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Variation in the AvrSr35 gene determines Sr35 resistance against wheat stem rust race Ug99

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Puccinia graminis f. sp. tritici (Pgt) causes wheat stem rust, a devastating fungal disease. The Sr35 resistance gene confers immunity against this pathogen’s most virulent races, including Ug99. We used comparative whole-genome sequencing of genes. Since the discovery of Ug99, most of the wheat varieties grown in Europe, in Uganda in 1999 (1), Ug99 was virulent on most of the wheat varieties grown in Europe, Asia, and the United States, prompting research into the discovery of Ug99-effective resistance genes. Since the discovery of Ug99, Pgt surveillance identified new Ug99-derived strains virulent against additional wheat resistance genes (2).

Plant resistance genes (R) defend against an invading pathogen by detecting the corresponding pathogen avirulence factors (Avr), which are often secreted effector proteins. R genes encode receptors that trigger an immune response upon perception of pathogen Avr factors. This response results in localized cell death at the site of infection (hypersensitive response (HR)) (3–5). A pathogen lacking an Avr gene renders the corresponding plant R gene ineffective. Here, we identified the fungal Avr gene recognized by the Ug99-effective wheat stem rust resistance gene Sr35 (6) and investigated the origin of Sr35-virulent fungal isolates.

Using confocal microscopy of Pgt-infected leaf tissues from resistant (Sr35+) and susceptible (Sr35−) wheat lines, we demonstrated that Sr35 triggers a resistance response at the early stages of infection (Fig. 1) (7). In wheat line U6169 (Sr35+), the development of fungal infection hyphae stopped even before the formation of a haustorium, the structure with which the fungus extracts nutrients from its host plant. This early immune response is consistent with the lack of pronounced HR symptoms in Triticum monococcum accession G2919 used to identify the Sr35 gene (6) and suggested early expression of a fungal gene recognized by Sr35. To identify this Avr gene, we mutagenized the spores of the Pgt race RKQC (Sr35-avirulent isolate 98KST6A-1) with ethylmethane sulfonate (EMS). We isolated 15 Pgt mutants virulent to the Sr35 gene, suggesting that they carry mutations affecting the Sr35-specific Avr factor (tables S1 and S2) (7). Both microscopy and time-course RNA-sequencing (RNA-seq) analyses showed no obvious effects of these mutations on the Pgt mutants’ interaction with a wheat host compared to the wild-type Pgt isolate (figs. S1 and S2, and tables S3 to S5) (7), perhaps due to the functional redundancy of virulence factors that can compensate mutations in the AvrSr35 gene (8). The genome of the wild-type Pgt isolate was assembled, annotated using RNA-seq data (tables S4 and S6), and compared with Illumina reads generated for each of 15 independent Pgt mutants (table S7), resulting in the detection of 30,429 EMS-induced mutations (table S8 and data S1).

Only one gene (MF474174) carried mutations in each Pgt mutant; 12 mutants had nonsense mutations, one mutant carried a splice-site disrupting mutation, and two mutants had the same nonsynonymous mutation producing valine to isoleucine (V128I) substitution (Fig. 2A and table S9). This AvrSr35 candidate gene encoded a 578-amino acid protein with a predicted secretion signal peptide (fig. S3). The protein was larger than many previously identified effectors (9); it showed no similarity to proteins from other species within the protein databases, nor did it contain any detectable protein domains (fig. S4) (7). Gene expression analysis of a Pgt-infected susceptible wheat line showed increased amounts of the

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Fig. 1. Sr35 provides prehaustorial resistance against Pgt. Infected leaves of susceptible cultivar Morocco (Sr35−) and resistant line U6169 (Sr35+) were collected 28 and 48 hours after infection (HAI). (A and B) Fungal infection hyphae (IH) (stained blue) entered the leaf mesophyll tissue (stained red) through the plant stoma (S) in both wheat lines at 24 HAI. Fungal haustoria (H) developed only in susceptible Morocco (B). (C and D) Imaging at 48 HAI revealed fungal growth in susceptible Morocco (D) but no further fungal growth in U6169 (C). Imaging at 48 HAI revealed two presumably dead host cells (DC) with increased fluorescence in close proximity of the HM in U6169 (E); no dead cells were revealed in Morocco (F). Staining of nuclei with propidium iodide (red) was indicative of cell death [insert in (E)]. SSV, fungal substomatal vesicle; MC, mesophyll cells; HM, haustorial mother cells.
AvrSr35 transcripts in the leaf tissues over the course of infection (fig. S3).

To understand the origin of virulence to Sr35 in the field, we resequenced AvrSr35 from 12 Sr35-virulent and 15 Sr35-avirulent natural isolates (tables S10 and S11 and data S2). Phylogenetic analysis revealed two major clades (Fig. 2B). Clade A sequences had intact coding sequences and were found only in the Sr35-avirulent isolates, including 99KS76A-1 and Ug99, indicating that functional AvrSr35 is required for triggering Sr35 resistance against Ug99. Clade V sequences were preferentially found in the Sr35-virulent isolates, except for five Sr35-avirulent isolates (77ND82A, 72CA1A, 75-36-700-3, 69SD657C, and 74MN1049) that carry at least two AvrSr35 gene copies from both clades (7). Clade V had the miniaturized inverted transposable element (MITE) in exon 6, resulting in the premature stop codon. Because even less severe AvrSr35 truncations detected in the Pgt mutants (Fig. 2A) caused Sr35-avirulence function loss, this MITE insertion is predicted to produce a nonfunctional protein. These results suggest that transposon-mediated disruption of AvrSr35 resulted in the origin of natural Sr35-virulent Pgt isolates. It is likely that the loss of Avr factors is facilitated by transposon proliferation in the rust genomes, which display the higher abundance of mobile elements compared with other fungi (9, 10), contributing to the erosion of plant R genes conferring resistance to rusts.

The ability of the wheat Sr35 gene to recognize the fungal AvrSr35 and trigger HR was confirmed...
by coexpressing the wild-type and mutated AvrSr35 and Sr35 gene constructs (table S12) in Nicotiana benthamiana leaves (Fig. 3, fig. S5, and movie S1). Even though the coexpressed wild-type constructs induced HR, the coexpression of truncated AvrSr35 with wild-type Sr35 failed to induce HR. No HR was evident in the combination of wild-type AvrSr35 with either of the two loss-of-function sr35 alleles, one with mutation (K206L) in the P-loop domain required for HR (7) and another with three mutations found in the leucine-rich repeat (LRR) domain of the Ug99-susceptible M1120 mutant of diploid wheat accession G2919 (6). The expression of individual wild-type constructs also failed to elicit HR (fig. S6). Because loss-of-function mutations in either AvrSr35 or Sr35 resulted in both HR loss in N. benthamiana and a compatible interaction between Pgt and wheat, direct or indirect recognition of AvrSr35 by Sr35 appears to induce a resistance response in wheat. Consistent with this conclusion, the infiltration of the AvrSr35 protein caused HR in the leaves of a Sr35+ but not in the leaves of a Sr35− wheat lines (Fig. 3E).

In N. benthamiana cells, fluorescently tagged Sr35 and AvrSr35 proteins, either coexpressed together or expressed individually, colocalized in the same subcellular compartment (Fig. 4, A to C). Colocalization of coexpressed Sr35:GFP protein fusion and the endoplasmic reticulum (ER) marker suggest that Sr35 and AvrSr35 in N. benthamiana are likely associated with the ER (Figs. 4C and fig. S7). To investigate whether colocalization of Sr35 and AvrSr35 interact in planta, we used bimolecular fluorescent complementation (BiFC) (Fig. 4D and figs. S8 and S9). The complementary AvrSr35 and Sr35 fusion proteins coexpressed in N. benthamiana produced a fluorescent signal that is consistent with a protein-protein interaction. The nonsynonymous mutations (sr35M1120 allele) affecting the LRR domain reduced the fluorescent signal intensity implicating the LRR domain in the Sr35-AvrSr35 interaction. These results suggest that the Ug99-susceptibility of wheat mutant M1120 (6) is associated with the inability of sr35M1120 to interact effectively with AvrSr35. The coinmunoprecipitation of epitope-tagged Sr35 and AvrSr35 expressed in N. benthamiana leaves supported the BiFC results, indicating that these proteins are capable of interacting in plant cells (fig. S10).

The identification of AvrSr35 and AvrSr50 (17) provides valuable tools for molecular surveillance and early detection of virulent fungal pathogen races, which can inform the deployment of resistance genes to prevent epidemics. AvrSr35 can also be used to confirm the expression of the functional Sr35 protein in the resistance gene cassettes, allowing for Sr35 to be quickly pyramided alongside other R genes. As more corresponding R-Avr gene pairs are identified, this information can guide the selection of complementary R genes targeting multiple avirulence factors to increase the durability of the deployed resistance gene pyramids and reduce the probability of spontaneous virulent Pgt strain origin.

Fig. 4. Sr35 and AvrSr35 proteins colocalize in plant cells and interact. (A) Coexpressed fluorescently tagged Sr35 and AvrSr35 colocalized in the N. benthamiana leaf epidermal cells. Scale bar, 20 μm. (B) In the N. benthamiana cells, the AvrSr35ΔSP:mRFP protein fusion accumulated in the ER strands (small arrows) and perinuclear space. Scale bar, 10 μm. (C) The Sr35(K206L):GFP protein fusion colocalized with the ER marker ER-mCherry in N. benthamiana cells (fig. S7). (D) Bimolecular fluorescent complementation showed interaction between Sr35 and AvrSr35 in the N. benthamiana cells. Compared with wild-type Sr35, the fluorescence intensity was significantly reduced in the cells expressing sr35M1120 (Tukey’s test adjusted P = 7.4 × 10−4) and negative control (Tukey’s test adjusted P = 8.7 × 10−5). Scale bar, 50 μm. N. nucleus.
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Fungal effectors of wheat stem rust

The fungal pathogen Ug99 (named for its identification in Uganda in 1999) threatens wheat crops worldwide. Ug99 can kill entire fields of wheat and is undeterred by many of the disease-resistance genes that otherwise protect wheat crops. Two papers describe two peptides secreted by the fungus as it attacks the wheat (see the Perspective by Moscou and van Esse). Chen et al. show that fungal AvrSr50 binds to the plant's immune receptor Sr50, and Salcedo et al. show that fungal AvrSr35 binds to Sr35. Successful binding activates the plant's immune defenses. Removing or inactivating these Avr effectors leaves the plant defenseless and susceptible to disease.

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