Title: Loss of AvrSr50 by somatic exchange in stem rust leads to virulence for Sr50 resistance in wheat

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Abstract: Race-specific resistance genes protect the global wheat crop from stem rust disease caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*), but often break down due to evolution of new virulent pathogen races. To understand virulence evolution in *Pgt* we identified the protein ligand (AvrSr50) recognized by the Sr50 resistance protein. A spontaneous mutant of *Pgt* virulent to *Sr50* contained a 2.5Mbp loss-of-heterozygosity event. A haustorial secreted protein from this region triggers *Sr50*-dependent defense responses *in planta* and interacts with the Sr50 protein. Virulence alleles of *AvrSr50* have arisen by DNA insertion and sequence divergence and our data provide molecular evidence that, in addition to sexual recombination, somatic exchange can play a role in the emergence of new virulence traits in *Pgt*.

One Sentence Summary:

An avirulence factor identified in the stem rust fungus is recognized by a wheat immune receptor to trigger resistance to disease.

Main Text:

Wheat is a staple crop that contributes 20% of human calorific intake, but its production is impacted by pathogens, including the fungus *Puccinia graminis* f. sp. *tritici* (*Pgt*) which causes stem rust disease (*1, 2*). Deployment of disease resistance genes by breeding provides cost-effective control of wheat rust diseases (*3*). Race-specific resistance is generally conferred by immune receptors of the nucleotide-binding leucine-rich repeat (NLR) class, which recognize pathogen effector proteins delivered into the host cell during infection, often known as avirulence (Avr) proteins (*4*). However, pathogen evolution to overcome resistance is a common occurrence and necessitates continued efforts to identify new resistance gene sources. The emergence of virulent races of *Pgt* in East Africa, particularly the Ug99 race group, has posed a threat to global wheat production (*2, 5*). A number of NLR-encoding rust resistance genes have been isolated from wheat (*6*). Here we identify one of the Avr proteins recognized by these receptors.

The *Sr50* resistance gene encodes an NLR protein and provides resistance against all race groups of *Pgt* worldwide, including Ug99 (*7*). We generated next generation sequence (NGS) data from *Pgt* isolate *Pgt*632, a spontaneous mutant with virulence to *Sr50* (*7*) (figure S1) and from its avirulent parental isolate *Pgt*279. Because the wheat-infecting uredinial stage of *Pgt* has a dikaryotic (n+n) genome with two haploid nuclei (*8*), *Pgt*279 is likely heterozygous for *AvrSr50* with the virulent derivative *Pgt*632 resulting from mutation of the dominant avirulence allele. We identified about 1.1 million heterozygous variants (single/multiple nucleotide variants [SNVs/MNVs] and small insertions and deletions; ~1% of sites) in each isolate compared to the reference genome PGTAus-pan (*9, 10*). Known *Avr* genes from the model flax rust fungus *Melampsora lini* encode secreted proteins expressed in haustoria, specialized structures that penetrate the host cell (*11, 12*). Although we did not identify any new non-synonymous variants in the 592 haustorial secreted protein (HSP) genes annotated in *Pgt* (*9*), 18 HSP genes showed loss-of-heterozygosity in *Pgt*632 (table S1, S2, figure S2). Mapping heterozygosity rates in *Pgt*632 and *Pgt*279 for each contig in the genome assembly revealed loss-of-heterozygosity in a region of at least 2.5Mbp spanning four full scaffolds and part of a fifth (figure 1A, S3 and table S3).

Loss-of-heterozygosity in *Pgt*632 could result from a deletion, which would halve the DNA copy number per dikaryotic genome of the affected region, or by somatic exchange between the two haplotypes, which would retain the DNA copy number. The normalized depth of sequencing read coverage for contigs in the loss-of-heterozygosity region was similar to the remainder of the genome in both *Pgt*632 and *Pgt*279 (table S4), suggesting no loss of DNA copy number. Likewise, there were no significant differences in coverage depth for individual gene loci between the isolates or genome regions (figure S4A-C), with a uniform read depth ratio between *Pgt*279 and *Pgt*632 close to one (figure 1B). Thus, we conclude that the loss of one haplotype in this region of the *Pgt*632 genome has been accompanied by duplication of the other haplotype. This was supported by quantitative PCR determination of relative copy number for shared and haplotype-specific sequences (figure S4D,E). Although it is not clear how genetic exchange occurs between the two separate haploid nuclei, which are thought to replicate independently (*13*), genetic evidence suggests that nuclear exchange and recombination between co-inoculated rust isolates can result in novel virulence combinations (*14, 15*). There is also evidence for nuclear fusion in *Pgt* (*16*) and somatic hybridization has been postulated as a mechanism underlying the emergence of new lineages in asexual rust populations (*17-19*).

The loss-of-heterozygosity region in *Pgt*632 contains 24 genes annotated as HSPs in the reference genome assembly, and the allelic variants of these genes missing from *Pgt*632 but present in *Pgt*279 are candidates to encode *AvrSr50*. Twenty-one of these genes showed two allelic types in *Pgt*279 with SNV frequencies close to 0.5 (figure S5), and only a single sequence variant in *Pgt*632. The two allele sequences of these genes were extracted from the NGS data (table S5) and the *Pgt*279-specific allele was used to generate *in planta* expression constructs. Another three HSP genes are part of multigene families and sequences representing these genes were obtained by DNA amplification, with all 20 variants retained for functional screening as it was not possible to assign them to haplotypes. In total, 41 unique HSP proteins (table S6) were expressed in *Nicotiana benthamiana* as cytosolic proteins lacking their signal peptides along with the Sr50 resistance protein. A single *AvrSr50* candidate, HSP#8 (HSGS210|asmbl_13131|m.9539), triggered a cell death response when co-expressed with *Sr50* (figure 2A, S6). Co-expression with the related Sr33 resistance protein produced no response (figure 2A, S7A), confirming the specificity of this recognition event, which was also observed in *N. tabacum* (figure 2B). A recombinant *Barley stripe mosaic virus* (*20*) expressing the *AvrSr50* candidate was unable to infect wheat plants containing *Sr50* (figure 2C), but retained virulence on susceptible wheat, confirming *AvrSr50* recognition by *Sr50* in wheat and showing that the *Sr50* resistance response is effective against virus infection. The AvrSr50 protein interacted with the Sr50 protein, but not with Sr33, in a yeast-two-hybrid assay (figure 2D, S7B). The 133 amino acid AvrSr50 protein has no homology to known proteins detected by either sequence or structure modelling searches, including in related *Puccinia* species.

A discontinuity in mapping of *Pgt*632 NGS reads to the *Pgt* genome sequence suggested that the alternative (virulence) allele of *AvrSr50* is disrupted by a DNA insertion. *De novo* assembly of *Pgt*632 sequencing reads resulted in two separate contigs containing the 5' and 3' regions of *AvrSr50*, each fused to an unrelated sequence, and the presence of this insertion was confirmed by DNA amplification (figure S8, S9, supp file 1). Examination of NGS data for genomes of other Australian *Pgt* isolates avirulent on *Sr50* (*9*) showed that 21-0, 326-1,2,3,5,6 and 194-1,2,3,5,6 were each heterozygous for the same two allelic variants of *AvrSr50* as in *Pgt*279, while rust strain 126-5,6,7,11 contained two alleles with identical coding sequence to the avirulence allele of *AvrSr50* but distinguished by SNVs in the 5' and 3' regions (figure S10). Sequencing of RNA from wheat infected with these isolates identified transcript sequences only for the avirulence alleles (figure S10), indicating that the virulence allele carrying the insertion sequence is not expressed.

We also examined *AvrSr50* diversity by amplification and sequencing from additional global *Pgt* races avirulent on *Sr50* (figure 2E, S9). Two North American isolates (pathotypes MCCFC, DFBJ) were homozygous for the avirulence allele, while another (SCCL) was heterozygous, containing this allele and another, which encoded a protein differing from AvrSr50 only by a single amino acid in the signal peptide region, with no effect on the predicted secretion. Thus this latter is also likely to be an avirulence allele. This allele is also identical in sequence to the virulence allele in *Pgt*632, but without the insertion sequence, suggesting it was the progenitor of this allele. An African isolate of the Ug99 group (TTKSK) contained a similar allele with one further conservative amino acid difference in the mature peptide, along with one copy of the insertion-disrupted virulence allele and is thus heterozygous for *AvrSr50* avirulence. One isolate (race QCMJC) collected from the alternate sexual host barberry and virulent on *Sr50* (*7*) contained one copy of the insertion-disrupted virulence allele along with another allele encoding a divergent protein with 12 amino acid differences from AvrSr50. We also extracted

another allelic variant from published NGS data for North American isolate RKQQC (*21*), which encoded a protein with 9 amino acid differences from AvrSr50. The RKQQC variant was recognised by Sr50 in *N. benthamiana* and in yeast, while the QCMJC variant was not (figure 2F,G and S11), consistent with the virulent phenotype of this isolate. The correlation between yeast protein interaction and induction of cell death *in planta* suggests that recognition specificity is mediated by direct interaction.

Analysis of the expression profiles of *Pgt* secreted protein genes in different infection stages detected 8 distinct clusters (figure 3A, S12, S13, table S7). *AvrSr50* is present in Cluster number 8, which contains genes showing high relative expression in haustoria versus germinated spores, and expression throughout infection. Cluster 4 shows a similar profile, but with smaller relative expression changes. Both clusters are over-represented for genes encoding predicted nuclear localized effectors and genes unique to *Pgt* or *Puccinia* species (table S8). Thus, the genes in these expression clusters are likely to be enriched for effectors involved in host manipulation during infection. Many plant pathogenic fungi and oomycetes display a two-speed genome with rapidly evolving genes, such as those encoding effectors, located in repeat-rich regions (*22*). However, clusters 4 and 8 were not significantly enriched for genes located close to repeat elements in the genome, consistent with the distribution of effector gene candidates in the *P. coronata* and *P. striiformis* genomes (*23*,*24*)**.** The AvrSr50 protein shows nucleo-cytosolic distribution when expressed in *N. benthamiana* (figure S14) and co-expression with the auto-active coiled-coil domains of Sr33 and Sr50 resulted in suppression of their cell-death signaling activity in tobacco (figure 3B). This suppression was also observed with 11 other HSP genes including HSP#18 (figure 3B), and may reflect a function of these effectors in suppressing defense responses during infection of wheat.

The identification of *AvrSr50* here and *AvrSr35* by Salcedo et al (*25*), will support resistance gene deployment strategies as the *Avr* gene sequences can be used as molecular markers to survey the spatio-temporal distribution of these genes in *Pgt* populations and anticipate the evolution of virulence. For instance, heterozygosity for *AvrSr50* may predispose certain *Pgt* lineages to more rapid evolution of virulence towards *Sr50*. This information can help prioritise resistance genes for deployment in different geographic locations.

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Fig. 1. Loss-of-heterozygosity associated with virulence of *Pgt*632 on *Sr50*. (**A**) Heterozygosity rates of genomic contigs 323-402 in scaffold 4 of the PGTAus-pan assembly in *Pgt*279 (orange) and *Pgt*632 (blue). (**B**) Ratio of coverage depth of reads from *Pgt*632 relative to *Pgt*279 for 1000 genes from scaffold 1 (green) and genes from scaffold 4 either outside (278 genes, blue) or within (724 genes, red) the loss-of-heterozygosity region.

Fig. 2. The AvrSr50 effector is recognized by Sr50. (**A**) Transient expression in *N. benthamiana* of Sr50:HA or Sr33:HA with YFP:AvrSr50 or YFP alone. Images taken 4 days post-infiltration (dpi). (**B**) Transient co-expression in *N. tabacum* of Sr50:HA with AvrSr50, YFP:AvrSr50, HSP#18 or YFP alone. Images taken 2 dpi. (**C**) Infection of wheat lines Gabo, Gabo-1DL.1RS (contains *Sr50*), Fielder and transgenic Fielder expressing *Sr50* with the *Barley stripe mosaic virus* expression vector containing either AvrSr50 or a non-coding multiple cloning site (MCS). (**D**) Growth of yeast strains co-expressing Sr50 or Sr33 fused to the GAL4 DNA binding domain (BD) with AvrSr50 or HSP #5 fused to the GAL4 activation domain (AD) on control media lacking leucine and tryptophan (-LW) or selective media additionally lacking histidine (-LWH). Self-interaction of the flax L6 protein TIR domain is a positive control (*26*). A 10-fold dilution series is shown. (**E**) Amino acid sequence of AvrSr50 and variants found in *Pgt* isolates of races SCCL, TTKSK, RKQQC and QCMJC. (**F**) Transient expression in *N. benthamiana* of Sr50:HA with YFP tagged AvrSr50 wildtype (WT) and variant alleles from races RKQQC and QCMJC. Image taken 4 dpi. (**G**) Growth of yeast strains co-expressing BD:Sr50 with AvrSr50 wildtype , RKKQC and QCMJC variants on selective media.

Fig. 3. *AvrSr50* is expressed early in infection and can suppress cell death responses. (**A**) Clustering analysis of *Pgt* secretome expression profiles. Blue color intensity indicates relative expression levels (relative *rlog* transformed counts) in haustoria (H), germinated spores (Sp) and infected leaves at 2, 3, 4, 5, 6 and 7 days post-infection. (**B**) Transient expression of the Sr33 and Sr50 coiled-coil (CC) domains with either YFP, AvrSr50 or HSP#18 in *N. tabacum* leaves. Image taken 4 dpi.

Supplementary Materials:

Materials and Methods

Figures S1-S14

Tables S1-S9

References (*27-48*)