facilitating plantarflexion and inversion of the foot (*7*, *8*). (iii) The astragalus and the navicular have a saddle joint, as well characterized for Cretaceous and Paleogene multituberculates (*7*–*10*), permitting not only side-to-side but also dorsi- and plantarflexion of the navicular to the astragalus (Fig. 4C, curved arrow), enhancing mobility of the midtarsal joint of the hind foot in general (*7*–*10*). (iv) The entocuneiform-metatarsal joint (Fig. 4A) is formed by a deep saddle of the entocuneiform, reciprocated by a curved groove on metatarsal 1 (Fig. 4D), identical in the Jurassic *Rugosodon* as in all later multituberculates (*8*, *10*). This joint facilitates rotation of metatarsal 1 to the entocuneiform (Fig. 4E), amplifying to an even greater dorsoventral excursion for phalanges of the hallux (pedal digit 1), as pulled by the peroneus longus and the extensor digitorium hallucis, both originating near the enlarged parafibula at the knee joint (Fig. 3A). The saddle on the entocuneiform allowed abduction of metatarsal 1, although that element is not habitually abducted as preserved in situ in *Rugosodon* (Fig. 3, B and D). The mobility of metatarsal 1 in multituberculates is a unique condition not seen in other Mesozoic mammals.

Despite a great taxonomic diversity and a wide range of feeding adaptations over the long history of multituberculates (*1*, *2*), the morphology of their ankles is remarkably conserved (*7*). The highly mobile tarsal joints are well suited for foot functions on uneven substrates (including arboreality) (*7*) and are apparently versatile enough to be retained in fossorial (*1*) and saltatorial forms (*9*). Major diversifications of multituberculates in the Cretaceous and Paleogene have a structural underpinning in ankle bones of their common ancestor of the Jurassic, for which *Rugosodon* provides fresh fossil evidence.

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Acknowledgments: We thank Z. Zeng, D. Liu, X. Xia (BMNH), M. A. Klingler, and A. M. Isch for assistance for research and graphics, and J. R. Wible and M. R. Dawson for improving the manuscript. Authors benefited from discussion with R. L. Cifelli, W. A. Clemens, M. R. Dawson, G. Hahn, J. A. Hopson, Y.-Q. Liu, T. Martin, P. C. Sereno, and J. R. Wible. Support was received from Ministry of Land Resources and Ministry of Science and Technology of China 973 Project Funding (C.-X.Y. and Q.J.), Scientific Commission of Beijing and Beijing Museum of Natural History (Q.-J.M.), NSF, Carnegie Museum, and University of Chicago (Z.-X.L.).

Supplementary Materials

www.sciencemag.org/cgi/content/full/341/6147/779/DC1
Supplementary Text
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18 March 2013; accepted 9 July 2013
10.1126/science.1237970

Identification of Wheat Gene *Sr35* That Confers Resistance to Ug99 Stem Rust Race Group

Cyrille Saintenac,[1]*† Wenjun Zhang,[2]† Andres Salcedo,[1] Matthew N. Rouse,[3] Harold N. Trick,[1] Eduard Akhunov,[1]‡§ Jorge Dubcovsky[2,4]‡§

Wheat stem rust, caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*), is a devastating disease that can cause severe yield losses. A previously uncharacterized *Pgt* race, designated Ug99, has overcome most of the widely used resistance genes and is threatening major wheat production areas. Here, we demonstrate that the *Sr35* gene from *Triticum monococcum* is a coiled-coil, nucleotide-binding, leucine-rich repeat gene that confers near immunity to Ug99 and related races. This gene is absent in the A-genome diploid donor and in polyploid wheat but is effective when transferred from *T. monococcum* to polyploid wheat. The cloning of *Sr35* opens the door to the use of biotechnological approaches to control this devastating disease and to analyses of the molecular interactions that define the wheat-rust pathosystem.

The fungus *Puccinia graminis* f. sp. *tritici* (henceforth *Pgt*) is the causal agent of wheat stem rust, a devastating disease responsible for major outbreaks and large losses of wheat yields in the past. The deployment of *Pgt* resistance genes, combined with the eradication of the alternative host (barberry), provided an effective control of this disease for the past 50 years (*1*). However, the widely deployed *Pgt* resistance gene *Sr31* was overcome by a previously uncharacterized race of *Pgt* identified in Uganda in 1999 and designated Ug99 (or TTKSK, according to the North American system for *Pgt* race nomenclature) (*2*). A decade later, six previously unidentified Ug99-related *Pgt* races, some showing a broader virulence spectrum, have been detected and have spread to the wheat-growing regions of Africa, Yemen, and Iran (*3*). Roughly 90% of the wheat varieties grown worldwide are susceptible to Ug99 and related races, representing a serious threat to global food security (*3*). The Borlaug Global Rust Initiative was launched in 2005 to coordinate international efforts to fight Ug99 (www.globalrust.org). The identification and characterization of Ug99-resistance genes *Sr35* (in this

[1]Department of Plant Pathology, Kansas State University, Manhattan, KS 66506, USA. [2]Department of Plant Sciences, University of California, Davis, CA 95616, USA. [3]Cereal Disease Laboratory, U.S. Department of Agriculture (USDA)–Agricultural Research Service, St. Paul, MN 55108, USA. [4]Howard Hughes Medical Institute (HHMI), Chevy Chase, MD 20815, USA.

*Present address: Genetics Diversity and Ecophysiology of Cereals, Institut National de la Recherche Agronomique–Université Blaise Pascal, UMR1095, 5 Chemin de Beaulieu, 63039 Clermont-Ferrand, France.
†These authors contributed equally to this work.
‡These authors contributed equally to this work.
§Corresponding author. E-mail: eakhunov@ksu.edu (E.A.); jdubcovsky@ucdavis.edu (J.D.)

study) and *Sr33* [in a companion paper (*4*)] are part of these efforts.

The stem rust resistance gene *Sr35* was identified in previous screens for resistance to *Pgt* in the diploid wheat species *Triticum monococcum* (*5*, *6*). The genome of *T. monococcum*, designated A^m, is closely related to the genome of *T. urartu*, the diploid donor of the A genome in tetraploid (*T. turgidum*, pasta wheat) and hexaploid wheat (*T. aestivum*, bread wheat) (*7*). *Sr35* was prioritized for cloning because it confers near immunity against Ug99, Ug99-related races, and the TRTTF group of races from Africa, Yemen, and Pakistan; the TRTTF group has a broad but different virulence profile from the Ug99 race group (*3*, *8*). *Sr35* was also selected because previous studies have confirmed that this gene is effective against the same virulent races when it is transferred to hexaploid wheat by crossing and recombination (*5*, *8*).

The gene *Sr35* was previously mapped on the long arm of chromosome $3A^m$ in *T. monococcum* (*8*). In this study, we used 4575 recombinant gametes (1925 F_2 and 725 BC_1F_1 plants) and seven molecular markers derived from the colinear region in *Brachypodium distachyon* (Fig. 1A) to map *Sr35* between markers *AK331487* [0.02 centimorgans (cM)] and *AK332451* (0.98 cM) (Fig. 1B). We then used the closest proximal markers *AK331487* and *SFGH* (*S-formylglutathione hydrolase-like*) to screen a *T. monococcum* bacterial artificial chromosome (BAC) library of the *Sr35*-resistant accession DV92 (*9*). The 23 selected BAC clones were assembled by fingerprinting into a single contig that spanned the *Sr35* locus (Fig. 1, C and D; fig. S1; and table S1).

We sequenced three overlapping BACs covering the *Sr35* region (*10*) and annotated the 307,519–base pair (bp) sequence (KC573058). This sequence includes a cluster of coiled-coil, nucleotide-binding, leucine-rich repeat (LRR) (henceforth, CNL) disease resistance genes, including five intact genes (*CNL1*, *CNL2*, *CNL4*, *CNL6*, and *CNL9*), two pseudogenes (*pCNL3* and *pCNL10*), and three small gene fragments (*pCNL5*, *pCNL7*, and *pCNL8*) (Fig. 1E). A phylogenetic tree of the complete *CNL* genes showed that *CNL4* and *CNL9* are the most closely related members of this cluster (fig. S2). The annotated sequence also includes two unrelated genes (*SFGH* and *APGG1*) and two pseudogenes (*pABC* and *pAP2*) (Fig. 1E). Additional markers developed from this sequence were used to delimit the *Sr35* candidate region to a 213-kb segment including candidate genes *APGG1*, *CNL4*, *CNL6*, and *CNL9* (Fig. 1E and table S1).

We sequenced these four candidate genes in a *T. monococcum* collection including 24 Ug99-resistant accessions carrying *Sr35* and 25 susceptible accessions without *Sr35*. We identified two resistant (R1 and R2) and six susceptible haplotypes (S1 to S6, table S2, primers in tables S3 to S5). The two resistant haplotypes differ in a short *CNL4* region with a 6-bp deletion and four single-nucleotide polymorphisms (SNPs) but show no differences in *APGG1*, *CNL6*, and *CNL9*. All susceptible accessions have mutations in *CNL9*, and among them, five have mutations only in *CNL9*, which suggests that this gene is necessary to confer resistance to Ug99. Among these five susceptible accessions, three of them share three close SNPs that result in amino acid changes at positions 854, 856, and 858 [RLWFT to HLRFS (R, Arg; L, Leu; W, Trp; F, Phe; T, Thr; H, His; S, Ser)] in the C-terminal region of the LRR domain (Fig. 2A). The same three SNPs are present in the closely related *CNL4* gene, suggesting a conversion event.

To validate the previous results, we mutagenized the *Sr35*-resistant accession G2919 with ethylmethanesulfonate (*10*). Out of 1087 M_2 mutant families screened with race RKQQC, we identified two mutant families segregating for susceptibility, which were validated with races Ug99 and TRTTF (Fig. 2B). Sequencing of the four candidate genes in these susceptible plants confirmed the presence of mutations only in *CNL9*. The first mutant ($cnl9^{1296}$) contained a G-to-A mutation that resulted in a premature stop codon at position 856 (Fig. 2A) and truncated the last 64 amino acids. In the progeny of a cross between $cnl9^{1296}$ and the resistant parental line G2919 (33 F_2 plants), homozygocity for the mutation cosegregated with susceptibility to Ug99.

The second susceptible mutant ($cnl9^{1120}$) showed the same three SNPs detected in accession PI428167-2 (RLWFT to HLRFS) (table S2). To test if this was the result of seed contamination or cross-pollination, we used genotyping-by-sequencing (*11*) to estimate the level of polymorphisms among $cnl9^{1296}$, $cnl9^{1120}$, and the nonmutagenized line G2919 (table S6 and supplementary text). We show that $cnl9^{1120}$ has the level of mutations and the ratio of homozygous-to-heterozygous loci expected from a mutagenized plant. Therefore, a spontaneous gene conversion between *CNL4* and *CNL9* is the most parsimonious explanation for the three linked mutations in $cnl9^{1120}$. Two of these amino acid positions (856 and 858) overlap with 15 amino acids located in the C-terminal half of the LRR domain of CNL9 that show evidence of positive selection (fig. S3, A and B, and table S7). Concisely, mutants $cnl9^{1296}$ and $cnl9^{1120}$ confirmed that *CNL9* is necessary for the *Sr35*-mediated resistance and that the distal region of the LRR domain is critical for *Sr35* function.

To determine if *CNL9* is sufficient to confer resistance to Ug99, we generated transgenic hexaploid wheat plants expressing the *CNL9* gene under the control of its native promoter (*10*). Out of four putative T_0 transgenic plants, only one, designated #1123, showed consistent expression of the transgene (fig. S4) and cosegregation between the presence of the transgene and resist-

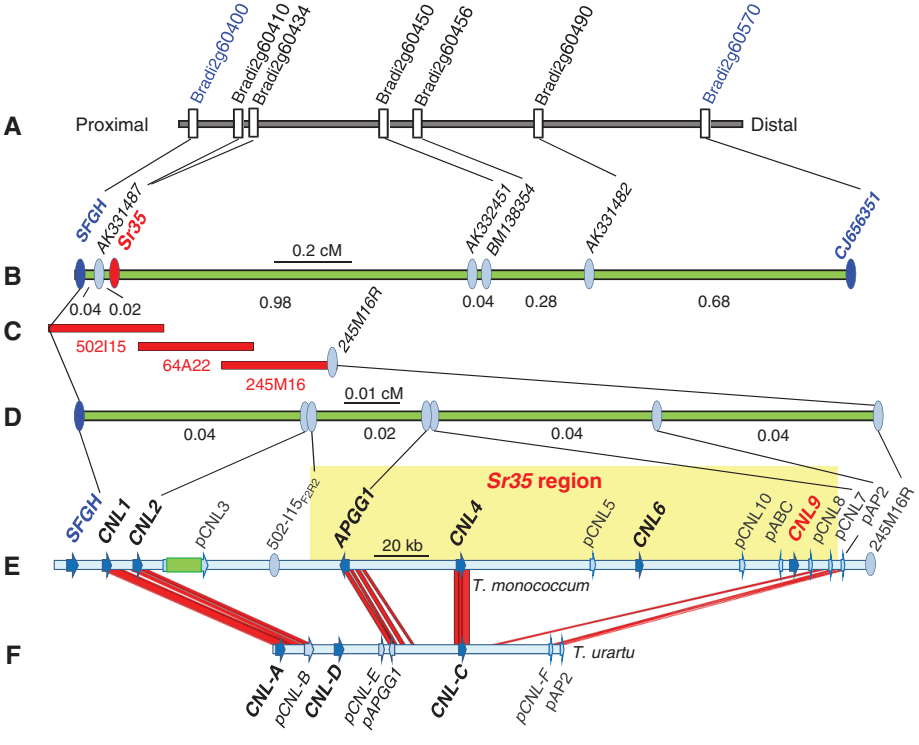

Fig. 1. Genetic and physical maps of *Sr35*. (**A**) A 174-kb colinear region of *Brachypodium* (*8*). Only genes for which a wheat orthologous gene was found in databases are represented here. (**B**) Genetic map of the *Sr35* locus. (**C**) Screening the DV92 BAC library with proximal markers *SFGH* and *AK331487* (only BACs from the minimum tilling path are shown). (**D**) High-density map. (**E**) Graphical representation of the *T. monococcum* annotated sequences (KC573058). The letter "p" before the gene name denotes a pseudogene (*pCNL3* has an inserted retroelement). The *Sr35* candidate gene region is highlighted in yellow. (**F**) Comparison of *T. monococcum* DV92 and *T. urartu* G1812 (KC816724) orthologous regions (92% identity threshold).

ance to Ug99 and RKQQC in the T_1 and T_2 progeny (Fig. 2C, fig. S5, and table S8). In contrast, all #1123 T_1 and T_2 plants were susceptible to the *Sr35*-virulent race QTHJC, regardless of the presence or absence of the transgene (Fig. 2C and fig. S5). This result suggests that the *CNL9* transgene has the same race specificity as *Sr35*. Taken together, the natural variation, mutant, and transgenic results demonstrate that *CNL9* is *Sr35*.

With the use of rapid amplification of cDNA ends (*10*), we found that the *CNL9* transcripts have a 196-bp 5′ untranslated region (UTR) and a 1526-bp 3′UTR that includes three introns (fig. S6A). The three introns in the 3′UTR were also detected in all *T. urartu*–, *T. turgidum* cv. *durum*–, and *T. aestivum*–related *CNL* genes for which we were able to obtain both genomic and transcript data (table S9). Both *CNL* homologs from *B. distachyon* (table S9) also have two introns in the 3′UTR, indicating that this structural feature is conserved in this disease resistance cluster. Exons 3 and 4 from the *B. distachyon CNL* genes correspond to exons 4 and 5 from the *T. monococcum CNL9* homolog.

Transcript levels of *CNL9* in leaves from G2919 plants inoculated with *Pgt* race RKQQC (*10*) were 40-, 81-, and 411-fold higher than those of candidate genes *APGG1*, *CNL4*, and *CNL6*, respectively (Fig. 2D), but did not significantly differ from mock-inoculated plants at different time points (Fig. 2E). With the use of isoform-specific primers (fig. S6B and table S5), we found that ~8% of the *T. monococcum CNL9* transcripts were represented by an alternative splicing variant that retained the second intron in the 3′UTR (Fig. 2E). We also detected transcripts with and without the same intron in *T. turgidum* (table S9). The ratio between the two *CNL9* transcript isoforms did not show changes in *T. monococcum* G2919 plants mock-inoculated and inoculated with *Pgt* race RKQQC (Fig. 2E). This finding suggests that the relative proportion of the two alternative splice forms is not affected by the presence of the pathogen. Previously reported alternative splicing events in *CNL* genes do not involve introns in the 3′UTR (*12*–*18*), which might be a distinctive feature of this particular group of *CNL* genes.

So far, *Sr35* has not been reported in *T. urartu* or polyploid wheat species. To better understand the reasons for this absence, we performed a comparative analysis of the *T. monococcum* (KC573058) and *T. urartu* (KC816724) colinear regions, which diverged less than 1 million years ago (*19*). The *T. monococcum* region encompassing genes *CNL6* and *CNL9* and pseudogenes *pCNL5*, *pCNL8*, *pCNL10*, and *pABC* is absent in *T. urartu* (Fig. 1F). Conversely, the *T. urartu* region including *TuCNL-D* and pseudogene *pCNL-E* is missing in *T. monococcum*. Large insertions and deletions have been found in other colinear intergenic regions of the *T. monococcum* and *T. urartu* genomes (*19*, *20*). The large and repetitive genomes of wheat show higher rates of insertion and deletions than the human genome (*19*).

A screen of 41 *T. urartu* accessions and 19 wild tetraploid wheat *T. turgidum* ssp. *dicoccoides* accessions (table S10) revealed no orthologs of *TmCNL9* (fig. S7). Gene *TuCNL-H* from *T. urartu* accession G1545 from Iran encoded the same RWT amino acids found in CNL9 at positions 854, 856, and 858, but the rest of the sequence was different and clustered with a separate set of *CNL* genes (fig. S7). Because *T. urartu* is the donor of the A genome to the polyploid wheat species (*7*), it is not surprising that *CNL9* homologs have not been detected in the genomic sequence of *T. aestivum* (www.wheatgenome.org/) or in the transcriptome of *T. turgidum* (wheat.pw.usda.gov/GG2/WheatTranscriptome/) (fig. S2).

The absence of *Sr35* in the tested pasta and bread wheat varieties highlights the value of wheat landraces and wild relatives as a reservoir of currently unknown resistance specificities. It also suggests that *Sr35* has the potential to improve stem rust resistance in a wide range of wheat germplasm. Our transgenic experiments additionally indicate that the transfer of *CNL9-Sr35* to hexaploid wheat is sufficient to confer effective

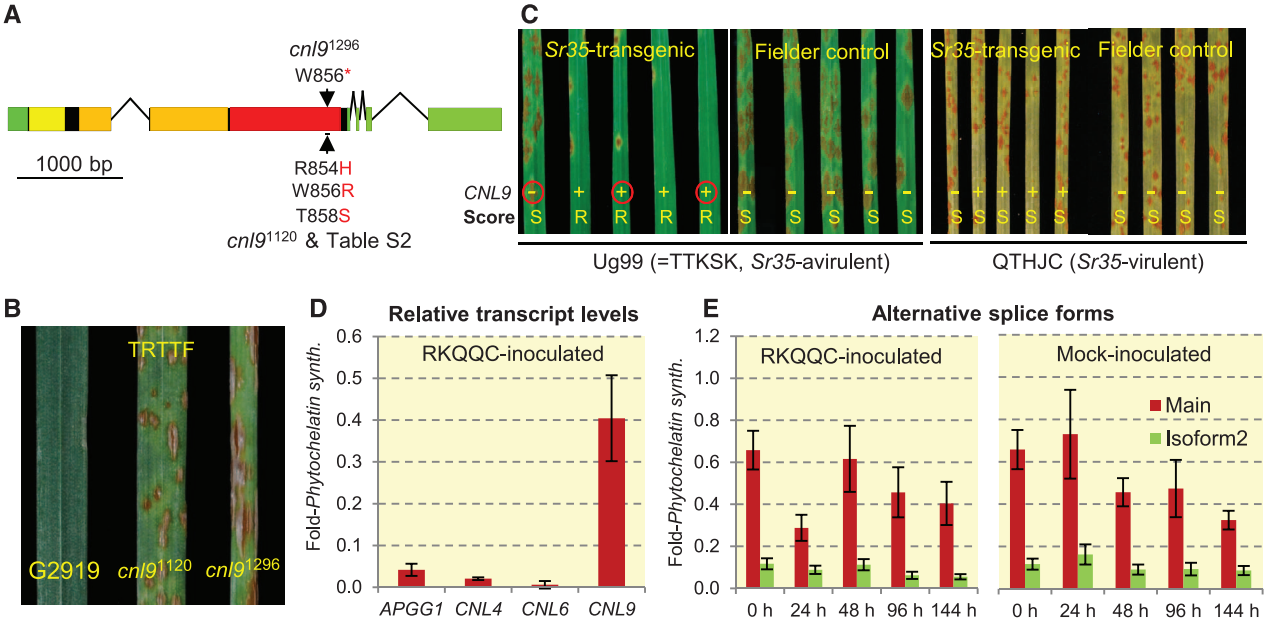

Fig. 2. Functional validation of the *CNL9* gene. (**A**) *CNL9* gene structure. Green, UTR; black, coding exons; yellow, coiled-coil domain; orange, nucleotide-binding domain; red, LRR domain; arrows, amino acid changes in susceptible induced mutants $cnl9^{1296}$ (W856*) and $cnl9^{1120}$ or natural mutants (table S2, RLWFT to HLRFS). (**B**) Infection types produced on *T. monococcum* G2919 and *CNL9* mutants $cnl9^{1120}$ and $cnl9^{1296}$ inoculated with *Pgt* race TRTTF. G2919 carries both *Sr35* and *Sr21* resistance genes, so we selected a race (TRTTF) that is virulent to *Sr21* and avirulent to *Sr35* to validate the mutations in *Sr35*. (**C**) Infection types on seedlings of T_1 lines from event #1123 segregating for the *CNL9* transgene. Plants carrying the *CNL9* transgene (+) were resistant to Ug99 (R), and plants without the transgene (-) were susceptible (S) (table S8). When inoculated with *Sr35*-virulent race QTHJC, all plants were susceptible, suggesting similar race specificity between the transgenic and natural *Sr35*. Red circles indicate available progeny tests in fig. S5. S, susceptible; R, resistant. (**D**) Relative transcript levels of candidate genes *APPG1*, *CNL4*, *CNL6*, and *CNL9* (main isoform) in G2919 6 days after inoculation with race RKQQC. (**E**) Transcript levels of the *CNL9* main isoform (red) and isoform two (green, retained intron) in mock- or race RKQQC–inoculated G2919 plants. Leaves were collected at 0, 24, 48, 96, and 144 hours after inoculation. Transcript levels are expressed relative to the *Phytochelatin synthase* internal control using the $2^{-\Delta Ct}$ method. Error bars in (D) and (E) denote SEM based on six biological and two technical replicates.

levels of resistance to Ug99. In contrast, some *CNL* genes (for example, wheat leaf rust resistance gene *Lr10*) require the presence of additional *CNL* genes to provide resistance (*21*).

CNL proteins mediate recognition of pathogen-derived effector molecules, as well as host proteins altered by the pathogen, and they subsequently activate host defenses. These proteins have an ancient origin and are encoded by one of the largest, most variable multigene families in plants (*22*). Members of this family confer resistance to a wide range of pathogens and pests. Remaining challenges are to identify which genes are responsible for resistance to a specific pathogen and to understand the signal transduction pathways involved in the plant resistance response. This information is particularly important in the case of Ug99, which now threatens the major wheat-producing areas in Asia (*3*).

The identification of *Sr35* and of *Sr33* in a companion paper (*4*) opens the door to transgenic approaches to control this devastating pathogen. *Sr35* shows a strong hypersensitive reaction to the TTKSK and TRTTF race groups when introgressed into hexaploid wheat but is susceptible to some *Pgt* races and, therefore, should not be deployed alone. In contrast, *Sr33* is resistant to all races tested so far (*23*, *24*) but confers only moderate resistance to the Ug99 race group when introgressed alone in hexaploid wheat. On the basis of these complementary characteristics, it might be beneficial to combine these two genes, by either crossing and recombination or transforming wheat with a cassette including both genes. The insertion of multiple resistance genes in a single locus can accelerate breeding efforts to pyramid multiple sources of resistance, which is a reasonable strategy to increase the durability of available resistance genes.

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Acknowledgments: This project was supported by Agricultural and Food Research Initiative grants 2011-68002-30029 (Triticeae-CAP) and 2012-67013-19401 from the USDA National Institute of Food and Agriculture, by the Borlaug Global Rust Initiative, and by support to J.D. from the HHMI and the Gordon and Betty Moore Foundation grant GBMF3031. We thank J. Nirmala, S. Sehgal, M. Padilla, S. Chao, K. Jordan, H. Lee, and D. Burdan for excellent technical support; B. Bowden and J. Dvorak for providing critical materials; K. Krasileva, A. Akhunova, and C. Li for valuable suggestions; and the University of California, Davis, and Kansas State University Genomic facilities. We also thank M. Pumphrey for his collaboration in the initial stages of the project, including design and initial development of the high-resolution mapping and TILLING populations. Sequences have been deposited in GenBank under accession numbers KC573058, KC816724, KF113354 to KF113357, and KC876115 to KC876121. Author contributions are listed in the supplementary materials.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1239022/DC1
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11 April 2013; accepted 10 June 2013
Published online 27 June 2013;
10.1126/science.1239022

The Gene *Sr33*, an Ortholog of Barley *Mla* Genes, Encodes Resistance to Wheat Stem Rust Race Ug99

Sambasivam Periyannan,[1] John Moore,[1] Michael Ayliffe,[1] Urmil Bansal,[2] Xiaojing Wang,[4,6] Li Huang,[4] Karin Deal,[3] Mingcheng Luo,[3] Xiuying Kong,[5] Harbans Bariana,[2] Rohit Mago,[1] Robert McIntosh,[2] Peter Dodds,[1] Jan Dvorak,[3] Evans Lagudah[1]*

Wheat stem rust, caused by the fungus *Puccinia graminis* f. sp. *tritici*, afflicts bread wheat (*Triticum aestivum*). New virulent races collectively referred to as "Ug99" have emerged, which threaten global wheat production. The wheat gene *Sr33*, introgressed from the wild relative *Aegilops tauschii* into bread wheat, confers resistance to diverse stem rust races, including the Ug99 race group. We cloned *Sr33*, which encodes a coiled-coil, nucleotide-binding, leucine-rich repeat protein. *Sr33* is orthologous to the barley (*Hordeum vulgare*) *Mla* mildew resistance genes that confer resistance to *Blumeria graminis* f. sp. *hordei*. The wheat *Sr33* gene functions independently of *RAR1*, *SGT1*, and *HSP90* chaperones. Haplotype analysis from diverse collections of *Ae. tauschii* placed the origin of *Sr33* resistance near the southern coast of the Caspian Sea.

Stem rust [*Puccinia graminis* f. sp. *tritici* (*Pgt*)] of wheat is a major threat to global food security. Continued adaptation of the pathogen necessitates continued development of new *Pgt*-resistant wheat varieties (*1*). A *Pgt* race, Ug99 or TTKSK, identified in Uganda in 1999, was virulent on 90% of wheat cultivars grown globally, including those carrying the stem rust resistance (*Sr*) *31* resistance gene, which hitherto had been widely deployed and effective for more than 30 years (*2*, *3*). Ug99 and subsequent mutational derivatives that overcame additional resistance genes raised concerns of a disease epidemic that could devastate wheat crops, which provide 20% of the world's caloric intake. More than 50 *Pgt* resistance (*R*) loci, including those introgressed from wild relatives such as *Aegilops tauschii*, have been cataloged in wheat.

The *Pgt R* gene, *Sr33*, discovered from *Ae. tauschii*, the diploid progenitor of the D genome in hexaploid wheat (*4*, *5*), was introgressed into common wheat (*Triticum aestivum*, genomes AABBDD). There, *Sr33* provides a valuable, intermediate level of resistance to diverse *Pgt* races, including the Ug99 lineage (*6*). To isolate the *Sr33* gene, we used a single-chromosome substitution genetic stock, CS1D5405, which has chromosome 1D of wheat cv Chinese Spring (CS) replaced by the corresponding chromosome bearing *Sr33*

[1]Commonwealth Scientific and Industrial Research Organization (CSIRO) Plant Industry, General Post Office Box 1600, Canberra, ACT 2601, Australia. [2]The University of Sydney Plant Breeding Institute–Cobbitty, PB4011, Narellan, NSW 2567, Australia. [3]Department of Plant Sciences, University of California, Davis, CA 95616, USA. [4]Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717, USA. [5]Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China. [6]State Key Laboratory of Crop Stress Biology for Arid Areas and College of Life Sciences, Northwest Agriculture and Forestry University, Yangling, Shaanxi 712100, China.

*Corresponding author. E-mail: evans.lagudah@csiro.au