Identification and characterization of \( \text{Sr22b} \), a new allele of the wheat stem rust resistance gene \( \text{Sr22} \) effective against the Ug99 race group

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Summary

Wheat stem (or black) rust, caused by \( \text{Puccinia graminis} \) f. sp. \( \text{tritici} \) (\( \text{Pgt} \)), has been historically among the most devastating global fungal diseases of wheat. The recent occurrence and spread of new virulent races such as Ug99 have prompted global efforts to identify and isolate more effective stem rust resistance (Sr) genes. Here, we report the map-based cloning of the Ug99-effective \( \text{SrTm5} \) gene from diploid wheat \( \text{Triticum monococcum} \) accession PI 306540 that encodes a typical coiled-coil nucleotide-binding leucine-rich repeat protein. This gene, designated as \( \text{Sr22b} \), is a new allele of \( \text{Sr22} \) with a rare insertion of a large (13.8-kb) retrotransposon into its second intron. Biologic transformation of an \( \sim 112\)-kb circular bacterial artificial chromosome plasmid carrying \( \text{Sr22b} \) into the susceptible wheat variety Feilder was sufficient to confer resistance to stem rust. In a survey of 168 wheat genotypes, \( \text{Sr22b} \) was present only in cultivated \( \text{T. monococcum} \) subsp. \( \text{monococcum} \) accessions but absent in all tested tetraploid and hexaploid wheat lines. We developed a diagnostic molecular marker for \( \text{Sr22b} \) and successfully introgressed a \( \text{T. monococcum} \) chromosome segment containing this gene into hexaploid wheat to accelerate its deployment and pyramiding with other \( \text{Sr} \) genes in wheat breeding programmes. \( \text{Sr22b} \) can be a valuable component of gene pyramids or transgenic cassettes combining different resistance genes to control this devastating disease.

Introduction

Wheat is an important cereal crop that contributes a substantial proportion of the calories and proteins consumed by humankind. Reducing yield losses inflicted by pathogens can contribute to grain yield improvements that are required to feed a growing world population. \( \text{Puccinia graminis} \) f. sp. \( \text{tritici} \) (\( \text{Pgt} \)), the causal agent of wheat stem rust (or black rust), has historically been a devastating fungal disease of tetraploid and hexaploid wheat. In the past, this pathogen was effectively controlled by growing resistant wheat varieties and eradicating alternate host (\( \text{Berberis vulgaris} \)) plants around cereal fields (Roelfs, 1982, 1985; Singh et al., 2015).

After the year 1998, this disease became a major concern again after the emergence and spread of the \( \text{Pgt} \) race TTKSK (Ug99) and its variants (henceforth the Ug99 race group), which were virulent on the majority of resistance genes deployed worldwide, including resistance genes \( \text{Sr31} \) and \( \text{Sr38} \) (Pretorius et al., 2000; Singh et al., 2006, 2011). In recent years, additional highly virulent \( \text{Pgt} \) races unrelated to Ug99, such as TKTTF, TKTTF and TTRTF (Olivera et al., 2012, 2015; Patpour et al., 2017; Tesfaye et al., 2020), have been detected in outbreaks in Africa (Olivera et al., 2015), Asia (Shamanin et al., 2016, 2018) and Europe (Bhattacharya, 2017; Olivera et al., 2017). Due to the threat of these new virulent \( \text{Pgt} \) races, there is an urgent need to identify and isolate new effective \( \text{Sr} \) genes to diversify the sources of resistance in wheat breeding programmes.

Over 60 stem rust resistance genes \( (\text{Sr1}–\text{Sr61}) \) have been assigned official designations (Chen et al., 2020, Zhang et al., 2020), among which a large proportion were introgressed from wild wheat relatives (Singh et al., 2015). The diploid wheat species \( \text{Triticum monococcum} \) (einkorn, genome \( \text{A}^\text{a} \)), comprising of the domesticated \( \text{T. monococcum} \) ssps. \( \text{monococcum} \) and the wild \( \text{T. monococcum} \) ssp. \( \text{agrioloides} \), is closely related to \( \text{T. urartu} \) (genome \( \text{A}^\text{b} \)), the donor of the A genome in polyploid wheat (Dvorak and McGuire, 1988). \( \text{T. monococcum} \) harbours several valuable rust resistance genes, including the leaf rust resistance genes \( \text{LiTm16} \) (Sodkiewicz and Strzembicka, 2008) and \( \text{Li63} \) (Kolmer and Anderson, 2010); the stripe rust resistance loci \( \text{QYrmt.pau-2A} \) and \( \text{QYrbl.pau-5A} \) (Chhuneja et al., 2008) and \( \text{Yr34} \) (Chen et al., 2017); and the stem rust resistance genes \( \text{Sr21} \) (Chen et al., 2015, The, 1973, Sr22...
Wheat gene Sr22b confers resistance to stem rust

The initial mapping of SrTm5 suggested that this gene was either a novel allele of Sr22 (TraesCS7A02G499600) or a tightly linked gene (Chen et al., 2018a). Since Sr22 is located on the long arm of chromosome 7A at 689.9 Mb (Chinese Spring RefSeq v1.0). The International Wheat Genome Sequencing Consortium, 2018), we developed Cleaved Amplified Polymorphic Sequence (CAPS) markers pkw4974 (689.9 Mb) and pkw5009 (688.2 Mb) (Table 1) flanking the Sr22 locus. Subsequently, we used these two markers to screen a population of 1132 plants (2264 gametes) from the cross PI 306540 × PI 272557, and we found 51 plants carrying recombination events within this region (2.7 Mb or 2.3 cM). Evaluations of progeny of these plants with race 34PKUSC confirmed that SrTm5 was located within this region. Using nine new markers spanning the 2.7 Mb (Table 1), we further delimited the SrTm5 candidate region to a 0.08-cM interval (140.4 kb). Cs RefSeq v1.0 coordinates) flanked by CAPS markers pkw4995 and pkw4999 (Figure 1b).

Only three complete genes (TraesCS7A02G499600, TraesCS7A02G499700 and TraesCS7A02G499800) were annotated in the Chinese Spring reference genome within this region (Figure 1a). To determine if additional genes were present in the orthologous region in T. monococcum, we screened the bacterial artificial chromosome (BAC) library of resistant parent PI 306540 using the two flanking markers (pkw4995 and pkw4999) and two markers completely linked to SrTm5 (TmSF3R4 and pkw4997). We obtained two overlapping BAC clones designated hereafter as Tm84C1 and Tm2677. Sequencing and annotation of these two selected BACs (Figure 1c; GenBank accession M237628) showed no additional genes in the SrTm5 candidate region in PI 306540 (146.5 kb) relative to Chinese Spring.

We designated the T. monococcum orthologues of Chinese Spring genes TraesCS7A02G499600, TraesCS7A02G499700 and TraesCS7A02G499800 as TmNLR1, TmPPR1 and TmFAR1, respectively. TmPPR1 encodes a protein containing pentatricopeptide repeat domains, whereas TmFAR1 encodes a far1-related sequence 5-like protein. We were not able to detect transcripts of these two genes in the leaves of SrTm5-resistant T. monococcum plants infected with Pgt (Figure S2), suggesting that these are unlikely candidate genes for SrTm5.

TmNLR1 is an orthologue of the cloned stem rust resistance gene Sr22 (TraesCS7A02G499600) (Steuernagel et al., 2016) and therefore an excellent candidate gene for SrTm5. In PI 306540, the TmNLR1 gene spans 19715 bp from start to stop codons, including the insertion of a 13.8-kb gypsy-like retrotransposon in the second intron (Figure 1d). Comparing the TmNLR1 genomic region with the full-length complementary DNA (cDNA) of TmNLR1, we determined that this gene contains four exons. The 2817 bp coding sequence encodes a typical CC-NBS-LRR protein containing 938 amino acids that were 95.7%–96.7% identical to six reported Sr22-resistant protein haplotypes (Figure S3).

Three lines of evidence support TmNLR1 as the best candidate for SrTm5. First, TmNLR1 is the only candidate gene that is expressed in infected leaves of the resistant parent. Second, the TmNLR1 allele from PI 306540 shares the diagnostic amino acids present in known Sr22-resistant alleles, whereas PI 272557 shares the diagnostic amino acids for the susceptible alleles (V381L, S605F/Y and G655D, BLOSUM62 scores = 1, −2 and −1, Table S2). Finally, sequencing of TmNLR1 in T. monococcum accession PI 277131-2, which was previously postulated to

(Gerechter-Amitai et al., 1971), Sr35 (McIntosh et al., 1984), Sr21 (Briggs et al., 2015) and Sr60 and SrTm5 (Chen et al., 2018a).

Triticum monococcum chromosomes can recombine with the A-genome chromosomes of polyploid wheat, particularly in the presence of the ph1b mutation (Dubcovsky and Luo, 1995). This feature has fuelled interest of scientists and breeders in the identification and isolation of stem rust resistance genes from this species and its transfer to commercial wheat cultivars. Among the six stem rust resistance genes derived from T. monococcum, four officially named ones (Sr21, Sr22, Sr25 and Sr60) have been successfully cloned and transferred into hexaploid wheat so far (Chen et al., 2020; Chen et al., 2018b; Saintenac et al., 2013; Steuernagel et al., 2016). The first three are Ug99-resistance genes encoding typical coiled-coil nucleotide-binding leucine-rich repeat (CC-NBS-LRR) proteins (Chen et al., 2018b; Saintenac et al., 2013; Steuernagel et al., 2016), whereas Sr60 encodes a different type of protein with two putative kinase domains (Chen et al., 2020).

Cultivated T. monococcum accession PI 306540 was identified as having a unique resistance response to five Pgt isolates (Rouse and Jin, 2011a, b), which was subsequently associated with the presence of stem rust resistance genes SrTm4, Sr21, Sr60 and SrTm5 (Briggs et al., 2015; Chen et al., 2018a, b). SrTm5 was previously mapped to the same region as Sr22 on the long arm of chromosome 7A, and showed good levels of resistance (IT = 2; to ≤1) to several Pgt races, including TTKSK, TTKS and MCCCFC (Chen et al., 2018a). Based on its mapped location and its different resistance profiles from Sr22, it was hypothesized that SrTm5 could be a novel allele of Sr22 or a tightly linked gene (Chen et al., 2018a).

In this study, we describe the map-based cloning of the stem rust resistance gene SrTm5, and confirm that it is a new allele of the cloned gene Sr22. SrTm5 was roughly 96% identical to the reported Sr22 proteins and showed a characteristic insertion of 13.8-kb retrotransposon in its second intron. We successfully introgressed a T. monococcum chromosome segment carrying SrTm5 into hexaploid wheat and developed a diagnostic molecular marker to accelerate its deployment in wheat breeding programmes.

Results

Assessment of stem rust responses

At the seedling stage, the SrTm5 monogenic line TmRS4-3 exhibited high levels of resistance (ITS = ; to ≤1) to Pgt races 34PKUSC, 34MTGSM and TTKSK, but was susceptible (ITS = 3+) to the other three races BCCBC, 21C3CTTMT and RTJM. By contrast, its sister line Tm5S7-57 without SrTm5 displayed susceptible infection types (ITS = 3+) to all the tested races (Figure S1a and Table S1). When inoculated with race 34PKUSC, selected F2 families from the SrTm5 segregating mapping population showed infection types that ranged from ≤1 to ≤3 in resistant plants, and from ≥3 to ≥4 in susceptible plants (Figure S1b).

To quantify the infected leaf area, we measured the percentage of the leaf area covered with Pgt pustules on six independent infected leaves of TmRS4-3 and TmS5-7 using the software ASSESS version 2. For SrTm5-avirulent races 34PKUSC, 34MTGSM and TTKSK, the average percentage was significantly lower (P < 0.001) in plants carrying SrTm5 than in those without the gene (Figure S1).
possess SrTm5 (Rouse and Jin, 2011a), confirmed the presence of a gene 100% identical to TmNLR1. Based on these results, we selected TmNLR1 for further functional characterizations.

Validation of TmNLR1 by transgenic complementation

To test if TmNLR1 was sufficient to confer resistance to Pgt, we transformed the Ug99-susceptible wheat variety Fielder with the PI 306540 circular BAC plasmid Tm84C1, which includes two transformed the Ug99-susceptible wheat variety Fielder with the transgene. All plants from T2 transgenic families (ranging from 1.3% to 9.2%) than in the non-transgenic plants close to a 1 : 1 segregation (Table S3). Taken together, these results suggest some segregation distortion against the transgenic line.

Transcript levels of TmNLR1 in all transgenic T1 families were significantly higher than in the susceptible control Fielder (P < 0.01), but only five of them (T1-Tm514, T1-Tm515, T1-Tm517, T1-Tm548 and T1-Tm554) were expressed at similar levels as in the introgression of the T. monococcum chromosome segment including SrTm5 into Fielder (positive control, see later) (Figure S4).

Roughly 25 T2 plants from each transgenic event and the untransformed control Fielder were challenged with Pgt race TTKSK (isolate 04KEN156/04). All plants from T2-Tm515-6 T2 plants perfectly co-segregated with the presence of the transgene (Figure S5). Measures of the percentage of leaf area covered by pustules was significantly lower in all transgenic T1 families were

Table 1 Primers used in the present study

| Marker         | ID in CS RefSeq v1.1 | Primer sequence 5′−3′ (Forward) | Primer sequence 5′−3′ (Reverse) | Size (bp) | Enzyme | Function       |
|----------------|----------------------|----------------------------------|----------------------------------|-----------|--------|----------------|
| pkw4974        | TraesCS7A02G497400   | GCCACTCCGAGCTGCCGCTCAAG         | ACCATTTCTCAGCCTGAGGTTC           | 619       | HaeIII | Fine mapping   |
| pkw4962        | TraesCS7A02G496200   | GTATGGAAATGAGAAGTTGAAAGAC        | CATATAAGAGCCCAAGAAAATGCCG         | 944       | MfeII  | Fine mapping   |
| pkw4984        | TraesCS7A02G498400   | CCACTTCCGCCAGCAAGAA             | CCCCAACTCCGACCTCTCATTA           | 607       | MboII  | Fine mapping   |
| Pkw4990        | TraesCS7A02G499000   | TGAAGAGGAAATGTCGGAA             | AGCTTGAGAGGACGTAGGAGA            | 970       | BsaI   | Fine mapping   |
| pkw4995        | TraesCS7A02G499500   | CTCAGAAAGCTGGTCAACAA            | GATCACTAGGACCTTCAA               | 900       | SspI   | Fine mapping   |
| Tm5F3R4        | TraesCS7A02G499600   | TGGAGAGGACGTCGGAAGA             | GTCGCTTCCCTGCGTTG                | 971       | PvuII  | Fine mapping   |
| TM5TF3R3       | TraesCS7A02G499600   | GGATTTAGGGTGCTGCGGA             | CCAACTACACCGACGCGAC             | 1137      | Accl   | Fine mapping   |
| pkw4997        | TraesCS7A02G499700   | TATGGCCAAAGGAGTGTGAA            | TACCACTCCGGAGAAGAAACCTG          | 709       | AccI   | Fine mapping   |
| pkw4999        | TraesCS7A02G499900   | GTGTCTCTGATGAGATTCAAC           | AGGCAGTCTTTAGGAGGAA             | 799       | AartI  | Fine mapping   |
| pkw5001        | TraesCS7A02G500100   | CGGTTGACATACCTTTCG              | TTCTTCTGAGCGGGGAGG               | 1448      | Fine mapping |
| pkw5003        | TraesCS7A02G500300   | CTGTGTCGTCGGACACCTCCTCC         | GTACCACTGCGGACGATA               | 675       | Smal   | Fine mapping   |
| pkw5009        | TraesCS7A02G500900   | TCTGCTGCTGGTCGTCGTCGTC         | GTCGCGCTGTGGTTCGTCGTCGTCGTC      | 1205      | SpII   | Fine mapping   |
| TM5TF2R2       | TraesCS7A02G499600   | GACATGAGACCTTCCCTGGTATTG        | CACATCTATACCCCTTCTTCATTACC       | 673       | MAS    |                |
| A1205F6R6      | TraesCS7A02G499600   | AAGAATCTGCTGGCCGAG             | AATCTTGAACACCCTTAAATGAAACTCG     | 108       | Expression analysis |
| HL-Fe1R60      | TraesCS7A02G499600   | GGTGACGGATGTTTCCGTTA            | GGTGACGGATGAAAGAAGA             | 109       | Expression analysis |
| 4997QF2R2      | TraesCS7A02G499700   | CCAAAAAGGAGTGAAGATGACTA         | ACGCATCATATCAGAAAGA             | 260       | Semi-quantitative PCR |
| 4998QF5R5      | TraesCS7A02G499800   | CATTCTAAGGGATGTGATGATTA         | ATTCGCTCCTTGGAGCTTTG            | 272       | Semi-quantitative PCR |
| TMSAF4R8       | TraesCS7A02G499600   | CTAGACAAACATCAACAGTAC           | GGTATCAATCCCAATCATCCTAATAT       | 1688      | Sequencing |
| TMSAF4R4       | TraesCS7A02G499600   | GTGTTCTCTCTCTCTCTCTGAATG        | ATCTATTGCTGCTGCTGCTGAAACTA       | 649       | Sequencing |
| caf2049        | –                    | TAATTGGATGGGTGGTGCAA             | CGTTGCTGAGTTGCTCTCGTCGTTG        | 1254      | Introgression |
| wmc205        | –                    | GTGCCGAAGAGACAGGAGT             | TATGGCACTGAGGAGCAGGAGG           | 1688      | Introgression |
| chf208        | –                    | TTTGCGACATACAGCAGTTT             | AAAATGATCCCCCGTGGGT             | 1688      | Introgression |
| gwm206        | –                    | GCCCTCTGACCAAAATC               | CGCAGTACACGAGGCCC               | 1688      | Introgression |
| barc108       | –                    | GGCGTGTGTTTCTCTGGAATATCAATCTCAA | GCGAAATGTGACGGTGTCGCTACCTGTTG   | 1688      | Introgression |
| barc312       | –                    | ACGATCACCGAATGTCGTTGA          | CGCGTGTGTTTCTCTAAGCTATG          | 1688      | Introgression |
| wmc1790       | –                    | AATTAAAGATATAGCCCGCCTATCATCACA | CGACACACGCTACCCGCC             | 1688      | Introgression |

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The progeny of the other five transgenic families displayed susceptible reactions similar to Fielder in all plants suggesting that the resistance gene was broken or damaged during the bombardment insertion. These transgenic families were discarded for further analysis.

To test if the transgenic plants had the same resistance profile as the natural SrTm5 gene in monogenic line TmR54-3, we inoculated transgenic family T 2Tm514-2 (homozygous for the transgene) with another two Pgt races RTJRM and 21C3CTTM, which are virulent on SrTm5 in T. monococcum. Plants from T 2Tm514-2 showed susceptible reactions similar to Fielder when challenged with SrTm5-virulent races RTJRM and 21C3CTTM (Figure S6) but were resistant when challenged with TTKSK (Figure 2), suggesting similar race specificity between the transgene and natural SrTm5 in T. monococcum.

Taken together, the map-based cloning and transgenic complementation results demonstrate that SrTm5 is an allele of the cloned gene Sr22. Based on its different resistance profiles (Table S4), we designated the R1 (Schomburgk/Pl 660256) and R4 (Pl 190945) haplotypes as allele Sr22a, and SrTm5 as allele Sr22b. This nomenclature has been approved by the Catalogue of Gene Symbols for wheat.

**Effect of Pgt inoculation on transcript levels of Sr22b**

We analysed Sr22b transcript levels relative to ACTIN in the monogenic line TmR54-3 by qRT-PCR. We found no significant transcriptional differences between plants inoculated with Sr22b-avirulent Pgt race 34PKUSC and mock inoculated with water at 1, 3 and 6 days post inoculation (dpi) (Figure 3), suggesting that Sr22b is not induced by the presence of the Pgt pathogen. We also compared the transcript levels of Sr22a in T. monococcum accession Pl 190945 and Sr22b in T. monococcum line TmR54-3 before inoculation and found no significant differences between them (Figure S7).

**Figure 1** Map-based cloning of SrTm5. (a) Collinear region on chromosome arm 7AL of Chinese Spring (RefSeq v1.1). Arrows represent genes. (b) High-density genetic map of SrTm5 using 2264 segregating gametes. (c) Predicted genes in the SrTm5 candidate region constructed with two overlapping BACs from the resistant parent Pl 306540. Dotted lines in arrows indicate deleted partial gene coding regions in BACs. (d) Gene structure of SrTm5 in Pl 306540. Black rectangles indicate exons and black lines indicate introns; the purple inverted triangle in the second intron indicates the insertion of a retrotransposon.

**Figure 2** Gene TmNLR1 confers resistance when transferred into the susceptible wheat variety Fielder. (a) Reactions to Pgt race TTKSK (isolate 04KEN156/04) in Fielder control and three transgenic families T 2Tm514-2, T 2Tm515-6 and T 2Tm517-1. S, susceptible; R, resistant. (b) The average percentage of the leaf area covered by Pgt pustules was measured using the software ASSESS v.2. More than 20 independent T 2 plants were evaluated. Error bars are standard errors of the mean.
**Sr22b is present only in *T. monococcum***

The dominant marker TM5TF2R2 was designed based on the special polymorphism (the insertion of repetitive sequence in the second intron) that differentiates Sr22b from the cloned Sr22-resistant haplotypes and all susceptible alleles. The forward primer was designed in the second intron and the reverse primer in the inserted retrotransposon. Amplification with PCR marker TM5TF2R2 at an annealing temperature of 60 °C generates an amplicon of 673 bp only when the gene Sr22b is present (Figure S8). Using this marker, we evaluated a collection of 165 wheat accessions, including 89 accessions of *T. monococcum*, 23 of *T. turgidum* and 53 of *T. aestivum*. PCR products were present only in 13 (14.6%) of the *Triticum monococcum* accessions but were absent in all tetraploid and hexaploid wheat lines tested in this study (Table S5). These observations were consistent with Sanger sequencing results using two pairs of primers TMSA6F6R8 and TMSA4f4R4 (Table 1), which were designed to amplify the LRR region of Sr22. The 13 *T. monococcum* accessions with the retrotransposon insertion, all carry the Sr22b haplotype in the LRR coding region, whereas all the other accessions have different haplotypes in the coding region and lack the retrotransposon insertion.

We then used the TM5TF2R2 marker to explore the presence of Sr22b in *T. monococcum* accessions PI 355538, PI 362610 and PI 377668 from the Balkans (Table S6), which were previously postulated to carry an unknown Pgt resistance gene different from Sr21 based on their different resistance reactions to races BCCBC and MCCFC (Chen et al., 2018b). We found that these three lines have Sr22b, which can explain their resistance to Pgt race MCCFC but susceptibility to BCCBC. This was confirmed by phenotyping 48 plants with race 34PKUSC in three F2 populations derived from crosses between PI 355538, PI 362610 and PI 377668 and the susceptible accession PI 272557. Genotyping with marker TM5TF2R2 showed that all plants in which the 673-bp fragment was amplified were resistant, whereas all plants without PCR products were susceptible. Moreover, we sequenced the coding regions of Sr22 from PI 355538, PI 362610 and PI 377668, and found that they were all 100% identical to Sr22b in PI 306540. These results confirmed that the resistance to MCCFC and 34PKUSC in these accessions was conferred by Sr22b.

**Introgression of Sr22b into hexaploid wheat background**

Figure 4a describes the crosses involved in the generation of the Sr22b introgression into hexaploid wheat. The diagnostic marker TM5TF2R2 and the closely linked CAPS marker pkw4974 (Table 1) were used for monitoring the presence of *T. monococcum* chromatin during backcrosses and for the final selection of BC3F2 plants homozygous for Sr22b. We confirmed the absence of stem rust resistance genes Sr13, Sr60, Sr21 and SrTm4 from the parental lines using diagnostic or closely linked markers (Briggs et al., 2015; Chen et al., 2020; Chen et al., 2018b; Zhang et al., 2017).

To determine the size of the 7A· chromosome region introgressed into hexaploid wheat, we first screened lines PI 306540, Kronos and Fielder for polymorphisms using 23 SSR markers distributed along chromosome 7A. We obtained seven polymorphic markers (Table 1) and determined their physical locations in the Chinese Spring reference genome (Refseq v1.0; Figure 4b). We genotyped 13 BC3F1 plants with markers TM5TF2R2 and pkw4974, and detected five plants with the 7A· introgression. BC3F1 plants 1, 3, 4 and 5 carried the 7A·L alleles for all the tested markers extending from 47.4 Mb to 689.9 Mb suggesting that they are disomic 7A· (7A substitution lines (Intro. 1 henceforth). The *T. monococcum* segment in plant number 2 extended from 446.9 Mb (barc108) to 689.9 Mb (TM5TF2R2), indicating a translocation of part of the long arm (referred hereafter as Intro.2, Figure S9). All these plants exhibited good levels of fertility when self-pollinated.

Homologous BC3F1 plants from these introgression lines challenged with Chinese Pgt race 34MTGSM showed good levels of resistance, whereas the recurrent parent Fielder and its sister line lacking Sr22b were completely susceptible (Figure 4c). Small amounts of BC3F1 seeds from the introgression lines are available by request from the senior authors. After the seed is increased, it will be deposited in the National Small Grain Collection in the United States and in the Chinese Crop Germplasm Resources Information System (CGRIS) in China.

**Discussion**

In this study, we confirmed that SrTm5 is a new allele of Sr22, officially designated as Sr22b. The stem rust resistance gene Sr22 was previously identified to encode a coiled-coil nucleotide-binding leucine-rich repeat protein, which confers broad-spectrum resistance to commercially important *P. graminis* races, including the Ug99 race group (Steuernagel et al., 2016). Sr22b and Sr22a both confer strong levels of resistance to Pgt races TTKSK (Ug99), TTKST, MCCFC, 34MTGSM and 34PKUSC, but differ in that Sr22b is susceptible to races BCCBC, 21C3CTTTM, RTJRM, QFSCC, TRTFF and TTTTF and Sr22a is not (Table S4). These results suggest that the Sr22a allele (R1 and R4 haplotypes) confers a broader resistance to tested Pgt races than Sr22b (Table S4). We currently do not know whether the other four Sr22-resistant haplotypes (R2, R3, R5 and R6, Figure S3) have different resistance profiles because monogenic lines are not available for these haplotypes.

The different Pgt resistance profiles of Sr22a and Sr22b were associated with more than 30 polymorphisms, located mostly within the LRR region (Figure S3). The LRR domain of plant NLR genes is known to play a major role in pathogen recognition specificity, and diversifying selection drives higher levels
of sequence variation (Dodds et al., 2006; Jiang et al., 2007; Krasileva and Dahlbeck, 2010). The different resistance profiles of Sr22a and Sr22b provide a useful tool to study the recognition mechanisms between Sr22 and the corresponding Avr proteins.

Insertions of large retrotransposons into functional genes is not a rare phenomenon in wheat, and can result in loss of function if inserted in the coding region. Insertions in introns may or may not have functional effects in the expression of the gene. For example, the gene Zfp69 is disrupted by a inserted retrotransposon in its intron, which generates a truncated mRNA (Scherneck et al., 2009) and insertion of retrotransposons into the intron of Maize waxy gene caused alternative splicing (Varagona and Purugganan, 1992). Unlike these genes, the large retrotransposon insertion in the intron of Sr22b did not affect its expression levels or function (Figure S7). We used this distinctive retrotransposon insertion in Sr22b to develop a diagnostic marker for this allele.

The complete coding region, UTRs and the inserted retrotransposon of Sr22b were too large to clone into a binary vector for Agrobacterium-mediated transformation, so we performed biolistic transformation using the circular BAC plasmid Tm84C1, which carries the 103.4-kb genomic fragment of PI 306540 and the 8.1-kb vector backbone sequence. Transformation with DNA fragments or circular plasmids larger than 100 kb has been previously reported in several plant species, such as tobacco (Wang et al., 2015), potato (Ercolano et al., 2004) and rice (Wang et al., 2015), but we are not aware of similar examples in wheat. Very large genes transformed by bombardment can be broken and disrupted (Liu et al., 2019; Makarevitch and Svitashev, 2003; Svitashev et al., 2002), which can explain the five confirmed transformation events that were susceptible to Pgt.

Fortunately, three independent events showed strong levels of resistance after infection with Pgt race TTKSK, indicating that the whole Sr22b gene was integrated into the plant genome in these transgenic lines. We observed a significant segregation distortion against the transgene both in T1 and T2 families (Table S3), but the distortion was not that strong, and we were able to recover plants homozygous for the different transformation events that showed stable resistance to Pgt.

Sr22b was successfully introgressed into the common wheat variety Fielder, where it conferred good levels of resistance to Pgt (Figure 4). However, the sizes of the T. monococcum introgression are quite large, including the whole 7Aa chromosome or most of the long arm of chromosome 7Aa (Figure S9). More work will be needed to reduce the length of the introgressed T. monococcum chromosome segment to minimize potential linkage drag. Fortunately, recombination between the A and Aa chromosome can be restored to normal levels through using the ph1b mutation (Dubcovsky et al., 1995). The diagnostic marker for Sr22b and the flanking SSR markers (Figure S9; Table 1) will be useful tools to develop shorter T. monococcum introgression lines carrying Sr22b.

Sr22b is only present in few cultivated T. monococcum accessions but absent in all tested polyploid wheats, indicating that it has the potential to improve Ug99 resistance in a wide range of modern wheat cultivars. However, since Sr22b is susceptible to several Pgt races, it would be necessary to combine with other resistance genes to provide a broader virulence
spectrum. Sr genes that are susceptible to race TTKSK but effective to other Pgt races could be considered as candidates for combination with Sr22b. Examples of these complementary genes include Sr60 (Chen et al., 2020), SrB155B1 (Nirmala et al., 2017), SrTRTTF (Hiebert et al., 2017) and Sr9e (Olivera et al., 2012).

The cloning of SrTrm5 demonstrated that it is a new allele of Sr22 and brings close to completion the characterization of all previously mapped stem rust resistance genes in T. monococcum (Sr21, Sr22, Sr35 and Sr60). The only mapped gene that has not been cloned yet is the recessive resistance gene SrTrm4 (Briggs et al., 2015). This information expands our understanding of the role of different stem rust resistance gene combinations in the adaptation of diploid wheat to this damaging rust pathogen and provides an entry point to understand the recognition specificity of different Sr22 alleles to different Pgt races and effectors. From a practical point of view, the identification of Sr22b, its transfer to hexaploid wheat and the reliable diagnostic marker developed in this study provide a useful tool to diversify the Sr genes deployed in modern wheat breeding programmes.

Methods

T. monococcum materials and mapping populations

As a source of SrTrm5, we used T. monococcum subsp. monococcum accession PI 306540, which was collected in Romania and that was previously shown to express the high levels of resistance to different Pgt races (Rouse and Jin, 2011a). PI 306540 was crossed with T. monococcum cultivated accession PI 272557, which does not carry any known Sr genes (Rouse and Jin, 2011b). Since PI 306540 carries multiple Sr genes, we selected F2 families segregating only for SrTrm5 from the cross PI 306540 × PI 272557 (Chen et al., 2018a). A total of 2264 segregating gametes were used to construct a high-density genetic map of SrTrm5. From this population, we selected the monogenic F2 line TmR54-3 homozygous for SrTrm5 (without any of the other resistance genes) and the sister control line TmS57-57 carrying no stem rust resistance gene.

A collection of 92 accessions of T. monococcum, 23 of T. turgidum and 53 of T. aestivum obtained from the US Department of Agriculture National Small Grains Collection (USDA-NSGC, https://npgrswb.ars-grin.gov/gringlobal/search) or the Chinese Crop Germplasm Resources Information System (CGRIS, http://www.cgris.net/cgris_english.html) were used to test the presence/absence of SrTrm5.

Stem rust evaluation

Previously, infection types of SrTrm5 to multiple Pgt races were reported, including TTKSK (isolate 04KEN156/04), TTKST (06KEN19v3), MCCFCF (59KS19), QTHJC (75ND717C), QFCSC (06ND76C), SCCSC (09ID73-2), TTTTF (01MN84A-1-2), TRTTF (06YEM34-1) and TKTTF (13ETH18-1 and 13GER15-1) (Chen et al., 2018a). In this study, stem rust seedling tests were carried out in three institutions: Peking University Institute of Advanced Agricultural Sciences, Weifang, China; USDA-ARS Cereal Disease Laboratory, Minnesota, USA; and University of California, Davis, USA. Selected sister lines TmR54-3 and TmS57-57 were re-evaluated with race TTKSK (04KEN156/04). To expand the resistance profile of SrTrm5, we also evaluated these lines with North American race BCCBC (09CA115-2) and Chinese races 34MTGSM (20GSA1), 21C3CTTTM (20GH13), RTJRM (mutant strain, 20A511) and 34PKUSC (19A508) (Li et al., 2016, 2018; Zhao et al., 2015). The origin and virulence/avirulence profiles of these Pgt races are presented in supplemental Table S1. Procedures for inoculation and scoring infection types (ITs) were as previously reported (Rouse et al., 2011; Stakman and Stewart, 1962).

For plants carrying critical recombination events in the high-density map, we preformed progeny tests including at least 25 progenies. These plants were inoculated with Chinese Pgt race 34PKUSC, and the percentage of the leaf area covered with pustules was estimated using the software ASSESS version 2.0 (American Phytopathological Society, St Paul, MN, USA) as reported previously (Lamari, 2008).

BAC library screening and sequencing

A non-gridded BAC library from PI 306540 with roughly 5× genome equivalents was available at the Wheat Molecular Genetics Laboratory, University of California, Davis (Chen et al., 2020). A PCR screening was performed using increasingly diluted library samples following the manufacturer’s instruction (Ampliton Express Inc., Pullman, WA). Screening of the BAC library with PCR markers pkw4995, TmS5F3R4, pkw4997 and pkw4999 yielded two positive BAC clones Tm84C1 and Tm2677. High quality BAC DNAs were extracted using Qiagen Large-Construct Kits (Qiagen, Hilden, Germany) and sequenced with Wideseq at Purdue University (https://purdue.ibalsolutions.com/landing/808). Repetitive elements were identified and annotated using the Cereal Repeat Sequences Database (https://wheat.pw.usda.gov/ITM/Repeatst/blastrepeatsth3.html). Candidate genes were annotated using the published reference genomes (The International Wheat Genome Sequencing Consortium, 2018; Walkowiak et al., 2020), and confirmed using the BLASTN/BLASTX searches available at National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). Expression profiles were determined with the Wheat Expression Browser (expVIP, http://www.wheat-expression.com/).

Wheat transformation

Bacterial artificial chromosome clone Tm84C1 containing 103 429 bp of T. monococcum PI 3065040 genomic sequence (GenBank accession MZ327628) was cloned into vector pCC1BAC (8128 bp). The cloned T. monococcum region carries complete genes TmB3 and TmNLR1 and a partial sequence of gene TmPPR1 (missing 30% of the distal coding region). Biolistic transformation was performed using a PDS1000/He particle bombardment system (Bio-Rad, Hercules, CA, USA). The cloned BAC Tm84C1 was co-transformed with plasmid pAH2C20, which carries bialaphos (BAR) selectable marker gene. BAC DNAs were mixed in a 1 : 1 (1 : 1 for BAC DNA and pAH2C20) molar ratio prior to bombardment. Transformation was performed using the Ug99-susceptible spring wheat variety Fielder by biolistic bombardment as described previously (Zhang et al., 2015).

Positive transgenic plants were identified using dominant or codominant PCR markers TmS5F3R4, TM5ST2R2 and TM5ST3R3 (Table 1). Expression levels of TmNLR1 in transgenic plants were assessed by quantitative real-time PCR (qRT-PCR) with primer pairs HL-F61R60. About 25 T2 transgenic seeds from each transgenic event were germinated and tested for their responses to Pgt race TTKSK (Ug99).

qRT-PCR analysis

Plants from SrTrm5 monogenic line TmR54-3 were mock inoculated or Pgt inoculated in two independent chambers under the same environmental condition: 22 °C day/20 °C night and 16 h
light/8 h dark. Total RNAs were extracted from leaves of different plants collected immediately after inoculation (0 h) and 1, 3 and 6 days post inoculation (dpi) using Spectrum Plant Total RNA Kit (Sigma-Aldrich, Saint Louis, MO, USA). First-strand cDNA was synthesized using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kits. qRT-PCR reactions were performed on a QuantStudio™ 5 real-time PCR system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using Fast SYBR GREEN reagents. PCR primers A120F6R6 (Table 1, 97% efficiency) were used to evaluate the effect of Pgt inoculation on SrTm5. Transcript levels were determined in four biological replicates and expressed as fold-\textit{ACTIN} levels as described previously (Pearce and Vanzetti, 2013).


text

\textbf{Introgression of }\textit{T. monococcum} \textbf{segments carrying }\textit{SrTm5} \textbf{into hexaploid wheat}

The diploid wheat accession PI 306540 (A\textsuperscript{m}A\textsuperscript{m}) was used for transferring \textit{T. monococcum} gene \textit{SrTm5} to hexaploid wheat variety Fielder using \textit{durum} wheat variety Kronos (AABB) as bridging species (The, 1973). The F\textsubscript{1} triplant plants from the cross of PI 306540 × Kronos were crossed with hexaploid wheat variety Clear White (UC1361), and the resulting F\textsubscript{2} plants were backcrossed three times to the recurrent spring common wheat line Fielder. PCR markers TM5TF2R2 and pkwd974 (Table 1) were used to validate the presence of the introgressed \textit{T. monococcum} segments during backcrossing. Five BC\textsubscript{2}F\textsubscript{1} plants carrying alien chromosome segments were self-pollinated and characterized with 23 simple sequence repeat (SSR) markers across chromosome 7A to analyse the length of introgressed \textit{T. monococcum} segments. Subsequently, we selected BC\textsubscript{3}F\textsubscript{2} plants homozygous for the introgressed \textit{T. monococcum} segment to generate seeds. The resulting progeny were inoculated with \textit{Pgt} race 34MTGSM.

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\section*{Conflicts of interest}

The authors declare that they have no conflict of interests.

\section*{Author contributions}

JL and MNR performed most of the experimental work; YW and CG designed the transgenic experiments. BL performed the biostatic transformation and obtained T\textsubscript{1} seeds. Leih contributed qRT-PCR and filled the gaps of BAC sequence; Hnal contributed primers development; TL performed part of the phenotyping experiments; WZ created the mapping population and contributed sequence analyses. SC analysed the data and wrote the first version of the manuscript. YW, SC and JD proposed and supervised the project, obtained the funding and generated the final version of the paper. All authors revised the manuscript and provided suggestions.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Reactions to six *Pgt* races 34PKUSC, 34MTGSM, TTKSK, BCBCB, 21C3CCTTM and RTJR.
Figure S2  Semi-quantitative PCR products from markers 4997QF2R2 (260 bp, TraesCS7A02G499700), 4998QF3R5 (272 bp, TraesCS7A02G499800) and ACTINF1R1 (ACTIN).

Figure S3  SrTm5 protein sequence analysis. Multiple sequence alignment between SrTm5 and reported Sr22-resistant and susceptible protein sequences (Steuernagel et al. 2016).

Figure S4  Transcript levels of TmNLR1 in transgenic T1 families (three positive plants per event, n = 3).

Figure S5  Reactions to Pgt race TTKSK (Ug99) in transgenic family T2Tm51-6.

Figure S6  Transgenic family T2Tm514-2 homozygous for the transgene were inoculated with two SrTm5-virulent Pgt races RTJR and 21C3CTTM.

Figure S7  Transcript levels and infection types of Sr22a and Sr22b in T. monococcum background.

Figure S8  PCR products from the Sr22b diagnostic marker TM5TF2R2.

Figure S9  Markers across chromosome 7A were used to analyse the length of introgressed T. monococcum segments.

Table S1  Avirulence/virulence formulae of Pgt races, and their responses to SrTm5.

Table S2  Comparison of SrTm5 protein with polymorphisms that discriminate perfectly between Sr22-susceptible and -resistant haplotypes from Steuernagel et al. (2016).

Table S3  Segregation ratios in T1 and T2 transgenic families detected using PCR markers Tm5F3R4, TM5TF2R2 and TM5TF3R3 (Table 1).

Table S4  Resistance profiles of Sr22b (=SrTm5) and Sr22a (haplotypes R1 and R4) to multiple Pgt races.

Table S5  A collection of 92 accessions of T. monococcum, 23 of T. turgidum and 53 of T. aestivum was used to test the presence of Sr22b.

Table S6  Geographic distribution of T. monococcum accessions, and their reactions against Pgt races TTKSK, MCCFC and 34PKUSC.