Extensive Genetic Variation at the *Sr22* Wheat Stem Rust Resistance Gene Locus in the Grasses Revealed Through Evolutionary Genomics and Functional Analyses

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In the last 20 years, severe wheat stem rust outbreaks have been recorded in Africa, Europe, and Central Asia. This previously well controlled disease, caused by the fungus *Puccinia graminis* f. sp. *tritici*, has reemerged as a major threat to wheat cultivation. The stem rust (*Sr*) resistance gene *Sr22* encodes a nucleotide-binding and leucine-rich repeat receptor which confers resistance to the highly virulent African stem rust isolate Ug99. Here, we show that the *Sr22* gene is conserved among grasses in the Triticeae and Poaceae lineages. Triticeae species contain syntenic loci with single-copy orthologs of *Sr22* on chromosome 7, except *Hordeum vulgare*, which has experienced major expansions and rearrangements at the locus. We also describe 14 *Sr22* sequence variants obtained from both *Triticum boeoticum* and the domesticated form of this species, *T. monococcum*, which have been postulated to encode both functional and nonfunctional *Sr22* alleles. The nucleotide

sequence analysis of these alleles identified historical sequence exchange resulting from recombination or gene conversion, including breakpoints within codons, which expanded the coding potential at these positions by introduction of nonsynonymous substitutions. Three *Sr22* alleles were transformed into wheat cultivar Fielder and two postulated resistant alleles from Schomburgk (hexaploid wheat introgressed with *T. boeoticum* segment carrying *Sr22*) and *T. monococcum* accession PI190945, respectively, conferred resistance to *P. graminis* f. sp. *tritici* race TTKSK, thereby unequivocally confirming *Sr22* effectiveness against Ug99. The third allele from accession PI573523, previously believed to confer susceptibility, was confirmed as nonfunctional against Australian *P. graminis* f. sp. *tritici* race 98-1,2,3,5,6.

Keywords: plant biotechnology, resistance gene, *Sr22*, stem rust, Ug99, wheat

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Stem rust, caused by *Puccinia graminis* f. sp. *tritici*, is one of the most devastating fungal diseases of wheat. Historically, this disease has caused dramatic, widespread crop failures leading to significant yield losses worldwide (Singh et al. 2015). The deployment of resistant cultivars and, in some locations, the eradication of the alternate host, common barberry (*Berberis vulgaris*), has kept the disease under control over the past several decades (Peterson 2001). However, in Uganda in the 1998–99 growing season, the *P. graminis* f. sp. *tritici* race TTKSK (Ug99) emerged, which was virulent on the majority of the world’s wheat cultivars, including those carrying the previously highly effective stem rust (*Sr*) resistance gene *Sr31*, thereby raising major concerns for global food security (Pretorius et al. 2000). Subsequently, Ug99 and variants thereof have spread throughout eastern and southern Africa, Yemen, and Iran (Nazari et al. 2009; Patpour et al. 2016; Pretorius et al. 2010; Singh et al. 2015). Moreover, this race has evolved rapidly and 13 TTKSK variants have been identified with virulence on *Sr24*, *Sr36*, and *SrTnp* (Fetch et al. 2016; Patpour et al. 2016; Pretorius et al. 2012;

Singh et al. 2015; Terefe et al. 2018). In more recent years, additional stem rust outbreaks have been reported in Europe (Berlin 2017; Bhattacharya 2017; Olivera Firpo et al. 2017), Asia (Shamanin et al. 2016), and Africa (Olivera et al. 2015) caused by other highly virulent *P. graminis* f. sp. *tritici* races unrelated to the Ug99 lineage. Collectively, Ug99 and these newer *P. graminis* f. sp. *tritici* races pose a significant threat to global food security.

Fungicide application only partially controls wheat stem rust disease. The deployment of resistance (*R*) genes is the most economical and environmentally sustainable control method. In all, 60 *Sr* resistance genes have been designated to date (Chen et al. 2018a; McIntosh et al. 2017; Zhang et al. 2020) and 11 major, dominant *Sr* genes have been cloned (Arora et al. 2019; Chen et al. 2018b, 2020; Mago et al. 2015; Periyannan et al. 2013; Saintenac et al. 2013; Steuernagel et al. 2016; Zhang et al. 2017; Zhang et al. 2020). These cloned *Sr* genes encode intracellular immune receptor proteins containing nucleotide-binding and leucine-rich repeat domain (NLR) proteins (in the case of *Sr13*, *Sr21*, *Sr22*, *Sr26*, *Sr33*, *Sr35*, *Sr45*, *Sr46*, *Sr50*, and *Sr61*) or a tandem kinase (*Sr60*). In contrast only two *P. graminis* f. sp. *tritici* avirulence (*Avr*) genes have been cloned that each encode a corresponding pathogen effector molecule recognized by the *Sr35* and *Sr50* NLR proteins, respectively (Chen et al. 2017; Salcedo et al. 2017).

Recent genetic and genomic studies have provided insights into the molecular evolution of NLR genes and the molecular mechanisms that generate sequence diversity in these genes. Genome sequencing has shown that most NLRs exist as members of small clusters of sequence-related paralogs, such as the lettuce *Dm3/13* (Meyers et al. 1998b) and tomato *I2* clusters (Ori et al. 1997). In potato, 73% of mapped NLR genes are grouped into 63 clusters in the genome (Jupe et al. 2012), with similar NLR clusters observed in the *Arabidopsis* and rice genomes (Bai et al. 2002; Meyers et al. 1999). These gene clusters are thought to provide a reservoir of genetic variation to generate new resistance specificities by recombination or gene conversion (Ellis et al. 2000; Hulbert et al. 2001; Michelmore and Meyers 1998). Extensive historical recombination between paralogs within *R* gene clusters have been observed in tomato (Parniske et al. 1997), *Arabidopsis* (Meyers et al. 1998a), and flax (Dodds et al. 2001; Ellis et al. 1999) based on mosaic patterns of conserved sequence segments. Genetic analyses revealed that recombination played a central role in the evolution of new specificities at the *M*, *N*, and *P* loci in flax (Dodds et al. 2001); the *Rp1* rust resistance complex in maize (Hulbert 1997); and the *Cf-4/Cf-9* Milky Way locus in tomato (Parniske and Jones 1999; Parniske et al. 1997). In some cases, NLR genes exist as simple loci containing a single gene such as the *Arabidopsis* *RPM1* (Stahl et al. 1999) and *RPP13* loci (Bittner-Eddy et al. 2000) and the flax *L* locus (Ellis et al. 1999).

Unequal recombination at *R* gene clusters has been observed relatively frequently at the maize *Rp1* locus (Sun et al. 2001) and, more rarely, at the lettuce *Dm3* locus (Michelmore and Meyers 1998). Tandem duplication of paralogous genes by unequal crossover has been an important mechanism in generating sequence variation at *Arabidopsis* NLR loci (Meyers et al. 2003). Gene duplication can also explain copy number variation existing between haplotypes such as that observed at the lettuce *Dm3* cluster (Kuang et al. 2004) and potato *MLB* cluster (Jupe et al. 2012). At loci containing a single NLR, diversifying selection coupled with recombination between allelic variants can also result in the evolution of new alleles. This was observed in the flax *L* locus, in which frequent interallelic recombination events produced at least a dozen novel alleles (Dodds et al. 2006). Very rarely, ectopic recombination between different clusters, even between those located on different chromosomes, may also occur (Parniske and Jones 1999). Consequently, although *R* gene clusters usually contain closely related sequences, individual genes may be more

related to sequences from other clusters within the genome than to members of the same cluster.

The *Sr22* gene was recently cloned using a mutagenesis and sequence capture approach from a hexaploid wheat introgression line, Schomburgk, in which the gene had been introgressed into chromosome 7A from the diploid A genome relative, *Triticum boeoticum* (Steuernagel et al. 2016). This gene confers broad-spectrum resistance to multiple races of *P. graminis* f. sp. *tritici*, including the Ug99 race group (Steuernagel et al. 2016). Previously (Steuernagel et al. 2016), we identified 14 *Sr22* sequence variants in *T. boeoticum* and *T. monococcum*, some of which were believed to confer functional *Sr22* resistance (Rouse and Jin 2011a). Herein, we describe several more *Sr22* alleles, provide evidence of historical sequence exchange in some alleles, and demonstrate that two of these alleles also confer functional *Sr22* resistance. We also inspected grass genomes and transcriptomes and found that *Sr22* likely arose in the last common ancestor of the Triticeae and Poeae lineages, and then evolved as a single gene locus, except in barley and oat, where the locus experienced pronounced independent expansions.

RESULTS

The majority of Triticeae genomes contain single-copy orthologs of *Sr22* on chromosome 7.

To understand the evolutionary history of *Sr22*, we conducted a comprehensive search for *Sr22* homologs in 40 genomes and 122 transcriptomes covering a total of 80 plant species, 76 of which belong to the monocot lineage (Fig. 1A; Supplementary File S1). Phylogenetic analysis recovered *Sr22* orthologs specifically in the genomes and transcriptomes of the Triticeae and Poeae species examined (Fig. 1B; Supplementary File S2). The *Sr22* protein tree largely mirrored the phylogeny of the species studied here. In the Triticeae species, all but *Hordeum vulgare* possessed single-copy orthologs of *Sr22*. Microsynteny analysis of the Triticeae genomes revealed the presence of single-copy orthologs of *Sr22* on chromosome 7 in each of the subgenomes of *T. aestivum* (7A, 7B, and 7D), *T. turgidum* (7A and 7B), and *T. diccoides* (7A and 7B) wheat, as well as in the genomes of the wheat wild relative species *Aegilops tauschii* (on chromosome 7D) and *T. urartu* (not assigned to a chromosome because the available assembly is not chromosome level) (Fig. 2; Supplementary File S1; Supplementary File S3). In the case of *H. vulgare*, we identified an *Sr22* syntenic region on 7H containing multiple NLR loci (several of which were orthologous to *Sr22*) interspersed with other non-NLR genes, likely the result of local gene duplication and rearrangement events. To confirm that this was not due to assembly errors and to check whether this was a cultivar-effect specific to the *H. vulgare* ‘Morex’ or more broadly conserved in *Hordeum* spp., we conducted microsynteny analysis of other *Hordeum* accessions and found similar expansion of the *Sr22* locus in *H. vulgare* ‘Golden Promise’ as well as *H. vulgare* var. *nudum* (Supplementary Fig. S2; Supplementary File S3). We also observed a similar expansion of the *Sr22* locus in the genomes of the diploid oat progenitors *Avena atlantica* and *A. eriantha* from the Poeae lineage (Fig. 2; Supplementary File S3). On the other hand, although our searches uncovered other NLRs homologous to *Sr22* in *Brachypodium distachyon* and the *Oryza* spp. studied here, orthologs of *Sr22* were not found in these species (Supplementary File S2). Although *Sr22* orthologs were not detected, regions exhibiting conservation of microsynteny to those in the Triticeae species investigated above could be identified in *Oryza sativa* and *B. distachyon* (Fig. 2). Of the 20 genes flanking the *Sr22* ortholog in the 7D subgenome of *T. aestivum*, homologs of five and six of these

Fig. 1. Orthologs of *Sr22* can be found in a wide range of species in the Triticeae and Poeeae grass lineages. **A**, Phylogenetic representation of the plant and monocot lineages from which species were examined for the presence of *Sr22* orthologs. Relationships between the lineages depicted are based on previous reports (Soreng et al. 2017; The Angiosperm Phylogeny Group et al. 2016) (Angiosperm Phylogeny Website). Numbers of species studied in each lineage and the type of dataset used are indicated in the figure. **B**, Maximum-likelihood phylogenetic tree of the *Sr22* clade containing proteins from the Triticeae and Poeeae lineages. The tree was constructed from an alignment corresponding to the NB-ARC1-ARC2 region of *Sr22* using 38 known nucleotide-binding and leucine-rich repeat domain (NLR) proteins as an outgroup. The query sequence used to retrieve these orthologous proteins was the *Triticum aestivum* Schomburgk *Sr22* protein (highlighted in pink). Orthologs retrieved from genomes are shown in darker shades with square taxon markers while those retrieved from transcriptomes are shown in lighter shades with diamond markers. Triticeae *Sr22* proteins are shown in blue while Poeeae *Sr22* proteins are shown in orange. Where more than one sequence was retrieved from a transcriptome without isoform information, the sequences are sequentially numbered starting from T1. Branch support values from 1,000 bootstrap replicates are indicated on the tree, which was constructed using the JTT+I+G4 evolutionary model.

genes were found in contiguous regions on chromosomes 1 and 6 of *B. distachyon* and *O. sativa*, respectively (Fig. 2; Supplementary File S3). We further examined this through searches on the pangenomes of *Brachypodium* spp. and rice covering 54 and 3,010 accessions (Supplementary File S1) respectively, but again did not find any *Sr22* orthologs (Supplementary File S4). The above findings suggest that the ancestral *Sr22* gene likely evolved in the last common ancestor of the Triticeae and Poaceae lineages. However, the strength of this hypothesis is limited by the lack of genomic resources for key grass lineages such as the Bromelaceae, a sister lineage to the Triticeae. As high-quality genomes of further grass species from evolutionarily important nodes become available, more comprehensive analyses can be carried out to test this hypothesis.

Historical recombination at the *Sr22* locus.

In total, 14 *Sr22* variants were previously PCR amplified and sequenced from *T. monococcum* and *T. boeoticum* accessions postulated to carry *Sr22* (Steuernagel et al. 2016). Two of these sequence variants were excluded from further analysis due to possible germplasm redundancy. Screening of another 25 accessions identified an additional eight sequence variants from these two species. Six of these eight sequences were identical to those previously observed, bringing the total number of unique

Sr22 alleles identified to 14. A possible new allele of *Sr22*, *SrTm5*, which has been recently mapped and characterized from *T. monococcum* accession PI306540 (Chen et al. 2018a), was also included in the analysis.

Nucleotide sequence comparison of these 15 sequences identified 136 informative polymorphic sites (IPS) (i.e., shared by at least two alleles) in the 2,841-bp *Sr22* open reading frame (ORF) (Fig. 3). Based on sequence similarity, eight of these genes could be assigned into three groups (i.e., group 1 = IG44855, Schomburgk, and IG44857; group 2 = PI289605, PI330550, and PI190945; and group 3 = PI573523 and IG44878) while the remaining seven alleles had no strong sequence affiliation with other variants. Group 1 shared greater than 99% sequence identity with each other whereas greater sequence variation was observed in the other two groups (Fig. 3). Several blocks of sequences containing conserved IPS (>3) that deviated from the consensus sequence were shared between at least two *Sr22* variants (Fig. 3, gray and black boxes). PI289605, PI330550, PI190945, PI573523, and IG44878 share almost continuous sequence identity from position 2,400 to 2,572 but PI289605 and PI330550 are most closely related to Schomburgk from position 276 to 2,272. This obvious mosaic pattern is indicative of sequence exchange between *Sr22* alleles resulting from recombination or gene conversion as observed

Fig. 2. *Sr22* is a conserved nucleotide-binding and leucine-rich repeat domain (NLR) found on chromosome 7 in Triticeae species. Genomic regions of Triticeae species containing genes orthologous to *Sr22* show microsynteny conservation. *Sr22* orthologs were not detected in *Oryza sativa* and *Brachypodium distachyon* but regions exhibiting conservation of microsynteny to the region containing *Sr22* in the Triticeae species investigated above could be identified. Genes are colored according to their predicted functional annotations. *Sr22* orthologs are colored green. Full-length NLR genes are shown with solid outlines while partial NLR genes are shown with dashed outlines (as predicted by NLR-Annotator). Gene families and model species' homologs are numerically coded for each gene. A question mark implies an unknown gene family or homolog.

for the tomato *Hcr9* genes (Parniske and Jones 1999; Parniske et al. 1997) and *Arabidopsis RAC1* and *RPP13* (Serra et al. 2018). A similar mosaic was seen for the PI190945 sequence which is most closely related to IG44857 from position 284 to 2,272 but then shares an IPS sequence signature with PI289605 and PI330550 from position 768 to 2,809. Six potential intracodon recombination breakpoints were detected, all of which resulted in nonsynonymous codons (Fig. 3, red arrows).

Confirmation of *Sr22* variant function in stable wheat transgenics.

Previously, a screen of 1,061 *T. monococcum* accessions for resistance to five *P. graminis* f. sp. *tritici* races with widely different virulence profiles (TTKSK [Ug99], TRTTF, TTTTF, QFCSC, and MCCFC) identified 55 accessions that were resistant to all five races. Of these, 15 accessions were postulated to carry functional alleles of *Sr22* based on infection type similarities with known monogenic *Sr22* lines (Rouse and Jin 2011b). Three *Sr22* alleles, two of which were predicted to be functional, were transformed into wheat cultivar Fielder and tested for resistance function. This study focused on the coding sequence of the *Sr22* genes. Therefore, to exclude phenotype

differences potentially arising from differences between the regulatory sequences of these variants, a common synthetic promoter and terminator was used in each case.

A 9.7-kb sequence encoding the Schomburgk *Sr22* sequence (Steuernagel et al. 2016) was fused to 5' promoter and 3' terminator sequences of the *Sr33* gene (Periyannan et al. 2013) (Table 1). The *Sr33* gene, although sequence unrelated to *Sr22*, is similar in that it also encodes a major, race-specific wheat stem rust NLR gene. Ten independent T₀ transgenic Fielder plants carrying this sequence (PC132) were all found to be resistant to the Australian wheat stem rust race *P. graminis* f. sp. *tritici* 98-1,2,3,5,6 (Table 2; Fig. 4). Moreover, the infection type was comparable with those seen on Fielder wheat transgenics containing an *Sr22* transgene with endogenous regulatory sequences (construct PC103) (Steuernagel et al. 2016) (Tables 1 and 2; Fig. 4).

To facilitate more high-throughput allele testing, a Golden Gate-compatible construct (PC127) encoding *Sr33* 5' and 3' regulatory sequences and a "domesticated" *Sr22* ORF were produced and introduced into Fielder wheat. Ten independent PC127 T₀ families were tested with Australian *P. graminis* f. sp. *tritici* race 98-1,2,3,5,6 and found to be resistant, with an

Fig. 3. Extensive historical intragenic recombination between *Sr22* variants. Only informative polymorphic sites (IPS) (a polymorphic nucleotide which is shared between at least two sequences within the alignment) of the nucleotide sequences of the 15 *Sr22* variants are shown. This reduces the total length of the sequence alignment from 2,841 nucleotides to 136. Vertical numbers refer to nucleotide position within the global alignment of 2,841 nucleotides. The domain structure of the *Sr22* protein according to Steuernagel et al. (2016) is indicated in the upper panel. Red arrows indicate possible intracodon recombination breakpoints which resulted in new amino acids at these positions. The almost continuous sequence affiliations within the variants of IG44855, Schomburgk, and IG44857 (upper); PI289605, PI330550, and PI190945 (middle); and PI573523 and IG44878 (bottom) are boxed. Sequence patches of at least three consecutive IPS that deviate from the consensus sequence and are shared between at least two *Sr22* variants are highlighted by black and gray boxes. Patches shared with IG44855, Schomburgk, IG44857, PI330550, PI289605, and PI190945 are shown by white letters in black boxes. Patches shared with all variants except PI330550, Westonia, and PI352504, are highlighted by gray boxes. Cons = consensus sequence (modal value shared by more than 1 residue is indicated by "+" symbol in the display for the simple reason that it is not possible to display multiple characters in a single character space); dots indicate nucleotides identical to the consensus and dashes indicate gaps in the alignment.

infection type similar to the equivalent undomesticated synthetic construct (PC132), confirming that the domestication process had not altered gene function (Table 2; Fig. 4).

Golden Gate-based constructs using the same *Sr33* regulatory sequences were then developed for *Sr22* alleles from accessions PI190945 (construct PC130) and PI573523 (construct PC146), which had been postulated to carry functional and nonfunctional *Sr22* alleles, respectively (Table 3) (Rouse and Jin 2011a). Ten independent T₀ families were again tested for each construct with plants containing construct PC130 resistant to *P. graminis* f. sp. *tritici* race 98-1,2,3,5,6, whereas plants carrying construct PC146 were susceptible (Table 2; Fig. 4). Based on this result, we designated the resistant allele as *Sr22* and the susceptible allele as *sr22*. Finally, we produced two constructs that expressed the Schomburgk *Sr22* (PC126) and PI573523 *sr22* (PC131) alleles, respectively, each under the regulatory control of a maize polyubiquitin promoter (Table 1). In total, 10 T₀ families containing PC126 showed resistance against *P. graminis* f. sp. *tritici* race 98-1,2,3,5,6 (Table 2; Fig. 4). No appreciable difference in resistance was observed when the Schomburgk *Sr22* allele was expressed with the maize polyubiquitin promoter compared with its endogenous regulatory sequences. Therefore, the maize polyubiquitin promoter can also be used to express functional *Sr22*-mediated resistance. The 10 T₀ families containing construct PC131 were also tested with *P. graminis* f. sp. *tritici* race 98-1,2,3,5,6 (Table 2; Fig. 4), where a marginal decrease in susceptibility, from 4 to 3+, was observed compared with Fielder transgenics expressing the wild-type *sr22* PI573523 sequence (Table 2; Fig. 4).

***Sr22* wheat transgenics confer resistance against *P. graminis* f. sp. *tritici* race TTKSK (Ug99).**

Sr22 is a valuable resistance gene conferring effective resistance against the virulent African *P. graminis* f. sp. *tritici* race TTKSK, also known as Ug99 (Singh et al. 2006). To

confirm that *Sr22* and its allele in PI190945 is sufficient to confer resistance against Ug99, we isolated homozygous T₂ lines carrying either Schomburgk (PC126, PC127, PC128, and PC103) or PI190945-derived *Sr22* gene constructs (PC129 and PC130). These T₂ lines were found to confer resistance against *P. graminis* f. sp. *tritici* race TTKSK (Table 4; Fig. 5).

DISCUSSION

Disease resistance genes can occur as simple single-gene loci with allelic variation between accessions, or as complex multigene clusters containing related paralogs with scope for copy number variation between accessions. These configurations enable new alleles and genes to be generated by intergenic or intragenic recombination. For the *Sr22* gene locus studied here, we uncovered evidence for both of these configurations in grasses.

Through a comprehensive phylogenetic analysis, we found that the *Sr22* gene locus exhibits a clustered distribution in the grass species *H. vulgare*, *A. atlantica*, and *A. eriantha*. The expansions in these species in the Triticeae and Poeae lineages are likely to have happened independently, as indicated by the *Hordeum* and *Avena* *Sr22* orthologs resolving into separate clades (Fig. 1B). Although the orthology relationships between these Poeae and Triticeae *Sr22* loci are clear, the regions containing the *Avena* *Sr22* orthologs exhibit very little synteny to the Triticeae *Sr22* genomic regions, suggesting that the *Sr22* orthologs in these two clades undertook vastly different trajectories since these lineages last shared a common ancestor.

On the other hand, analysis of the *Sr22* locus in wheat and its wild relatives revealed single-copy orthologs located on chromosome 7 in *T. aestivum*, *T. turgidum*, *T. diccoides*, *Aegilops tauschii*, and *T. urartu* (Fig. 2). Therefore, this locus appears to have remained as a simple, single-gene locus in wheat species since the separation of the A, B, and D lineages 7 million years ago (Marcussen et al. 2014). This physical organization in

Table 1. List of binary constructs with *Sr22* variants^a

Name	ID	<i>Sr22</i> variant	Promoter	Terminator	Addgene
pBW_0001	PC126	Schomburgk	Maize ubiquitin	<i>Sr22</i> _Schomburgk	102833
pBW_0002	PC127	Schomburgk	<i>Sr33</i>	<i>Sr33</i>	102834
pBW_0003	PC128	Schomburgk	<i>Sr33</i>	<i>Sr22</i> _Schomburgk	102835
pBW_0004	PC130	PI190945	<i>Sr33</i>	<i>Sr33</i>	102836
pBW_0005	PC131	PI573523	Maize ubiquitin	<i>Sr22</i> _Schomburgk	102837
pBW_0006 ^b	PC132	Schomburgk	<i>Sr33</i>	<i>Sr33</i>	102838
pBW_0007	PC146	PI573523	<i>Sr33</i>	<i>Sr33</i>	102839
...	PC103 ^c	Schomburgk	<i>Sr22</i> _Schomburgk	<i>Sr22</i> _Schomburgk	SP ^d
...	PC129	PI190945	<i>Sr22</i> _PI190945	<i>Sr22</i> _PI190945	SP ^d

^a All constructs are in pVecBarII vector with phosphinothricin as plant resistance.

^b Synthesized as native, undomesticated gene.

^c Published by Steuernagel et al. (2016).

^d Available from Sambasivam Periyannan upon request.

Table 2. Stem rust infection assays with Australian *Puccinia graminis* f. sp. *tritici* race 98-1,2,3,5,6 on *Sr22* variant T₀ families^a

Plant ID	Line	Plant A	Plant B	Plant C	Plant D	Plant E	Plant F	Plant G	Plant H	Plant I	Plant J
PC126	PC126_T1	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =
PC127	PC127_T1	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =
PC128	PC128_T1	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =
PC130	PC130_T1	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =
PC131	PC131_T1	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
PC132	PC132_T1	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =	2 =
PC146	PC146_T1	4	4	4	4	4	4	4	4	4	4
PC103 ^b	PC103_T1	2–	2–	2–	2–	2–	2–	NA	NA	NA	NA
Fielder	S control ^c	4	4	4	4	4	4	4	4	4	4

^a NA = not available.

^b Published by Steuernagel et al. (2016).

^c Susceptible control.

wheat species is similar to the *Arabidopsis Rpm1* (Stahl et al. 1999) and *RPPI3* (Bittner-Eddy et al. 2000) genes but in contrast to most resistance genes which are members of large clusters of gene paralogs such as the tomato *Cf-4* and *Cf-9* genes at the Milky Way locus (Parniske et al. 1997), the flax *M* locus (Anderson et al. 1997), the *Arabidopsis RPP5* locus (Noël et al. 1999), the RGC2 locus in lettuce (Kuang et al. 2004), and the maize *Rp1* locus (Sun et al. 2001). In the case of the complex barley *Mla* (powdery mildew) resistance locus (Wei et al. 2002), stem rust resistance occurs in haplotypes derived from both *Aegilops tauschii* (*Sr33*) (Periyannan et al. 2013) and rye (*Sr50*) (Mago et al. 2015). By analogy, it is conceivable that *Sr22* variants from outside the genus *Triticum* also provide stem rust resistance, although this hypothesis was not tested in the present study.

Nucleotide sequence alignment of the 15 *Sr22* alleles showed that the greatest sequence variation occurs in the leucine-rich repeat (LRR) domain. This is consistent with its proposed role in pathogen recognition specificity, which is believed to explain the higher degree of diversifying selection typically found in this part of the protein (Dodds et al. 2006; Krasileva et al. 2010). Comparison of these nucleotide sequences also showed evidence of extensive historical recombination between alleles based on obvious mosaic patterns of sequence conservation (Fig. 3). This “gene shuffling” most likely resulted from multiple, iterative intragenic sequence exchange events and is proposed to be a major mechanism in generating sequence variation from which new resistance specificities are selected during host–pathogen coevolution (Parniske et al. 1997). Because the wheat *Sr22* locus is

physically simple, with only a single gene member, the observed sequence variation between alleles is most likely due to intraallelic recombination, as observed for the *Arabidopsis RAC1* and *RPPI3* genes (Serra et al. 2018). Intraallelic recombination can also be unequal, particularly between tandem repeat domains such as in the LRRs (Dixon et al. 1998).

From 22 accessions that were initially identified, 8 contain the same *Sr22* allele. This may indicate that they were derived from a common parent (which was erroneously given different accession numbers) or that there is conservation of sequence at this locus among genetically distinct accessions. The generation of additional genotype data outside of the *Sr22* locus would be required to further elucidate this question.

In this study, we confirmed the function of three *Sr22* alleles by generating and phenotyping transgenic wheat plants (Figs. 4 and 5). As postulated, two of these *Sr22* alleles were found to confer resistance against *P. graminis* f. sp. *tritici* race 98-1,2,3,5,6 and TTKSK. We used multisegment Golden Gate assembly (Weber et al. 2011) to rapidly generate constructs and negate potential expression differences that could arise from variation in allele regulatory sequences. We used the *Sr22* promoter and terminator, *Sr33* promoter and terminator, and maize ubiquitin promoter to test three allelic variants of *Sr22*. All of these regulatory elements were undomesticated, apart from the *Sr22* terminator. In the future, these modular components can be used for allele mining and functionality testing of candidate stem rust resistance genes identified by map-based approximation (e.g., TACCA [Thind et al. 2017]), mutational genomics (e.g., MutRenSeq [Steuernagel et al. 2016]) and

Fig. 4. Phenotypic reaction of transgenic *Sr22* variants against Australian *Puccinia graminis* f. sp. *tritici* race 98-1,2,3,5,6. **A**, Schematic overview (not shown to scale) of the constructs used in stem rust disease resistance assays. Binary construct containing (i) *BsaI*-domesticated *Sr22* Schomburgk (*Sch*) driven by 5′ maize ubiquitin (*Ubi*) and *BsaI*-domesticated 3′ *Sr22* regulatory elements (*tSch*), respectively; (ii) *BsaI*-domesticated *Sr22* Schomburgk driven by 5′ and 3′ *Sr33* regulatory elements (*pSr33* and *tSr33*, respectively); (iii) *BsaI*-domesticated *Sr22* Schomburgk driven by 5′ *Sr33* and *BsaI*-domesticated 3′ *Sr22* regulatory elements, respectively; (iv) *BsaI*-domesticated *Sr22* PI190945 driven by 5′ and 3′ *Sr33* regulatory elements; (v) *BsaI*-domesticated *Sr22* PI573523 driven by 5′ maize ubiquitin and *BsaI*-domesticated 3′ *Sr22* regulatory elements, respectively; (vi) native *Sr22* Schomburgk driven by 5′ and 3′ *Sr33* regulatory elements; (vii) *BsaI*-domesticated *Sr22* PI573523 driven by 5′ and 3′ *Sr33* regulatory elements; and (viii) native *Sr22* Schomburgk driven by its 5′ and 3′ regulatory elements (*pSch* and *tSch*, respectively). Nonnative four-nucleotide linkers in pBW_0001, pBW_0002, pBW_0003, pBW_0004, pBW_0005, and pBW_0007 were introduced immediately before the start codon, in the middle of the coding region, and immediately after the stop codon. White arrows correspond to the removed *BsaI* sites. **B**, Stem rust infection assays using the Australian *P. graminis* f. sp. *tritici* race 98-1,2,3,5,6 on representative seedlings of primary transgenic lines (T_0) of *Sr22* variants and comparison with the susceptible control Fielder.

MutChromSeq [Sánchez-Martín et al. 2016]), or association genetics (e.g., AgRenSeq [Arora et al. 2019]).

MATERIALS AND METHODS

Phylogenetic analysis to identify *Sr22* orthologs.

The coding sequence of the *Sr22* gene from wheat cultivar Schomburgk was translated and the predicted amino acid sequence was used as a query for BLASTP searches against the predicted protein sequences derived from the genomes and transcriptomes of 80 plant species (Supplementary File S1). The taxonomic status of the 80 plant species used here was determined using the Angiosperm Phylogeny Group IV system APG IV (The Angiosperm Phylogeny Group et al. 2016) (Tropicos database and the Angiosperm Phylogeny Website) for non-Poaceae and, for Poaceae species, the system presented by Soreng et al. (2017) was used. A representative phylogeny of the different plant clades studied here (Fig. 1A) was made using TreeGraph version 2 (Stöver and Müller 2010) and edited in Adobe Illustrator. Predicted protein sequences for the genomes used were downloaded from the respective genome database (Supplementary File S1) whereas, for the transcriptomes used here, predicted proteins were obtained using TransDecoder version 5.5.0 (Haas et al. 2013). The top 25 hits from each species in the genome dataset and the top 3,000 hits from the combined transcriptome data were obtained and aligned with MUSCLE version 3.8.425 (Edgar 2004) using default parameters. An initial tree was constructed with this alignment as input using FastTree version 2.1.11 (Price et al. 2010)

with the WAG model to identify putative *Sr22* homologs. In the above preliminary phylogeny, the *Sr22* clade was identified and the tree was manually curated to retain only the longest isoform for genes where multiple isoforms were picked up by the BLAST searches. Because no orthologs of *Sr22* were found in the proteomes of *Aegilops tauschii* and *T. diccoides*, a further search in the genomes of these species was conducted as detailed in the “Manual identification of *Sr22* homologs” section below. BLASTP searches on the pangenomes of *Brachypodium* spp. and rice were also conducted using the translated coding sequences of the wheat Schomburgk *Sr22* gene to check whether any *Sr22* orthologs could be found in these pangenomes. The top 50 hits from each pangenome were combined with the sequences obtained from the Triticeae, rice, and *Brachypodium* genomes in the initial search and aligned using MUSCLE version 3.8.425, and a phylogeny was constructed with IQTree version 1.6.10 (Nguyen et al. 2015) using default options. The JTT+F+R8 evolutionary model was found to best explain the evolution of these sequences, and branch support was assessed using 1,000 bootstrap replicates for UFBoot and SH-aLRT.

In *H. vulgare*, several proteins orthologous to *Sr22* with consecutive identifications (IDs) were found. To ensure that these were not artifacts of the gene prediction process, the *H. vulgare* genomic region containing these genes was manually curated (as detailed in the “Manual curation of syntenic region in *H. vulgare*” section below) to collect only high confidence gene annotations. The resultant curated *Sr22* homologous sequences were then used to construct an alignment using MUSCLE version 3.8.425. The

Table 3. Stem rust resistance score for selected diploid accessions of *Triticum boeoticum* and *T. monococcum* and hexaploid wheat cultivars^a

Species, accession (<i>Sr22</i> allele)	<i>Sr22</i> ^b	Infection type							
		Australian <i>Puccinia graminis</i> f. sp. <i>tritici</i>			Rouse and Jin 2011b				
		17-1,2,3,7 = 142	98-1,2,3,5,6	194-1,2,3,5,6	TRTTF	TTKSK	TTTTF	QFCSC	MCCFC
<i>T. monococcum</i>									
PI289605	+	NA	NA	NA	2–	2–	2–	;1	;1
PI355523	–	NA	NA	NA	3+	3	4	3+	4
PI330550	+	NA	NA	NA	2	2	2/2+	;1–	;1
PI190945	+	NA	NA	NA	1;	1	2–;	;1–	;1
W3534	+	22–	NA	NA	NA	NA	NA	NA	NA
DV92	–	NA	NA	NA	NA	NA	NA	3+	NA
PI573523	–	NA	NA	NA	2–	;N	2	;2–	;1–
PI272557	–	NA	NA	NA	3+	4	4	4	4
PI362553	+	NA	NA	NA	2–	1	2–	;2 =	;1
PI362554	+	NA	NA	NA	2–	;1	2–	;2–	;1
PI355522	+	NA	NA	NA	1;	;1	1;/2–	;12–	;1
<i>T. boeoticum</i>									
IG44855	+	2 =	2 =	1+	NA	NA	NA	NA	NA
IG44857	+	2 =	2–	NA	NA	NA	NA	NA	NA
IG44921	+	2 =	2 =	1+	NA	NA	NA	NA	NA
IG44878	–	3+	NA	NA	NA	NA	NA	NA	NA
PI352504	+	NA	NA	NA	2	2–	2	;2–	;1
IG44870	+	2 =	1	1	NA	NA	NA	NA	NA
IG44866	+	2 =	1	1–	NA	NA	NA	NA	NA
IG44868	+	2 =	NA	NA	NA	NA	NA	NA	NA
IG44919	+	2 =	2 =	NA	NA	NA	NA	NA	NA
<i>T. aestivum</i>									
Westonia	–	3+	NA	NA	NA	NA	NA	NA	NA
Schomburgk	+	2–	NA	NA	NA	NA	NA	NA	NA
GM Schomburgk ^c	...	NA	2–	NA	NA	2	NA	NA	NA
GM PI190945 ^d	...	NA	2 = ^e	NA	NA	2 ^f	NA	NA	NA
GM PI573523 ^g	...	NA	4	NA	NA	NA	NA	NA	NA

^a NA = not available.

^b *Sr22* postulation.

^c Genetically modified (GM) (i.e., transgenic) Fielder with Schomburgk *Sr22* allele. Driven by native regulatory elements and published by Steuernagel et al. (2016).

^d GM Fielder with PI190945 *Sr22* allele.

^e Driven by *Sr33* regulatory elements.

^f Driven by native regulatory elements.

^g Driven by *Sr33* regulatory elements.

conserved NB-ARC regions in these sequences were extracted using the procedure detailed below. First, the sequences were aligned using HMMER3 HMMALIGN (Eddy 2009) to a previously published NB-ARC1-ARC2 HMM (Bailey et al. 2018) and any sites with greater than 95% gaps in the alignment were removed. Only sequences with more than 200 amino acid residues corresponding to this region in the alignment were then retained. The NB-ARC1-ARC2 regions from a curated set of 38 *R*-gene

Table 4. Stem rust infection assays with *Puccinia graminis* f. sp. *tritici* race TTKSK on *Sr22* variants T₂ families^a

Event, line	Rep 1		Rep 2	
	N	IT	N	IT
PC103-1	5	2	5	2
PC103-2	5	2	5	2
PC103-4	5	22–	4	22–
PC103-5a ^b	5	3+	5	3+
PC126-2	5	22+	5	2–
PC126-3	5	2–	5	2–
PC126-5 ^b	5	3+	5	3+
PC127-2	5	2–	5	22–
PC127-3	5	2–	5	2–
PC127-5	5	2–	5	2–
PC127-6 ^b	5	3+	5	3+
PC128-5	4	2–	5	22–
PC128-6	4	2–	5	22–
PC128-7 ^b	4	33+	4	33+
PC129-4	5	2	5	2
PC129-5	5	2	5	22–
PC129-6 ^b	5	33+	5	33+
PC130-2 ^c	5	2–	4	22–
PC130-4 ^c	3	22–	5	22–
Fielder	5	3+	5	3+

^a N = Number of tested plants and IT = infection type.

^b Null lines.

^c Published by Steuernagel et al. (2016).

proteins were added to provide outgroup references for the final phylogeny and an alignment was constructed using HMMER3 HMMALIGN (Eddy 2009) against the NB-ARC1-ARC2 HMM.

Based on this alignment, we then constructed a phylogenetic tree (Supplementary File S2) using the default options in IQTree version 1.6.10 (Nguyen et al. 2015) and assessing branch support with 1,000 bootstrap replicates for UFBoot and SH-aLRT using the JTT+F+R10 evolutionary model, which was found to be the best fit for these sequences. The sequences from the *Sr22* clade from this tree and the curated *R*-gene dataset (Bailey et al. 2018) were extracted and a phylogeny was generated (Fig. 1B) as described above using IQTree using the JTT+I+G4 evolutionary model, which was found to be the best fit for these sequences. The alignments used to generate the phylogenetic trees can be found in figshare.

Manual curation of syntenic region in *H. vulgare*.

For the genomic region on chromosome 7H in *H. vulgare* containing *Sr22* orthologs, several gene annotations were not supported by coding sequence annotations or spanned multiple coding sequences over a length of ≥ 100 kb. In addition, some annotations overlapped with respect to genomic positions. To obtain reliable gene predictions, the region was manually curated to keep only those genes with coding sequence support. Following this, the longest isoform for a given gene was used. If overlapping coding sequences were found to be of the same length, those containing start and stop codons were retained. The genomic region was also parsed using NLR-Annotator to identify loci in the region that potentially coded for an NLR (Steuernagel et al. 2018) (github NLR Annotator). A coding sequence was designated as encoding an NLR (full or partial) only if supported by NLR-Annotator. For *Sr22* homologs, if two overlapping coding sequences were of the same length and both had start and stop codons, the one with higher amino acid sequence identity to *Sr22* was selected.

Fig. 5. Phenotypic reaction of transgenic *Sr22* variants against *Puccinia graminis* f. sp. *tritici* race TTKSK. **A**, Schematic overview (not shown to scale) of the constructs used in stem rust resistance assays. Binary construct containing (i) *BsaI*-domesticated *Sr22* Schomburgk driven by 5' maize ubiquitin and *BsaI*-domesticated 3' *Sr22* regulatory elements, respectively; (ii) *BsaI*-domesticated *Sr22* Schomburgk driven by 5' and 3' *Sr33* regulatory elements, respectively; (iii) *BsaI*-domesticated *Sr22* Schomburgk driven by 5' *Sr33* and *BsaI*-domesticated 3' *Sr22* regulatory elements, respectively; (iv) native *Sr22* PI190945 driven by its 5' and 3' regulatory elements; (v) *BsaI*-domesticated *Sr22* PI190945 driven by 5' and 3' *Sr33* regulatory elements; and (vi) native *Sr22* Schomburgk driven by its 5' and 3' regulatory elements. Nonnative four-nucleotide linkers in pBW_0001, pBW_0002, pBW_0003, and pBW_0004 were introduced immediately before the start codon, in the middle of the coding region, and immediately after the stop codon. White arrows correspond to the removed *BsaI* sites. **B**, Stem rust infection assays using the *P. graminis* f. sp. *tritici* race TTKSK on representative seedlings of T₂ lines of *Sr22* variants and comparison with the susceptible control Fielder.

Manual identification of *Sr22* homologs in *Aegilops tauschii*, *T. diccoides*, and *T. urartu*.

For *Aegilops tauschii* and *T. diccoides*, orthologs of *Sr22* were not detected when examining the predicted protein sequences. Using the *Sr22* genomic sequence (from Schomburgk) as a query, we searched the genomes of these two species using BLASTN. The top hits were found to be from genomic regions on chromosome 7 in each of these species in their respective subgenomes. To obtain the corresponding *Sr22* ortholog from these regions, splice sites and start or stop codons were manually annotated through alignment and comparison with the *Sr22* coding and genomic sequences. In *Aegilops tauschii*, the annotation overlapped with the gene annotation with ID AET7Gv21218700. Thus, the *Sr22* homolog was renamed to AET7Gv21218700_[corrected] (reflected in nomenclature in Supplementary File S1).

For *T. urartu*, two proteins with consecutive gene IDs were predicted to be orthologous to *Sr22*. Examination of the alignment of these proteins to the *Sr22* protein sequence showed that, whereas one of these sequences aligned to the N-terminal region of *Sr22*, the other aligned to the C-terminal region. The genes encoding these proteins were found to be immediately adjacent on the genome, separated by a stretch of ambiguous bases (NNNNNNs). Thus, these two loci were determined as corresponding to a single NLR locus, fragmented due to assembly errors (reflected in nomenclature in Supplementary File S1).

Syntenic analysis of genomic regions containing *Sr22* homologs.

Ten genes on either side flanking the genomic regions containing *Sr22* orthologs in the genomes of *T. aestivum*, *T. turgidum*, *T. diccoides*, *Aegilops tauschii*, and *T. urartu* were obtained using the neighborhood conservation feature on Gramene. In *H. vulgare* 'Morex', *A. atlantica*, and *A. eriantha*, because several *Sr22* orthologous copies were identified, 10 genes flanking the first and last orthologous copy as well as the genes in between these two copies were obtained. The predicted functional annotation of these genes was determined using the model species homolog prediction feature in Gramene (for only *H. vulgare*), BLASTX searches against the NCBI nr protein database, and through domain identification using InterProScan. We also used NLR-Annotator to check whether any of these genes were either full-length or partial NLRs. For the *H. vulgare* 'Golden Promise', *H. vulgare* var. *nudum*, and *H. spontaneum* syntenic searches, 1 Mb of sequence around the top BLASTN hit to the *H. vulgare* 'Morex' *Sr22*-containing region was analyzed. The predicted functional annotation was determined in the same way as described above for Morex.

For *B. distachyon* and *O. sativa*, proteins orthologous to *Sr22* could not be located. To check whether these genomes contained any regions exhibiting conservation of micro-synteny to the genomic regions containing *Sr22* in the tribe Triticeae, the genes flanking the *Sr22* ortholog in chromosome 7D in *T. aestivum* 'Chinese Spring' were used. We selected 20 genes in the neighborhood of the *Sr22* ortholog in *T. aestivum* chr7D, 10 on either side. For each of these genes, we determined its homolog in *O. sativa* and *B. distachyon* using Gramene. We then examined the location of these homologs to check whether homologs of the *Sr22* gene neighborhood were found in a similar contiguous genomic stretch in these species. Functional identities of these homologs were obtained using the model species homolog prediction feature in Gramene, BLASTX searches against the NCBI nr protein database, and through domain identification using InterProScan. The regions showing microsynteny were

analyzed with NLR-Annotator to check for the presence of NLRs.

Acquisition and verification of *Sr22* sequence variants.

In total, 14 *Sr22* sequence variants were previously identified in Steuernagel et al. (2016) by PCR screening and sequencing of accessions of *T. boeoticum* (and its domesticated form *T. monococcum*), which had been postulated to carry either functional (*Sr22*) or nonfunctional (*sr22*) alleles of *Sr22*. In this study, further PCR screening of an additional 25 accessions and sequencing revealed another eight different sequences from these species.

Accessions of *T. boeoticum* were assessed for seedling infection types in response to *P. graminis* f. sp. *tritici* isolates 59KS19 (race MCCFC), 04KEN156/04 (race TTKSK, syn. Ug99), 09CA115-2 (race BCCBC), and 06ND76C (race QFCSC) according to previously described methods (Hundie et al. 2019).

Nucleotide sequence analysis of *Sr22* variants.

Nucleotide sequence alignment of *Sr22* variants was performed using Clustal Omega (Sievers et al. 2011) with the default parameters, except the order in which the sequences appear in the final alignment was set to "input sequence order". The alignment output was visualized and analyzed using Jalview version 2 (Waterhouse et al. 2009). Only informative polymorphic sites are shown in the final alignment, defined as those sites where two or more alternative nucleotides occur in at least two sequences within the alignment.

Generation of binary constructs carrying *Sr22*.

The maize ubiquitin and the *Sr33* promoter regions were used for designing *Sr22* chimeric constructs. *Sr22* coding regions (sequence from START to STOP codon, including both exons and introns) from three different sources, including *Sr22*-Schomburgk, *Sr22*-PI190945, and *sr22*-PI573523, were used for the designs. For the simplicity of construct synthesis, the coding regions were divided into two sections; namely, coding region 1 and 2. The *Sr22*-Schomburgk and the *Sr33* terminator regions were used in some constructs. All of these modular components were synthesized by a commercial DNA synthesis provider (Life Technologies Ltd.). The synthesized components were assembled by Golden Gate cloning (Weber et al. 2011) into the toolkit vector pICH47732 (Weber et al. 2011), then transferred to pVecBARII (a derivative of pWBvec8 in which the 35S hygromycin gene has been replaced with a 35S BAR selectable marker gene) (Wang et al. 1998) as *NotI* fragments. Prior to synthesis, all native *BsaI* sites were domesticated (i.e., removed by editing) without changing the predicted amino acid coding sequence within the exons of the coding regions, and without changing the intron splice donor or acceptor sites. The domestication was performed using the program Genious version 7.1.7. All modular components were domesticated and synthesized as level 0 modules by introducing *BsaI* sites at the start and at the end of each module. In addition to introduction of flanking *NotI* sites, the sequence AATG was added to link promoter and coding regions and the sequence GCTT was added to link coding regions and terminators. The sequence ACGT was added to link coding region 1 and 2. The sequences GGAG and CGCT were added to link left and right borders of the vector, respectively. The assembled level 1 module was transferred into the pVecBARII binary vector for wheat transformation. In addition, a gene which was not domesticated by *BsaI* site removal and was composed of the *Sr33* promoter fused to the *Sr22* coding region fused to the *Sr33* terminator, was designed and synthesized. All binary plasmids containing the desired insert were transformed by electroporation into *Agrobacterium tumefaciens* for wheat transformation.

Wheat transformation, stem rust inoculations, and phenotypic evaluations.

The *Sr22* binary constructs were introduced into wheat cultivar Fielder using the *Agrobacterium* transformation protocol described by Ishida et al. (2015) and phosphinothricin as a selective agent. Ten independent primary transgenic plants were recovered and grown in an automated growth cabinet with conditions of 16 h of light and 8 h of darkness and a constant temperature of 23°C. The plants were inoculated with the Australian *P. graminis* f. sp. *tritici* race 98-1,2,3,5,6 (Park 2016), which is virulent on Fielder at the fully developed third-leaf stage. After 24 h of incubation in a closed transparent plastic box under high humidity, the plants were restored to the original growth conditions and observed for rust development. Rust infection types were scored 14 days postinoculation.

For all six binary constructs, at least two homozygous T₂ transgenic plants were inoculated with the *P. graminis* f. sp. *tritici* race TTKSK (isolate 04KEN156/04) at the fully developed first leaf stage according to previously described methods for evaluating stem rust seedling infection types (Hundie et al. 2019). Infection types were scored 15 days postinoculation.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Angiosperm Phylogeny Website, version 14:

<http://www.mobot.org/MOBOT/research/APweb>

figshare: <https://figshare.com>

github NLR Annotator: <https://github.com/steuernb/NLR-Annotator>

Gramene database: <http://www.gramene.org>

Tropicos database: <https://www.tropicos.org/home>

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