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Wheat gene \textit{Sr60} encodes a protein with two putative kinase domains that confers resistance to stem rust

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Summary

- Wheat stem rust, caused by *Puccinia graminis* Pers. f. sp. *tritici* (*Pgt*), is a devastating fungal disease threatening global wheat production. Here, we report the identification of stem rust resistance gene *Sr60*, a race-specific gene from diploid wheat *Triticum monococcum* L. that encodes a protein with two putative kinase domains. This gene, designated *WHEAT TANDEM KINASE 2* (*WTK2*), confers intermediate levels of resistance to *Pgt*.
- *WTK2* was identified by map-based cloning and validated by transformation of a ~10-kb genomic sequence including *WTK2* into susceptible common wheat variety *Fielder* (*T. aestivum* L.).
- Transformation of *Fielder* with *WTK2* was sufficient to confer *Pgt* resistance. *Sr60* transcripts were transiently upregulated one day after *Pgt* inoculation, but not in mock-inoculated plants. The upregulation of *Sr60* was associated with stable upregulation of several pathogenesis-related genes.
- The *Sr60*-resistant haplotype found in *T. monococcum* was not found in polyploid wheat, suggesting an opportunity to introduce a novel resistance gene. We successfully introgressed *Sr60* into hexaploid wheat and developed a diagnostic molecular marker to accelerate its deployment and pyramiding with other resistance genes. The cloned *Sr60* can also be a useful component of transgenic cassettes including other resistance genes with complementary resistance profiles.

**Key words:** Wheat, *Triticum monococcum*, stem rust, resistance genes, *Sr60*, kinase proteins
Introduction

Wheat is a major source of calories and proteins for the human population. To achieve the increases in global wheat production required to feed a rapidly growing population, it is important to minimize yield losses generated by rapidly evolving fungal pathogens. Among these pathogens, *Puccinia graminis* f. sp. *tritici* (henceforth *Pgt*), the causal agent of wheat stem rust, is of particular concern. The recent appearance and spread of Ug99 (*Pgt* race TTKSK) and its variants (henceforth Ug99 race group), resulted in extensive yield losses in Africa and has recently expanded to the Arabic Peninsula and Iran (Nazari *et al.*, 2009; Singh *et al.*, 2015; Patpour *et al.*, 2016). Unfortunately, the Ug99 race group is not the only problem. A *Pgt* race unrelated to Ug99 called TKTTF was reported in Africa in 2013 (Olivera *et al.*, 2015) and a similar race was detected in outbreaks in Germany (Olivera *et al.*, 2017). Epidemics of stem rust have been recently reported in Sicily (Bhattacharya, 2017) and *Pgt* was detected in the UK after being absent for nearly six decades (Lewis *et al.*, 2018). These reports have prompted efforts to identify and isolate more Sr resistance genes, to combine them into wheat cultivars and to develop transgenic cassettes including multiple and diverse resistance genes.

Only ten of the 60 officially named stem rust resistance genes (Singh *et al.*, 2015; Chen *et al.*, 2018a) have been cloned so far and eight of them are race-specific genes encoding coiled-coil nucleotide-binding leucine-rich repeat (NLR) proteins. These eight genes include *Sr35* (Saintenac *et al.*, 2013), *Sr33* (Periyannan *et al.*, 2013), *Sr50* (Mago *et al.*, 2015), *Sr22* (Steuernagel *et al.*, 2016), *Sr45* (Steuernagel *et al.*, 2016), *Sr13* (Zhang *et al.*, 2017), *Sr21* (Chen *et al.*, 2018b) and *Sr46* (Arora *et al.*, 2018). Only the slow-rusting multi-pathogen resistance genes *Sr57/Lr34* (Krattinger *et al.*, 2009) and *Sr55/Lr67* (Moore *et al.*, 2015), encode different types of proteins, including a putative ATP-binding cassette (ABC) transporter and a hexose transporter, respectively.
The cloning of Sr resistance genes has contributed to the development of diagnostic markers and has accelerated the pyramiding and deployment of these resistance genes in wheat-breeding programs. However, a more extended set of genes is desirable to diversify the resistance gene pyramids and to extend their durability. Wheat wild and cultivated relatives are valuable sources of new stem rust resistance genes. For example, the diploid wheat species *Triticum monococcum* (genome A^m^) has contributed several *Pgt* resistance genes including *Sr21*, *Sr22* and *Sr35*, which have been cloned and transferred into polyploid wheat (Gerechter-Amitai *et al.*, 1971; The, 1973; McIntosh *et al.*, 1984; Saintenac *et al.*, 2013; Steuernagel *et al.*, 2016; Chen *et al.*, 2018b). *T. monococcum* chromosome segments can be transferred to hexaploid wheat using the presence of the *Pairing homeologous 1* mutation (*ph1b*) (Dubcovsky *et al.*, 1995).

Three additional Sr genes (*SrTm4*, *SrTm5* and *Sr60*) have been mapped in *T. monococcum*, but have not been cloned or transferred to hexaploid wheat so far (Rouse & Jin, 2011a; Briggs *et al.*, 2015; Chen *et al.*, 2018a). *Sr60*, discovered in *T. monococcum* accession PI 306540, is effective against races QFCSC, QTHJC and SCCSC. This gene was mapped on the distal region of chromosome arm 5A^m^, within a 0.44 cM region that does not include typical NLR genes in the orthologous genomic region in Chinese Spring (Chen *et al.*, 2018a).

In this study, we report the positional cloning of the wheat stem rust resistant gene *Sr60*, which is induced transiently one day after inoculation with *Pgt* race QFCSC. *Sr60* encodes a protein including two putative kinase domains that is sufficient to confer resistance to stem rust in transgenic wheat plants. We identified a single *Sr60*-resistant haplotype in *T. monococcum* that was not detected in any of the accessions of *T. urartu*, *T. turgidum* subsp. *dicocoides*, *T. turgidum* subsp. *dicoccon*, *T. turgidum* subsp. *durum* and *T. aestivum* tested so far. This result suggests that *Sr60* is a novel source of resistance to stem rust for durum and bread wheat. To accelerate the deployment of this gene, we backcrossed a small *T. monococcum* chromosome segment including *Sr60* into a high-yielding hard spring wheat.
Materials and Methods

Segregating populations and stem rust assays

A high-resolution genetic map of Sr60 was constructed using 4,046 segregating gametes from two crosses between diploid wheat lines (3,854 from PI 306540 × G3116 and 192 from PI 306540 × PI 272557). From the second population we selected two F₃ lines, one homozygous for the susceptible Sr60 allele (TmS57-57) and the other homozygous for the resistant allele (TmR57-32), and both lacking Sr21, SrTm4 and SrTm5 (Chen et al., 2018a).

Sr60 was previously reported to be effective against Pgt races QFCSC, QTHJC and SCCSC, but not against TTKSK, TTKST, MCCFC, TRTTF, TTTTF, and TKTTF (Chen et al., 2018a). Isolates and their virulence / avirulence profiles are presented in Table S1. During the last decade, QFCSC has been the predominant Pgt race in the U.S. (Long et al., 2010; Jin et al., 2014). Race SCCSC was first identified in Idaho, and has virulence to resistance gene Sr9e, which contributes towards stem rust resistance in durum wheat (Long et al., 2010). Race QTHJC was detected in of states of Alabama and North Dakota in 1997 (McVey et al., 2002).

Plants with recombination events in the candidate gene region were challenged with Pgt race QFCSC at the USDA-ARS Cereal Disease Laboratory according to previously described methods (Rouse & Jin, 2011b). Evaluations were performed at 25 °C during the day and 22 °C during the night with a 16 h photoperiod. Infection types (ITs) were recorded 12–14 days after inoculation (dpi). The image analysis software ASSESS v.2 was used to quantify the average sporulation areas as reported previously (Lamari, 2008). The fungal infection area at 5 dpi (visualized by a Zeiss Discovery V20 fluorescent dissecting scope) and the amount of fungal DNA relative to host DNA were used to compare the growth of the Pgt pathogen in the presence and absence of Sr60 using methods described before (Zhang et al., 2017).
Non-arrayed BAC library construction, screening and sequence annotation

A non-arrayed Bacterial Artificial Chromosome (BAC) library from the resistant parent PI 306540 was constructed using the BAC-MAPS pooling technology (Amplicon Express Inc., http://ampliconexpress.com/). The average clone size of this non-arrayed library was 120 kb and its coverage was roughly five genome equivalents. Individual BAC clones were identified by PCR of increasingly diluted library samples and DNAs of the selected clones were extracted using QIAGEN Large-Construct Kit (Qiagen, CA, USA). BAC clones were sequenced with WideSeq (https://purdue.ilabsolutions.com/landing/808). Gaps were filled by Sanger sequencing and contiguous sequences were generated using Galaxy (Bankevich et al., 2012; Afgan et al., 2016). The Triticeae Repeat Sequence Database was used to identify repetitive elements (http://wheat.pw.usda.gov/ITMI/Repeats/blastrepeats3.html). The coding regions were annotated using the IWGSC Chinese Spring RefSeq v1.1 annotation (https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Annotations/v1.1/), BLASTN/BLASTX searches in GenBank (http://www.ncbi.nlm.nih.gov/), and expression databases for T. monococcum (Fox et al., 2014), and polyploid wheat (https://wheat.pw.usda.gov/WheatExp/).

Full-length cDNA and 5’ and 3’ RACE of Sr60 candidate gene

Total RNAs were extracted from leaves of resistant parent PI 306540 using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). Rapid amplification of cDNA ends (RACE) was performed using the Invitrogen™ FirstChoice™ RLM-RACE Kit (Catalog number AM1700, Invitrogen). The amplification products from 3’ and 5’RACE reactions were cloned using the TOPO™ TA Cloning™ Kit (Invitrogen). Fifty colonies per reaction were sequenced by Sanger sequencing.
**qRT-PCR analysis**

Plants were grown in growth chambers at 25°C during the day and 22°C during the night with a photoperiod of 16 h. Plants were mock inoculated or inoculated with *Pgt* race QFCSC using previously published procedures (Rouse & Jin, 2011a). Samples from different plants were collected immediately before inoculation (0 h) and one, three, five and six days post inoculation (dpi). Total RNAs were extracted from leaves using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). RNase-Free DNase RQ1 (Promega) was used to remove DNAs from RNA samples. First strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Fast SYBR GREEN Master Mix and an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) were used to perform qRT-PCR reactions. Primers used to evaluate the transcript levels of the *Sr60* candidate gene are listed in Table S2, whereas primers used to quantify the expression of pathogenesis-related (*PR*) genes (*PR1*, *PR2*, *PR3*, *PR4*, *PR5* and *PR9 = TaPERO*) were described before (Zhang *et al.*, 2017). Transcript levels were expressed as fold-ACTIN levels using the formula $2^{(\text{Actin } CT - \text{Target } CT)}$ (Pearce *et al.*, 2013).

**Wheat transformation**

Restriction sites *XhoI* and *SpeI* were added to primers SR60TransF1 and SR60TransR2 (Table S2) for cloning the complete genomic region including *Sr60*. We cloned a 9,910-bp fragment from PI 306540 BAC clone Tm7588 using Phusion High-Fidelity DNA Polymerase (Catalog number M0530S, New England BioLabs Inc.). This fragment contains the complete *Sr60* coding region and introns (5,008 bp), as well as 2,403 bp upstream of the start codon and 2,499 bp downstream of the stop codon. We cloned the fragment into the *XhoI* / *SpeI* linearized binary vector pLC41Hm. *Agrobacterium tumefaciens*-mediated wheat transformation with this construct was carried out at plant transformation facility of UC Davis (http://ucdptf.ucdavis.edu/).
PCR primers HptmikiF/R and SR60F2/R2 (Table S2) were used to validate the presence of the transgene. qRT-PCR primers SR60RTF2/R2 (Table S2) were used to estimate Sr60 transcript levels in T₀ transgenic plants using ACTIN as the endogenous control. Twenty-five T₁ transgenic plants were inoculated with Pgt race QFCSC in a growth chamber at 25°C during the day and 22°C during the night (the whole experiment was replicated twice). Copy number of Sr60 insertions in each transgenic event was estimated based on the segregation ratio of T₁ plants and using a TaqMan copy number assay (Diaz et al., 2012; Zhang et al., 2017).

**Haplotype analysis**

The Sr60 candidate gene was sequenced in 47 T. monococcum accessions including PI 306540, PI 277131-2, PI 272557 and G3116. Infection types of these accessions to Pgt races TRTTF, TTKSK, TTTTF, QFCSC and MCCFC were reported before (Rouse & Jin, 2011b), and confirmed with race QFCSC for this study. Diagnostic markers from cloned genes Sr35 (Saintenac et al., 2013), Sr22 (Steuernagel et al., 2016) and Sr21 (Chen et al., 2018b) were used to determine the presence or absence of these genes. Gene postulation was based upon infection types and genotypes from these gene diagnostic markers. We tested the presence of the cloned Sr60 gene with the diagnostic marker Sr60F2R2 in 104 T. monococcum, 63 T. urartu, 49 T. turgidum ssp. dicocoides, 89 T. turgidum ssp. dicoccon, 94 T. turgidum ssp. durum and 43 T. aestivum accessions.

**Introgression of Sr60 in hexaploid wheat**

To introgress the distal region of chromosome arm 5A∗S including Sr60 (Chen et al., 2018a), we crossed PI 306540 (A∗mA∗m) with T. turgidum ssp. durum wheat variety Kronos (AABB, released by Arizona Plant Breeders in 1992). The triploid F₁ was crossed with UC Davis hard white spring common wheat variety Clear White and the resulting F₁ was backcrossed five times to the recurrent hard red spring breeding line UC12014-36 (UC1110/UC1037Gpc-B1, 2NS, Glu-D1d). The presence of the introgressed T. monococcum segment was followed during the
backcrossing using molecular markers developed in a previous study (Chen et al., 2018a). The BC$_3$F$_1$ plant was self-pollinated and BC$_3$F$_2$ plants homozygous for the introgressed *T. monococcum* segment were selected using molecular markers. Progeny of the BC$_3$F$_2$ plants were evaluated with race QFCSC to validate the presence/absence of *Sr60*. Kernel hardness was tested using a Single Kernel Characterization System (SKCS) at the California Wheat Commission Milling and Baking Lab (http://www.californiawheat.org/milling/).

**Results**

**Resistance response of *Sr60***

*Sr60* conferred intermediate levels of resistance (*IT = 2–22*) to *Pgt* races QFCSC, QTHJC and SCCSC (Fig. 1a). Smaller sporulation areas were observed at 14 dpi in the plants carrying *Sr60* than in the plants without this gene (*P < 0.001*; Fig. 1a). The average *Pgt* infection areas at 5 dpi using microscopy and fluorescent staining (Fig. 1b) were also significantly smaller in the lines with *Sr60* than in those without this gene (*P < 0.001*). In addition, the borders of the infected areas in the lines without *Sr60* showed a diffuse network of expanding hyphae, whereas those in the lines with *Sr60* were denser, suggesting that the hyphal growth was facing opposition from the host (Fig. 1b). Finally, we quantified the progression of the *Pgt* infection by measuring the ratio of *Pgt* DNA relative to wheat DNA. During the first two days after inoculation, fungal growth was negligible and no differences were detected between lines with and without *Sr60*. By contrast, significant differences were detected at 3 dpi (*P < 0.001*), 5 dpi (*P < 0.001*) and 6 dpi (*P < 0.001*), with slower growth detected in the lines carrying *Sr60* than in those without the gene (Fig. 1c). Since the presence of *Sr60* delayed but did not stop the *Pgt* infection, the resistance conferred by this gene was classified as partial resistance.
High-resolution genetic and physical maps of the *Sr60* region

In the previous study, we mapped *Sr60* within a 0.44 cM interval delimited by markers CJ942731 and GH724575 and completely linked to *LRRK123.1* in the distal region of chromosome arm 5A<sup>m</sup>S (Chen *et al.*, 2018a). Using the Chinese Spring reference genome (RefSeq v1.0), we determined that the two flanking markers define a region of 436.4 kb that contains 16 annotated genes (*TraesCS5A02G004500*-*TraesCS5A02G006000*, Fig. 2a). We developed five new markers in the region (Fig. 2b, Table S2) and mapped *Sr60* completely linked to markers *ucw530* and *ucw540*, within a 0.13 cM (74.5 kb) interval delimited by markers *ucw510* and *ucw550* (Fig. 2b). Flanking marker *ucw510* is located within an oxidoreductase gene (*SRG1*) that is not annotated in RefSeq v1.0 (it has a premature stop codon in CS), and the other flanking marker *ucw550* is located within gene *TraesCS5A02G005500*.

We used the two completely linked markers and the two closest flanking markers to screen the non-arrayed BAC library of the resistant *T. monococcum* accession PI 306540. Using the proximal flanking marker *ucw510* and the completely linked markers *ucw530* and *ucw540*, we detected two BAC clones designated Tm4266 and Tm7588. An additional BAC (Tm9510) was identified using the distal flanking marker *ucw550* (Fig. 2c). The sequences of BACs Tm7588 and Tm9510 revealed a 13,802-bp segment that was 100% identical, confirming that their ends were overlapping. In summary, these three BACs included both flanking markers and completed the physical map of the *Sr60* candidate region (Fig. 2c, GenBank accession MK629715).

Three complete genes were annotated within the 74.5 kb *T. monococcum* candidate-gene region (Fig. 2d). The first one, is orthologous to *TraesCS5A02G005100* which encodes a hypothetical protein with low identity to any other proteins (<40%). No transcript of this gene was detected in the leaves of *T. monococcum* (three-leaf stage) nor in RNASeq data from CS (WheatExp). These data, together with the absence of polymorphisms between the resistant
parent PI 306540 and the susceptible parents PI 272557 and G3116, suggest that
TraesCS5A02G005100 is an unlikely candidate gene for Sr60. The adjacent gene in Chinese
Spring, TraesCS5A02G005200, is not found in the T. monococcum BAC sequences (Fig. S1).
This gene has a reverse transcriptase domain and is likely a repetitive element.

The second gene in the T. monococcum region is an ortholog of Chinese Spring
TraesCS5A02G005300 and encodes a leucine-rich repeat receptor-like
serine/threonine-protein kinase. This gene is similar to Arabidopsis FEI 1, which is known to
play a role in the regulation of cell wall (Steinwand & Kieber, 2010). This T. monococcum
gene was designated as LRRK123.1 in our previous study (Chen et al., 2018a). The predicted
LRRK123.1 protein showed two amino acid polymorphisms between PI 306540 and PI
272557 (T395M and T484A; BLOSUM62 scores = -1 and 0, respectively), but none between
PI 306540 and G3116. Since the population generated from the last two accessions
segregated for Sr60 resistance, LRRK123.1 is an unlikely candidate for Sr60.

The third gene in T. monococcum is an ortholog of Chinese Spring TraesCS5A02G005400,
which encodes a protein with two putative kinase domains in tandem, and is designated here
as WHEAT TANDEM KINASE 2 (WTK2). Sequencing of the complete WTK2 region in the
susceptible parents revealed a deletion of four amino acids (SRAR at positions 714-717) in
both PI 272557 and G3116 relative to the resistant accession PI 306540. In addition, G3116
showed the insertion of a retrotransposon in the fourth exon, providing further evidence that
this gene is not functional in G3116.

We then compared the Sr60 region in PI 306540 with the recently released genomes of
tetraploid wheat Zavitan WEWSeq v1.0 (Avni et al., 2017) and hexaploid wheat Chinese
Spring RefSeq v1.0 (Appels et al., 2018). This comparison showed that the genes within the
candidate region are well conserved with the exception of WTK2, which is completely deleted
in Zavitan and has a deletion including the last two exons in Chinese Spring (Fig. 2e and Fig.
S1). This result is consistent with the absence of Sr60 resistance in these two accessions.
Finally, we sequenced *T. monococcum* accession PI 277131-2 previously postulated to carry Sr60 (Rouse & Jin, 2011a) and confirmed the presence of a gene 100% identical to WTK2 in PI 306540. Taken together, these results suggested that WTK2 was the best candidate for Sr60 among the genes detected within the 74.5 kb candidate gene region in *T. monococcum*.

**WTK2 gene structure and phylogenetic analysis**

We compared a full-length complementary DNA (cDNA) of WTK2 with the corresponding genomic sequence and determined that this gene has nine exons. WTK2 spans 5,008 bp from the starting ATG to the termination TAG, with a complete coding sequence of 2,175 bp (Fig. 2e). Using 5’ RACE, we identified the transcriptional start of WTK2 803 bp upstream from the start codon. The 5’-untranslated region (UTR) included one or two introns depending on the alternative splice forms (see alternative splicing section). Using 3’ RACE, we determined that the 3’ UTR is 255 bp long without any introns (Fig. S2).

The predicted WTK2 protein is 724 amino acids long and contains two putative protein kinase domains. The KinI and KinII domains of WTK2 have both eight conserved residues G52, K72, E91, H158, H164, D166, N171 and D184 found in functional plant protein kinase domains (Table S3) (Hanks *et al.*, 1988; Klymiuk *et al.*, 2018) suggesting that they may be functional kinases. However, since we have not demonstrated kinase activity, we will refer to these domains as putative kinase domains hereafter.

A neighbor-joining (NJ) analysis was performed to compare these two putative kinase domains to 184 putative kinase or pseudokinase domains used in a previous study of the WHEAT TANDEM KINASE 1 (WTK1) protein encoded by the stripe rust resistance gene Yr15 (Klymiuk *et al.*, 2018). The kinase domain I (KinI) of WTK2 was grouped together with HORVU6Hr1G025940.2 K1 from *Hordeum vulgare* and TraesCS5B02G005400.3 K1 from *Triticum aestivum* (Fig. S3), which were classified within the LRR_8B group (cysteine-rich kinases) by Klymiuk et al. (2018). The kinase domain II (KinII) of WTK2 was most similar
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displayed intermediate levels of resistance, whereas all Fielder plants displayed susceptible reactions (Fig. 3a). Measures of average pustule size in twelve independent plants at 14 dpi showed significantly smaller (~7-fold) sporulation areas \((P < 0.0001, \text{Fig. 3b})\) in the transgenic plants than in the Fielder control.

The ratio of \(Pgt\) DNA to wheat DNA in Fielder and transgenic family \(T_1\text{Sr60-009}\) (Fig. S5) was similar until one dpi, but became significantly lower in the transgenic plant at 3, 5 and 6 dpi (Fig. S5), similar to the \(T. monococcum\) time course in Fig. 1c. The pathogen/host DNA ratio at 6 dpi was lower in Fielder (ratio = 1.1) than in the susceptible \(T. monococcum\) (ratio = 2.6), but this may simply reflect the higher DNA content per nucleus in hexaploid Fielder than in diploid \(T. monococcum\) (~3-fold). The difference in DNA content does not affect the comparison between the DNA ratios in the susceptible and resistant controls within each species. Whereas this ratio is 2.6/1.3 = 2-fold higher in the susceptible than in the resistant diploid wheat, it is 1.1/0.2 = 5.5-fold higher in the susceptible Fielder than in the transgenic plants. The more effective resistance in the transgenic plants than in the natural resistance \(T. monococcum\) plants may reflect the higher number of resistance genes present in the selected transgenic family.

To test if one copy of the \(Sr60\) transgene was sufficient to confer resistance, we analyzed transgenic families \(T_1\text{Sr60-010}\) and \(T_1\text{Sr60-001}\) segregating for one and two independent transgenic copies, respectively (Table S4). The presence of the \(Sr60\) transgene was determined with PCR primers \(\text{Sr60F2/R2}\) and the level of resistance was estimated by measuring sporulation area in leaves inoculated with race QFCSC. The average sporulation area in lines carrying a \(WTK2\) transgene was 60% smaller than the combined lines without the transgene. The differences were significant both for family \(T_1\text{Sr60-010}\) and \(T_1\text{Sr60-001}\) \((P <0.0001)\). Since plants carrying one or more functional copies of the transgene were more resistant than the susceptible Fielder control and the sister lines without the transgene (Fig. S6), we concluded that one copy of the \(Sr60\) transgene was sufficient to confer resistance to \(Pgt\).
Finally, we inoculated transgenic plants from family $T_1{\text{Sr60-009}}$ (segregating for four independent transgenic copies) with races TTTTF and MCCFC that are virulent on $Sr60$ in $T. \textit{monococcum}$ and QFCSC that is avirulent. $T_1{\text{Sr60-009}}$ was clearly resistant to race QFCSC (Fig. 3), but susceptible to races MCCFC and TTTTF (Fig. S7). These results suggest that the transgene has a similar resistance profile as the natural $Sr60$ gene.

**Expression pattern of WTK2**

Transcript levels of $WTK2$ relative to $ACTIN$ were analyzed in $Sr60$-resistant $T. \textit{monococcum}$ line TmR57-32 (diploid) and $Sr60$-transgenic family $T_1{\text{Sr60-005}}$ (hexaploid) by qRT-PCR. The basal transcript levels in transgenic family $T_1{\text{Sr60-005}}$ were 2.5-fold higher than in $T. \textit{monococcum}$ line TmR57-32. As expected, no significant differences were detected between QFCSC-inoculated and mock-inoculated plants at the time of inoculation (0 h, Fig. S8). However, at 1 dpi $WTK2$ transcript levels were significantly higher ($P < 0.001$) in $Pgt$-inoculated plants than in mock-inoculated plants both in the $Sr60$-resistant $T. \textit{monococcum}$ (3.7-fold increase) and $T_1{\text{Sr60-005}}$ (2.5-fold increase, Fig. S8). In both species, this increase was transient and disappeared at later time points (3, 5 and 6 dpi). At these later time points, $WTK2$ transcript levels became significantly lower in $Pgt$-inoculated than in mock-inoculated plants in $T. \textit{monococcum}$ (Fig. S8a). Although $WTK2$ transcript levels also decreased in the transgenic hexaploid line after the peak at 1 dpi, they remained at similar levels as those in the mock-inoculated plants (Fig. S8b). These results suggest that $WTK2$ is transiently induced by the presence of $Pgt$.

Sequences from the 5’ RACE reactions showed the presence of four different alternative splicing forms at the 5’ UTR region of $WTK2$, designated hereafter as $WTK2$-1, $WTK2$-2, $WTK2$-3 and $WTK2$-4. Transcript levels of the four alternative splicing forms were evaluated using four isoform-specific qRT-PCR primers described in Table S2, and all four showed similar levels of transient induction at 1 dpi with race QFCSC (Fig. S9).
Transcript levels of *pathogenesis-related* (PR) genes

To evaluate the potential downstream genes involved in the *Sr60*-mediated resistance, transcript levels of six *PR* genes were analyzed in *Pgt*-inoculated and mock-inoculated resistant *T. monococcum* plants (line TmR57-32) at 1, 3, and 5 dpi (Fig. S10). Transcript levels of *PR1*, *PR4* and *PR5* were significantly higher (*P* < 0.05, Table S5) in *Pgt*-inoculated plants than in mock-inoculated controls at all three sampling times. Transcript levels of *PR2* and *PR9* were also significantly higher (*P* < 0.01, Table S5) in *Pgt*-inoculated than in mock-inoculated plants but only at 1 dpi. Finally, there was no significant difference in the transcript levels of *PR3* in the resistant *T. monococcum* plants between *Pgt*-inoculated and mock-inoculated plants. These results suggest that the rapid and transient upregulation of *WTK2* is associated with a rapid, significant and more extended upregulation of *PR* genes *PR1*, *PR4* and *PR5* that may contribute to the *Pgt* resistance observed in *Sr60* genotypes (Fig. 1 and S5).

Haplotype analysis of WTK2

We sequenced the complete *WTK2* gene from 47 *T. monococcum* accessions using four pairs of gene-specific primers (Table S2). Six accessions (PI 277130, PI 277135, PI 306545, PI 306547, PI 428158, and PI 435001) showed the same sequence in the gene region as the resistant lines PI 306540 and PI 277131-2 (GenBank accession MK629715). These accessions showed similar resistance reactions to race QFCSC and were classified as haplotype R1 (Table S6). We also identified eight different susceptible haplotypes, which were designated as S1 - S8 (GenBank accessions MK629708 to MK629714, Table S6). No PCR amplification was detected with any of the *WTK2* primers for haplotype S8, suggesting the presence of a deletion in these accessions. Similarly, *WTK2* was not detected in the reference genome of wild emmer wheat Zavitan (Fig. S1).

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Susceptible haplotypes S1 to S7 share a four amino acid deletion at positions 714-717 (SRAR). The susceptible parent PI 272557 and other six accessions were classified as haplotype S1 (MK629708), which differs from the resistance haplotype R1 only by the SRAR deletion (Fig. S11). The susceptible haplotypes S3 (MK629710) and S7 (MK629714) differ from the S1 haplotype by one amino acid change each. The haplotype S2 (G3116, MK629709) includes 19 T. monococcum accessions and is likely a non-functional gene since it is interrupted by the insertion of a retrotransposon in the fourth exon. Haplotypes S5 (MK629712) and S6 (MK629713) are similar to S2 and share eight common changes but differ by some unique changes in S2 and S5 (Fig. S11). Haplotype S4 (MK629711) shares three amino acid changes with haplotypes S5 and S6 but has two unique amino acid changes. Haplotype S6 has a 10-bp deletion in the coding region, which alters the reading frame, modifying the last 12 amino acids.

We designed a dominant marker based on the SRAR polymorphism that differentiates the resistant haplotype from all susceptible haplotypes found so far. PCR amplification with primers SR60F2/R2 (Table S2) at an annealing temperature of 58˚C produces a 585-bp fragment only when the resistant haplotype is present, and no specific amplification in all susceptible haplotypes (Fig. S12). Using this marker, we evaluated a collection of 442 wheat accessions, including 104 T. monococcum accessions, 63 T. urartu, 49 T. turgidum ssp. dicoccoides, 89 T. turgidum ssp. dicoccon, 94 T. turgidum subsp. durum and 43 T. aestivum. WTK2 was detected in T. monococcum but not in any of the other wheat species tested in this study (Table S7).

Introggression of WTK2 into hexaploid wheat.

The introgression of WTK2 from T. monococcum accession PI 306540 into common wheat breeding line UC12014-36 (Fig. 4a) was followed during five backcrosses using CAPs marker DK722976F5R5 (digested with HhaI) and the diagnostic marker Sr60F2R2. We also used molecular markers to confirm that parental Pgt resistance genes Sr13, Sr21, SrTm4 and SrTm5 (Briggs et al., 2015; Chen et al., 2018a) were not present in the selected line.
To characterize the 5A<sup>m</sup> chromosomal translocation into hexaploid wheat, we used 5A SSR markers (Table S8). The physical locations of these markers on CS chromosome arm 5AS were obtained from the reference genome (RefSeq v1.0). Marker <i>wms443</i> (11,313,921 bp) showed the <i>T. monococcum</i> allele, whereas marker <i>gwm154</i> showed the <i>T. aestivum</i> allele indicating that the 5A<sup>m</sup>S chromosome segment introgressed into UC12014-36+Sr60 was between 11.3 Mb and 21 Mb, or 1.6%-2.9% of the length of chromosome 5A (Fig. 4b, Table S8). This <i>T. monococcum</i> segment includes two PUROINDOLINE genes associated with grain softness (Tranquilli et al., 2002), located 971 kb distal to Sr60 based on RefSeq v1.0 coordinates. The grains of the UC12014-36 BC<sub>5</sub>F<sub>3</sub> lines carrying Sr60 were significantly softer (<i>P</i> < 0.0001) than those of the sister lines without the <i>T. monococcum</i> introgression, and showed no significant differences in kernel weight, diameter and moisture content (Table S9).

Introgression line UC12014-36+Sr60 exhibited intermediate levels (IT = 2–22+) of resistance to race QFCSC, whereas its sister line lacking Sr60 showed susceptible reactions (IT = 3) to the same race (Fig. 4c). Average pustule size in UC12014-36+Sr60 was significantly smaller than in its isogenic sister line UC12014-36 without the Sr60 introgression (<i>P</i> < 0.001, Fig. 4c). BC<sub>5</sub>F<sub>3</sub> seeds from the introgression line UC12014-36+Sr60 were deposited in the U.S. National Plant Germplasm System (National Small Grain Collection) as PI 689563.

**Discussion**

**Proteins with two kinase or pseudokinase domains are an emerging class of resistance genes**

To protect against pathogen infection, plants have evolved distinct resistance mechanisms including cell surface-localized receptors, intracellular immune receptors and quantitative (broad-spectrum) disease resistance (Jones & Dangl, 2006; Krattinger & Keller, 2016). Most of the race-specific resistance genes in plants identified so far encode NLR proteins, which act as intracellular immune receptors that recognize effector proteins or the modifications
these effectors induce in host proteins, and activate effector-triggered immunity (ETI) (Jones et al., 2016). By contrast, Sr60 encodes a protein containing two putative kinase domains.

To date, only three plant resistance genes, barley stem rust resistance gene Rpg1 (Brueggeman et al., 2002), wheat stripe rust resistance gene Yr15 (Klymiuk et al., 2018), and the proposed candidate for barley true loose smut resistance gene Un8 (Zang et al., 2015) were found to possess two tandem kinase (or pseudokinase) domains. The previous three genes have been reported to confer a broader-spectrum of resistance than Sr60, suggesting that protein structure is not sufficient to predict race specificity.

The kinase domain I of Yr15 (Klymiuk et al. 2018) and the kinase domain II of RPG1 (Brueggeman et al., 2002) show conserved residues at key amino acids required for kinase function, suggesting that they are functional kinases. The same key residues are more divergent in the other domains of these two proteins, which were classified as pseudokinases (Klymiuk et al. 2018). By contrast, the key amino acid residues for kinase function were conserved in both kinase domains in both Un8 (Zang et al., 2015) and WTK2 (Sr60), suggesting that they may both represent active kinase domains. The phylogenetic analysis of the individual kinase/pseudokinase domains of these four genes indicated that they represent independent domain fusions. A recent study has described 92 kinase/pseudokinase fusions across the plant kingdom (Klymiuk et al., 2018), suggesting that these fusions may be frequent evolutionary events, or may be favored for their involvement in immune responses.

Non-arginine-aspartate (non-RD) kinases, are a subclass of kinases that are often found in association with pattern recognition receptors and are frequently involved in early steps of the innate immune response (Dardick et al., 2012). In non-RD kinases, an uncharged residue replaces the conserved positively charged R residue in the activation loop. One unique characteristic of WTK2 is the fusion of non-RD (KinI) and RD (KinII) putative kinases. The kinase/pseudokinase domains from Rpg1, Un8 and WTK1 are all non-RD kinases. Previous
studies showed that some RD kinases are required for activation of non-RD kinases, such as the cooperation between the RD kinase IRAK4 and the non-RD kinase IRAK1 (Dardick et al., 2012). It would be interesting to investigate the mechanism by which an RD and a non-RD kinase interact within the same protein to confer resistance to Pgt.

**Sr60 is rapidly upregulated and down-regulated after Pgt infection**

Alternative splicing is important in the regulation of gene expression and in the responses to biotic or abiotic stresses (Barbazuk et al., 2008; Mastrangelo et al., 2012). Alternative splicing forms have been previously reported in pathogen-resistance genes, such as Mla (Halterman et al., 2003), Lr10 (Sela et al., 2012), Sr35 (Saintenac et al., 2013), Sr21 (Chen et al., 2018b) and Yr15 (Klymiuk et al., 2018), and are also reported here for Sr60. However, the four alternative splicing forms detected for WTK2 are all located in the 5’ UTR region and do not differ in transcriptional activation profiles (Fig. S9), suggesting that they may have similar functions.

An interesting characteristic of the Sr60 expression profile is its rapid transcriptional activation one dpi (Fig. S8). This result indicates that Sr60 is involved in an early event in the Pgt infection. However, when a resistance protein is directly involved in the recognition of a pathogen or a pathogen effector, the recognition does not necessarily affect its transcription profile (unless there is a positive feedback regulatory loop). The NLR stem rust resistance gene Sr35 (Saintenac et al., 2013) that recognizes the Pgt effector AvrSr35 (Salcedo et al., 2017) showed no transcriptional differences between plants inoculated with the pathogen or mock inoculated with water. Similarly, no transcriptional upregulation was detected after pathogen infection for the stripe rust resistance gene Yr15 (Klymiuk et al., 2018), or the stem rust resistance genes Sr13 and Sr21 (Zhang et al., 2017; Chen et al., 2018b). It would be interesting to investigate if Sr60 transcription is upregulated by a separate protein responsible for the detection of the Pgt pathogen.
Two days after its rapid transcriptional upregulation, Sr60 transcripts returned to basal levels in transgenic hexaploid wheat plants, or to levels that were significantly lower than those in the mock-inoculated plants in T. monococcum (Fig. S8). This result, together with the fact that the pathogen is still actively growing in the plant at the time when Sr60 is downregulated, suggests the possibility of a feedback loop that actively downregulates Sr60 transcription after the signal that triggers the activation of the immune response is transmitted.

The transient transcriptional upregulation of Sr60 was also reflected in a transient upregulation of pathogenesis-related genes PR2 and PR9. However, the transcriptional upregulation of PR1, PR4 and PR5 extended well beyond the Sr60 upregulation. This result suggests that once Sr60 transmits its signal (likely by phosphorylation of a downstream target), its presence is no longer required to maintain the activation of this subset of PR genes. The activation of PR genes could be associated with the partial resistance response observed for Sr60. A similar partial resistance response has been described for Sr21 and Sr13, which is also associated with the activation of PR genes. However, in the case of the last two genes, the transcriptional activation of all six PR genes remained high (Zhang et al., 2017; Chen et al., 2018b). These results suggest that Sr60 may operate through a different mechanism than Sr13 and Sr21.

**Detection and utilization of Sr60 in agriculture**

A comparison of resistant and susceptible haplotypes of WTK2 revealed that the presence of the four amino acids SRAR could be used as a diagnostic marker for the presence of the resistant haplotype (Fig. S11). Using a marker based on this polymorphism, we showed that WTK2 is present in roughly 7.7% of the T. monococcum varieties but is absent in all other diploid, tetraploid and hexaploid wheats. This result suggests that the incorporation of WTK2 has the potential to benefit a wide range of commercial wheat varieties and highlights the benefits of mining new resistance genes outside the primary wheat gene pool.
The value of Sr60 to increase stem rust resistance in hexaploid wheat was successfully validated by the introgression of this gene into the susceptible common wheat breeding line UC12014-36. However, additional studies will be necessary to test if Sr60 is effective in different polyploid wheat backgrounds, and to test potential pleiotropic effects. Although the size of the introgressed segment is relatively small (less than 2.9% of the total length of chromosome 5A), the linkage between Sr60 and the PUROINDOLIN genes will affect grain texture in the varieties in which this segment is introgressed (Tranquilli et al., 2002).

Although the increased softness may be an advantage for soft wheats, the linkage between Sr60 and grain softness should be broken to expand its deployment into hard wheat varieties used for bread. The linkage can be broken by editing loss-of-function mutations in the PUROINDOLIN genes using CRISPR-Cas9, which works with good efficiency in wheat (Wang et al., 2014). Alternatively, the two genes can be separated by recombination in the presence of the ph1b mutation, which restores normal levels of recombination between the A genome of T. aestivum and the Am genome of T. monococcum (Ph1) gene (Dubcovsky et al., 1995).

Finally, the direct incorporation of Sr60 into transgenic wheat plants would avoid this limitation, and has the additional advantage that multiple resistance genes can be incorporated in the same transgenic cassette. The resistance profile of Sr60 could complement well the resistance profiles of genes Sr35 (Zhang et al., 2010), Sr21 (Chen et al., 2015) and SrTm5 (Chen et al., 2018a), which are susceptible to race QFCSC but confer resistance to Ug99. The incorporation of Sr60 to these resistance cassettes can improve resistance to races QFCSC, QTHJC and SCCSC, which were identified in the United States (Dunckel et al., 2015). However, the ineffectiveness of Sr60 to virulent isolates of the Ug99-race group (Chen et al., 2018a) limits the value of this gene in regions where these new races have been established.
In summary, the identification of \(Sr60\) and the available diagnostic marker developed in this study can contribute to diversify the stem rust resistance genes deployed in wheat breeding programs. In addition, the unique structure of \(Sr60\) can provide insights into novel mechanisms of resistance that can diversity our tools against this devastating pathogen.

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Author contributions

SC performed the research, analyzed the data and wrote the first draft. MNR designed the phenotyping experiments. MNR and JB performed the phenotyping experiments. WZ contributed quantification sporulation areas and sequence analyses. YG contributed to the mapping. SC and XZ produced the \(Sr60\) introgression lines in hexaploid wheat. JD proposed and supervised the project, obtained the funding and generated the final version of the manuscript. All authors revised the manuscript and provided suggestions.
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Supplementary Figures and Tables

**Figure S1.** Comparative analysis of the \textit{Sr60} region.
**Figure S2.** Alternative 5’ splice sites of \textit{WTK2}.
**Figure S3.** Phylogenetic analysis of plant protein kinase domains.
**Figure S4.** Transcript levels of \textit{Sr60} in \textit{T\textsubscript{0}} plants.
**Figure S5.** Pathogen growth in genotypes Fielder and \textit{Sr60}-transgenic plants.
**Figure S6.** Average sporulation area in transgenic families inoculated with \textit{Pgt} race QFCSC.
**Figure S7.** Inoculation of transgenic plants with \textit{Sr60}-virulent \textit{Pgt} races TTTTF & MCCFC.
**Figure S8.** Transcript levels of \textit{Sr60} in \textit{Pgt}-inoculated and mock-inoculated plants.
**Figure S9.** Transcript levels of alternative splicing forms of \textit{WTK2} in \textit{Pgt}-inoculated plants.
**Figure S10.** Transcript levels of \textit{PR} genes in \textit{Pgt}-inoculated and mock-inoculated plants.
**Figure S11.** Protein polymorphisms among \textit{WTK2} haplotypes.
**Figure S12.** Amplification products from the \textit{Sr60} diagnostic PCR marker.

**Table S1.** Races of \textit{Pgt} used to inoculate wheat and their response to \textit{Sr60}.
**Table S2.** Sequences of primers used in the study.
**Table S3.** Key conserved residues in both KinI and KinII domains of \textit{WTK2}.
**Table S4.** Estimated \textit{WTK2} copy number in transgenic plants.
**Table S5.** Differences in transcript levels of \textit{PR} genes.
**Table S6.** Infection types of \textit{T. monococcum} accessions used for haplotyping.
**Table S7.** Wheat accessions used to test the presence of \textit{WTK2}.
**Table S8.** Markers used to determine the length of the introgressed chromosome segment.
**Table S9.** Kernel hardness of sister lines with and without the \textit{T. monococcum} introgression.
Figure Legends

Fig. 1 Resistance response of Sr60. (a) Infection types in *Triticum monococcum* F₃ lines homozygous for the susceptible (TmS57-57) or the resistant (TmR57-32) Sr60 allele in response to *Pgt* races QFCSC (isolate 06ND76C), QTHJC (isolate 75ND717C) and SCCSC (isolate 09ID73-2). Numbers listed below leaves are average pustule sizes (n = 4). (b) *Pgt* infection areas visualized by WGA-FITC staining. Leaves were collected at 5 days post inoculation (dpi), cleared with KOH and stained with WGA-FITC. Scale bars at the bottom of the four panels = 500 μm. (c) Fungal growth estimated from the ratio of *Pgt* DNA relative to wheat DNA (n=5). TmS57-57 and TmR57-32 were selected from the cross of PI 306540 × PI 272557. ***, P < 0.001. Error bars are standard errors of the mean (SEM).

Fig. 2 Map-based cloning of Sr60. (a) Colinear region in the Chinese Spring reference genome (RefSeq v1.0). Arrows indicate genes. (b) High-density genetic map of the 5AᵉS region containing Sr60. (c) Physical map of Sr60 constructed with three overlapping BACs from the Sr60-resistant diploid wheat accession PI 306540. (d) Genes annotated within the 74.5 kb candidate region (GenBank accession MK629715) (e) Gene structure of WTK2 in PI 306540 and Chinese Spring (CS). Orange rectangles indicate exons, black lines introns and dotted lines a deleted region in CS.

Fig. 3 WTK2 confers resistance when transferred into a susceptible wheat background. (a) Reactions to *Pgt* race QFCSC in Fielder and transgenic families T₁Sr60-005 and T₁Sr60-009. S, susceptible; R, resistant. Plants were grown at 25°C during the day and 22°C during the night. (b) The average pustule size was estimated using the image analysis software ASSESS v.2.0. Twelve independent T₁ plants from every transgenic event were evaluated. Error bars are standard errors of the mean (SEM).
Fig. 4. Introggression of WTK2 into hexaploid wheat. (a) Procedure for the production of *Triticum aestivum* (UC12014-36) - *T. monococcum* (PI 306540) introgression lines. Markers *DK722976F5R5* (digested with *HhaI*) and *Sr60F2R2* were used for monitoring the present of alien chromatin. (b) Markers on chromosome 5A were used to estimate the length of the introgressed chromosomal fragment from *T. monococcum* between 11.3 Mb and 21 Mb (blue color). (c) Infection types produced by sister introgression lines with and without *Sr60* (*BC_3F_3*). Plants were grown at 25°C during the day and 22°C during the night. The numbers listed on the right side of the leaves are average pustule sizes (n = 6 leaves). ***, P < 0.001. **
Fig. 1

Pathogen growth in *T. monococcum* lines

- **(a)** +Sr60  -Sr60  -Sr60  +Sr60  +Sr60  -Sr60
- **(b)** +Sr60  -Sr60
- **(c)**

- Fungal / host DNA
- Days post inoculation (dpi)

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Fig. 2
Fig. 3
Fig. 4