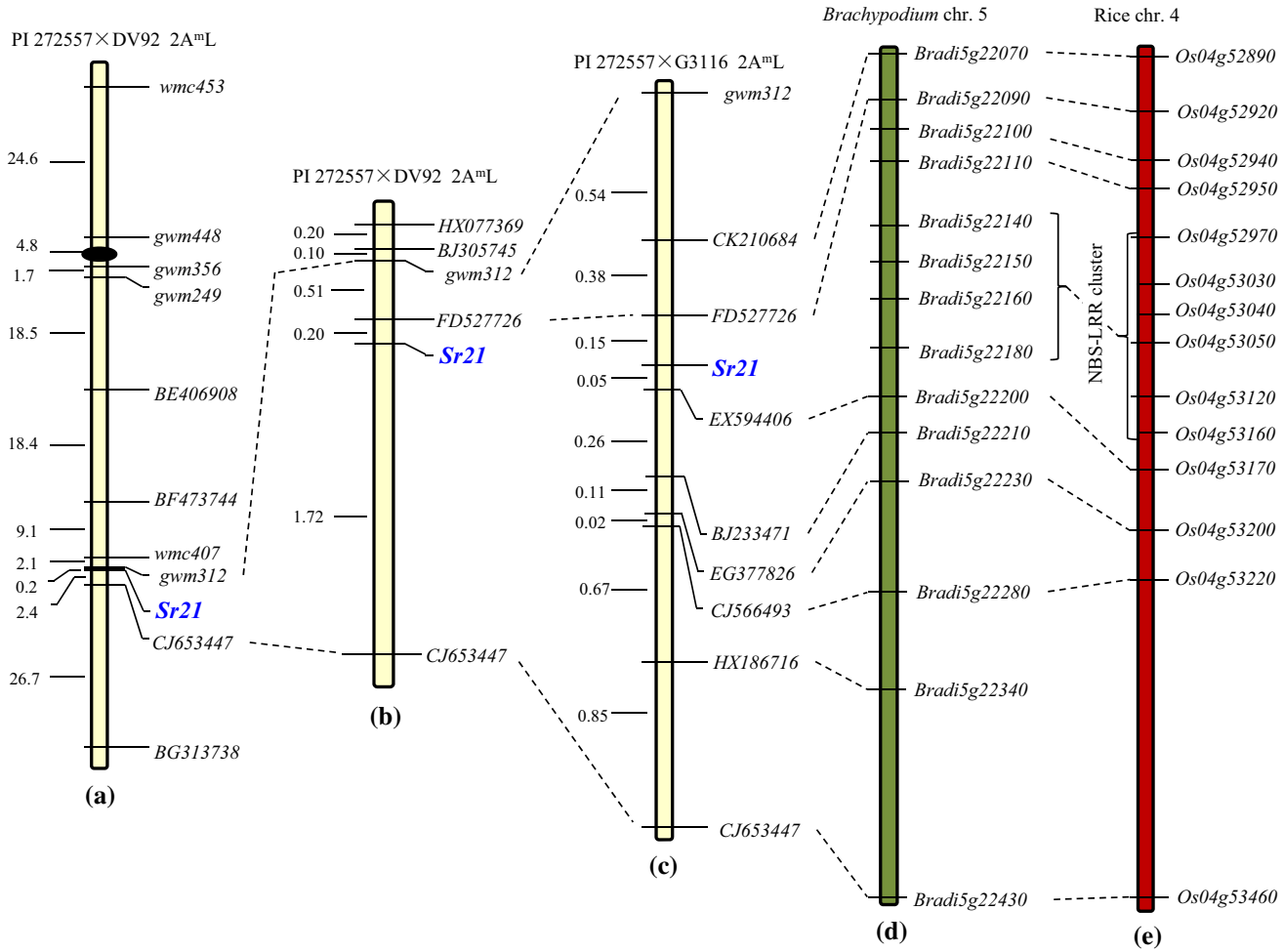


Fig. 3 Genetic maps for stem rust resistance gene *Sr21* and its comparison with genomic regions of *Brachypodium* and rice. **a** Initial map from PI 272557 × DV92 (204 F_2 plants); **b** high-density map from PI 272557 × DV92 (734 F_2 plants); **c** high-density map of PI 272557 × G3116 (2,850 F_2 plants); **d** colinear genomic region in *Brachypodium* chromosome 5; **e** colinear genomic region in rice chromosome 4. *Brackets* indicate groups of linked NBS-LRR genes

Table 3 Markers in *Sr21* region. Band sizes correspond to the *T. monococcum* PI 272557 allele followed by the DV92 or G3116 resistant allele (between parentheses)

Markers	Marker type	Forward primer (5′–3′)	Reverse primer (5′–3′)	Restriction enzyme	Ann. T (°C)	Expected size (bp) susceptible (resistant)
Markers in population PI 272557 × DV92						
wmc453	SSR	ACTTGTGTCCATAACCGACCTT	ATCTTTTGAGGTTACAACCCGA	–	61	187 (181)
gwm448	SSR	AAACCATATTGGGAGGAAAGG	CACATGGCATCACATTTGTG	–	60	135 (131)
gwm356	SSR	AGCGTTCTTGGGAATTAGAGA	CCAATCAGCCTGCAACAAC	–	55	150 (154)
gwm249	SSR	CAAATGGATCGAGAAAGGGA	CTGCCATTTTTCTGGATCTACC	–	55	210 (220)
BE406908	dCAP	GAGGGAGTAACCTTGATTTGCA	CAATCTTCTTCARTTGAGCTTCAAT	*Ssp*I	58	114 (91)
BF473744	dCAP	GGTATCCATTTTTTTGTTCTGGGTT	AAAACTTTGTTCACTCATGCCAAGTA	*Sca*I	58	124 (101)
wmc407	SSR	GGTAATTCTAGGCTGACATATGCTC	CATATTTCCAAATCCCCAACTC	–	61	135 (133)
HX077369	dCAP	AAATTTCACATGTA-CAAATAATATAACTCA	TTTGTAATAGATGTTTAGCTGGAAC	*Dde*I	55	150 (123)
BJ305745	dCAP	ATTGGTTTGGTTAATACTCTCT	AAAAATAAAATCTACTCCCTCTGGT	*Ava*II	54	240 (246)
gwm312	SSR	ATCGCATGATGCACGTAGAG	ACATGCATGCCTACCTAATGG	–	60	180 (182)
FD527726	CAP	CGGCATCAATAGGAGAAGA	TAGGATACGTGACCCAGGA	*BsrD*I	53	806 (754)
CJ653447	CAP	AACTAACCTACGCCCAATAA	TGAGCCATCAACTCCTTTC	*CviQ*I	52	1,193 (620)
BG313738	dCAP	CTGACTGCGCCTTATGTTGA	GTGCCCATGGCTTGATGGAGCCG	*Sca*II	56	392 (413)
Markers in population PI 272557 × G3116						
gwm312	SSR	ATCGCATGATGCACGTAGAG	ACATGCATGCCTACCTAATGG	–	60	180 (176)
CK210684	CAP	GCGTCAAGAACAAGTCCAAGC	ATCTGCACCTCCGCCACCT	*BaeG*I	57	600 (835)
FD527726	Indel	CGGCATCAATAGGAGAAGA	TAGGATACGTGACCCAGGA	–	53	806 (750)
EX594406	CAP	TCAACAACTTCAACAAGGC	AACAAGAGAACGAGCATCG	*Xmn*I	52	420 (566)
BJ233471	Indel	AAATCAAGAACCACAGAAC	CAATATCAATCAAGCAGAA	–	51	751 (701)
EG377826	Indel	TAACTCGTCCTCCCAGCAC	GAATAGCACATTTGAAACTCTCC	–	55	1,350 (1208)
CJ566493	CAP	GAAGATGGCGAGTAAGGGG	GCTTTCACAAAAGCTAGATAAGGT	*Acl*I	53	909 (720)
HX186716	Indel	AACATTTGGAGCTGTGTTG	GAAACCAGTGGCTTGTGAT	–	49	295 (324)
CJ653447	CAP	AACTAACCTACGCCCAATAA	TGAGCCATCAACTCCTTTC	*CviQ*I	52	1,193 (620)

CAP cleavage amplification polymorphism, *dCAP* degenerate cleavage amplification polymorphism, *Indel* insertion/deletion, *SSR* simple sequence repeat, *Ann. T* annealing temperature

Table 4 Flanking markers *FD527726* and *EX594406* of *Sr21* are colinear with a region of *Brachypodium* chromosome 5 including 12 genes. Annotations of 12 *Brachypodium* genes, corresponding wheat ESTs, and rice genes

Brachypodium[a]	Wheat EST	Annotation in *Brachypodium* (putative function)	Rice gene[b]
Bradi5g22090	*FD527726*	Uncharacterized protein	*Os04g52920*
Bradi5g22100	*CD887748*	Serine/threonine-protein phosphatase 6 regulatory subunit 3	*Os04g52940*
Bradi5g22110	*CJ961291*	Uncharacterized protein	*Os04g52950*
Bradi5g22120	–	Transcript 'BRADI5G22120.1' not found	–
Bradi5g22130	*HX199130*	Uncharacterized protein	(*Os04g33780*)[c]
Bradi5g22140[d]	*HX125831*	Fragment of XP_003581680, similar disease resistance protein RGA2-like	*Os04g52970*[d] *Os04g53030*[d] *Os04g53040*[d] *Os04g53050*[d] *Os04g53120*[d] *Os04g53160*[d]
Bradi5g22150[d]	*CJ564170*	Fragment of XP_003581680, similar disease resistance protein RGA2-like	
Bradi5g22160[d]	*CJ564170*	Pseudogene, similar disease resistance protein RGA2-like	
Bradi5g22180[d]	*CJ564170*	Pseudogene, similar disease resistance protein RGA2-like	
Bradi5g22170	*EX594406*	Transcription factor GTE7-like	*Os04g53170*
Bradi5g22190	–	Transcript 'BRADI5G22190.1' not found	–
Bradi5g22200	*EX594406*	Transcription factor GTE7-like	*Os04g53170*

[a] *Brachypodium* genome: http://www.modelcrop.org/

[b] Rice genome: http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/

[c] Most similar rice gene outside *Sr21* region

[d] Clusters of NBS-LRR genes. No clear orthologous relationships could be established

Sr21 colinear regions in other cereal genomes

To define the colinear regions in *Brachypodium* (Fig. 3d) and rice (Fig. 3e), we first identified orthologs to the flanking markers *FD527726* and *EX594406*. The proximal marker *FD527726* was orthologous to *Bradi5g22090* on *Brachypodium* chromosome 5 and to *Os04g52920* on rice chromosome 4. The distal marker *EX594406* was orthologous to *Brachypodium* gene *Bradi5g22200* and rice gene *Os04g53170* (Fig. 3d, e).

The orthologs to wheat *Sr21* flanking markers defined regions of 88 kb in *Brachypodium* chromosome 5 (Bd5: 24573330– 24661358) and 138 kb in rice chromosome 4 (Chr4: 31527861– 31665411). The 88 kb region in *Brachypodium* chromosome 5 includes twelve putative genes (*Bradi5g22090*– *Bradi5g22200*), 10 of which have orthologs in the colinear region in rice chromosome 4 (Table 4). The order of these genes was relatively conserved between rice and *Brachypodium* and their annotations are summarized in Table 4. These *Brachypodium* and rice genes include several typical resistance genes encoding NBS-LRR domains. In *Brachypodium*, *Bradi5g22140* and *Bradi5g22150* seem to encode complete proteins whereas *Bradi5g22160* and *Bradi5g22180* are partial sequences that correspond to genes with premature stop codons, and are likely pseudogenes. In rice, six complete NBS-LRR resistance proteins (*Os04g52970*, *Os04g53030*, *Os04g53040*, *Os04g53050*, *Os04g53120* and *Os04g53160*) were found in the corresponding genomic region.

Discussion

Effect of temperature and photoperiod on *Sr21* resistance to Ug99

Our data indicate that the disease reaction of different isolates of the Ug99 race group and *Sr21* monogenic lines is temperature sensitive. The difference in infection types from 16 to 20 °C (15 h photoperiod) was obvious, representing a range between high infection types to low infection types, respectively (Fig. 1). Also, the reaction was influenced by genetic background, as exemplified by the slightly higher reactions observed on *T. monococcum* deriv. compared to the other monogenic lines against isolates from the Ug99 race group.

The infection types of isolates from the Ug99 race group were similar to race MCCFC in most treatments, demonstrating that the Ug99 race group could be considered avirulent or virulent on plants carrying *Sr21* depending on the environmental conditions used for testing. Indeed, *Sr21* was temperature-sensitive for both MCCFC and Ug99 race group isolates, although the latter races tended to confer slightly higher infection types than race MCCFC. Previous studies of *Sr21* (Roelfs and McVey 1979) did not report temperature sensitivity. It is possible, that the previous study used *Pgt* isolates that were less temperature-sensitive than the isolates used in this study, since slight differences in avirulence among races have been reported previously (Watson and Luig 1968). Similar to our study, environmental effects on virulence were observed on the temperature- and light-sensitive gene *Sr6*, although the effect of

temperature was in the opposite direction (Forsyth 1956). In our study, the effect of photoperiod was small compared with the effect of temperature, but slight differences in IT were detected under different photoperiods at 20 °C. In particular, the reduction in linearized infection types to races of the Ug99 group between lines with and without *Sr21* was smaller under 20 h than under 15 h photoperiod (Fig. 2e, note that reduction in linearized ITs to race TTKSK is not significantly different from race QCCLL under this photoperiod). This suggests that *Sr21* may be less effective against the Ug99 race group at very long photoperiods (20 h).

Isolates previously classified as races TTKSK, TTKST, and TTTSK would be better classified in our study as races PTKSK, PTKST, and PTTSK, respectively, based on their relative low infections to *Sr21* under certain environments and in certain *Sr21* lines (e.g. in *T. monococcum* lines). However, since hexaploid wheat lines carrying only *Sr21* show relatively high levels of infection in the field in Kenya, we are hesitant to propose a formal change in the TTKSK nomenclature. Pretorius et al. (2010) discriminated among isolates of the Ug99 race group on the basis of reaction to *Sr21* (at temperatures ranging from 18 to 25 °C). However, isolates classified as both high (TTKSK) and low (PTKSK) infection on lines with *Sr21* in that study exhibited relatively low infection types on the diploid wheat accession CI 2433 (‘2’ and ‘;1’, respectively). In addition, diverse isolates including those previously classified as virulent on lines with *Sr21* displayed intermediate reactions to CI 2433 in the 16 °C treatment suggesting that this line may carry additional resistance gene(s) that confer partial resistance to *Pgt* under low temperatures. Additional genetic studies will be required to identify these genes.

High density mapping of *Sr21* and identification of candidate genes using comparative genomics approaches

Among the nineteen SSR and sequenced-based markers mapped on chromosome $2A^m$ in our *T. monococcum* populations, two are located in the short arm and seventeen in the long arm defining the position of the centromere within a 4.8 cM interval between markers *gwm448* (short arm) and *gwm356* (long arm). Using this information, *Sr21* was mapped 50.0 to 54.8 cM from the centromere, a much larger genetic distance than the 2.4 cM reported earlier (McIntosh and Bennett 1979). This difference can be explained by the different genetic backgrounds used in the two studies. In our study, *Sr21* was mapped in crosses among diploid *T. monococcum* accessions, where no restrictions on recombination are expected. By contrast, McIntosh and Bennett (1979) used a cross between two hexaploid lines, one of which carried the $2A^mL$ introgression segment with *Sr21*. Previous studies have shown that recombination between the A and A^m genome chromosomes is greatly reduced in the presence of the *Ph1b* gene (Dubcovsky et al. 1995; Zhang et al. 2010). The absence of restriction in recombination, the large segregating population used in the construction of the high-density map, and the high level of polymorphism found in the PI 272557 × G3116 cross (cultivated x wild type diploid wheat) allowed us to define a relatively small candidate region for *Sr21* of only 0.20 cM.

The orthologs for the flanking markers of the wheat *Sr21* candidate region define an 88 kb region in *Brachypodium* chromosome 5 and a 138 kb region in rice chromosome 4, which are known to be colinear with wheat chromosome 2 (The *Brachypodium* consortium initiative, 2010). Sequencing studies have shown that wheat is more closely related to *Brachypodium* than to rice (Kellogg 2001; Faricelli et al. 2010), so we prioritized the analysis of the 10 *Brachypodium* genes present between *Bradi5g22090* and *Bradi5g22200* (Fig. 3d). The two flanking genes are excluded as candidates for *Sr21* since their respective wheat orthologs are separated from *Sr21* by eight (*FD527726*) and three recombination events (*EX594406*) (Fig. 3c). *Bradi5g22120* and *Bradi5g22190* have no longer identifiers present in the Ensambl Plant Database (http://www.modelcrop.org/), suggesting that they corresponded to in silico predicted genes that were discarded in more recent annotations. This suggestion is reinforced by the absence of wheat ESTs or rice orthologs corresponding to these two *Brachypodium* hypothetical genes. For the *T. monococcum* orthologs of *Bradi5g22100*, *Bradi5g22110* and *Bradi5g22130*, we sequenced the complete coding regions and one kb of the promoter regions, but could not find any polymorphisms between DV92 and PI 272557. This result suggests that they are not good candidate genes for *Sr21*.

Bradi5g22140, *Bradi5g22150*, *Bradi5g22160* and *Bradi5g22180* are interesting candidate genes since they are annotated as typical plant disease resistance genes which encode proteins carrying a coiled-coil domain (predicted by COILS WEB site), a nucleotide-binding site (NBS, AAA superfamily), and leucine-reach repeat (LRR) domains (also referred as resistance gene analogs RGA). NBS-LRR genes are the most frequent class found among cloned disease resistance genes in cereals and other plant species (Feuillet et al. 2003; Huang et al. 2003; Periyannan et al. 2013; Saintenac et al. 2013). A cluster of NBS-LRR resistance genes was found also in the corresponding colinear region in rice suggesting that an ancient cluster of resistance genes is present in this chromosome region in grass genomes and is likely to be present in wheat.

Using the sequences of the four *Brachypodium* resistance genes, we identified four *T. aestivum* contigs on the long arm of chromosome 2A (IWGSC_2AL_contig 6323931, 6358826, 3612672, 6429167; http://wheat-urgi.versailles.inra.fr/Seq-Repository/BLAST). However, gene

deletions were observed in the resistant parent G3116 when sequencing PCR products from these four contigs. Moreover, we found no polymorphisms in the coding and regulatory regions of these genes between DV92 and PI 272557. These results suggest that they are unlikely candidate genes for *Sr21*. Clusters of NBS-LRR resistance genes are known to evolve at faster rates than other regions of plant genomes, with frequent duplications and deletions caused by ectopic recombination (Todd and Ronald 2000). Therefore, different resistance genes may be present in *T. monococcum* than in the colinear region in the A genome of *T. aestivum*, as described before for the NBS-LRR gene cluster including *Sr35* (Saintenac. et al. 2013). To test this hypothesis we are currently screening a BAC library from the resistant parent DV92 (Lijavetzky et al. 1999) using the closest molecular markers flanking *Sr21*. Once a physical map is completed, the region will be sequenced and annotated to identify the best candidate gene for *Sr21*.

Meanwhile, the markers linked to *Sr21* identified in this study can be used to deploy this gene in wheat breeding programs using marker-assisted selection (MAS). The *Sr21* flanking markers can be also used to reduce the size of the $2A^m$ chromosome segment introgressed into common wheat. Smaller introgressed segments can reduce linkage drag and the risk of unfavorable linked *T. monococcum* genes. However, since *Sr21* is temperature sensitive, susceptible to several *Pgt* races, and has limited effect to the Ug99 race group when deployed alone in hexaploid wheat, it is important to deploy *Sr21* in combination with other *Pgt* resistance genes. In spite of this limitation, *Sr21* can contribute an additional layer of resistance to several *Pgt* races and can help to diversify the sources of resistance deployed by wheat breeding programs.

Author contribution statement SC, MR, and WZ performed most of the experimental work and analyzed the data, EA, YJ and YW contributed ideas and materials, SC and MR wrote the first version of the manuscript and all authors reviewed the manuscript. MR designed the temperature and photoperiod sensitivity component of the project. JD designed and directed the mapping component of the project, contributed to data analysis, revised the manuscript and wrote its final version.

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Conflict of interest The authors declare that they have no conflict of interest.

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