Supplementary Information

Allelic variation of Avr genes in highly virulent strains explains severe wheat stem rust epidemics

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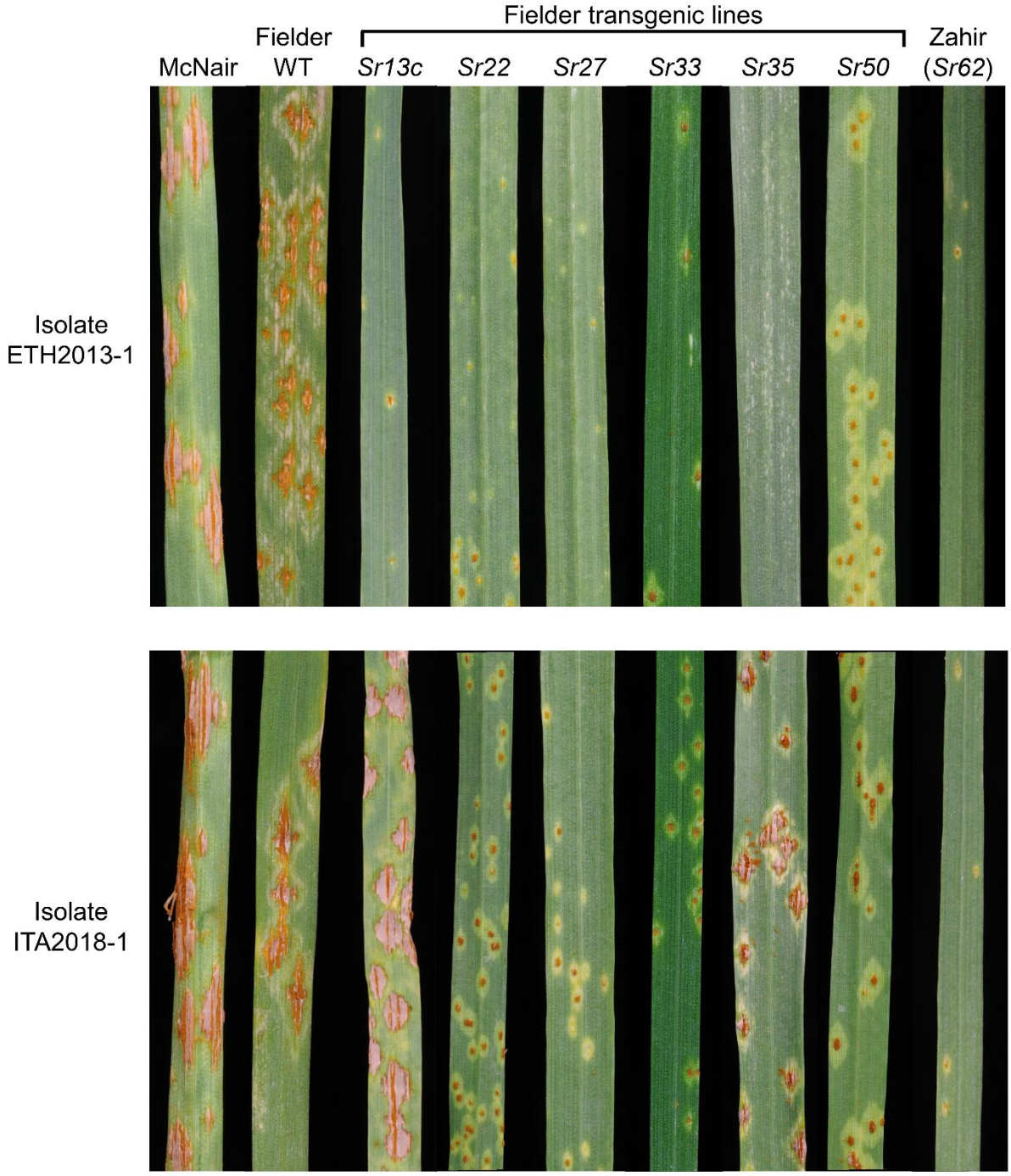
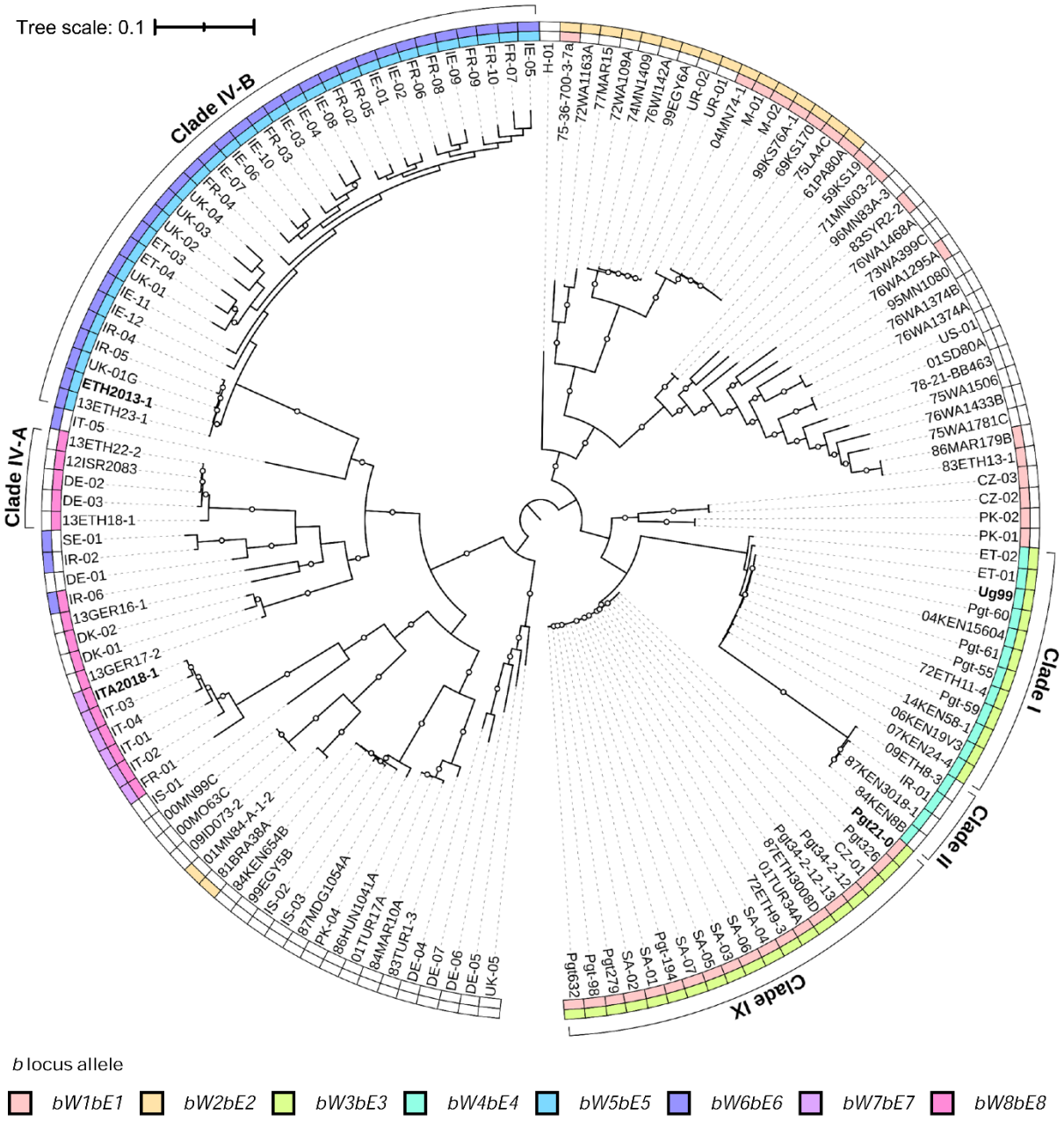
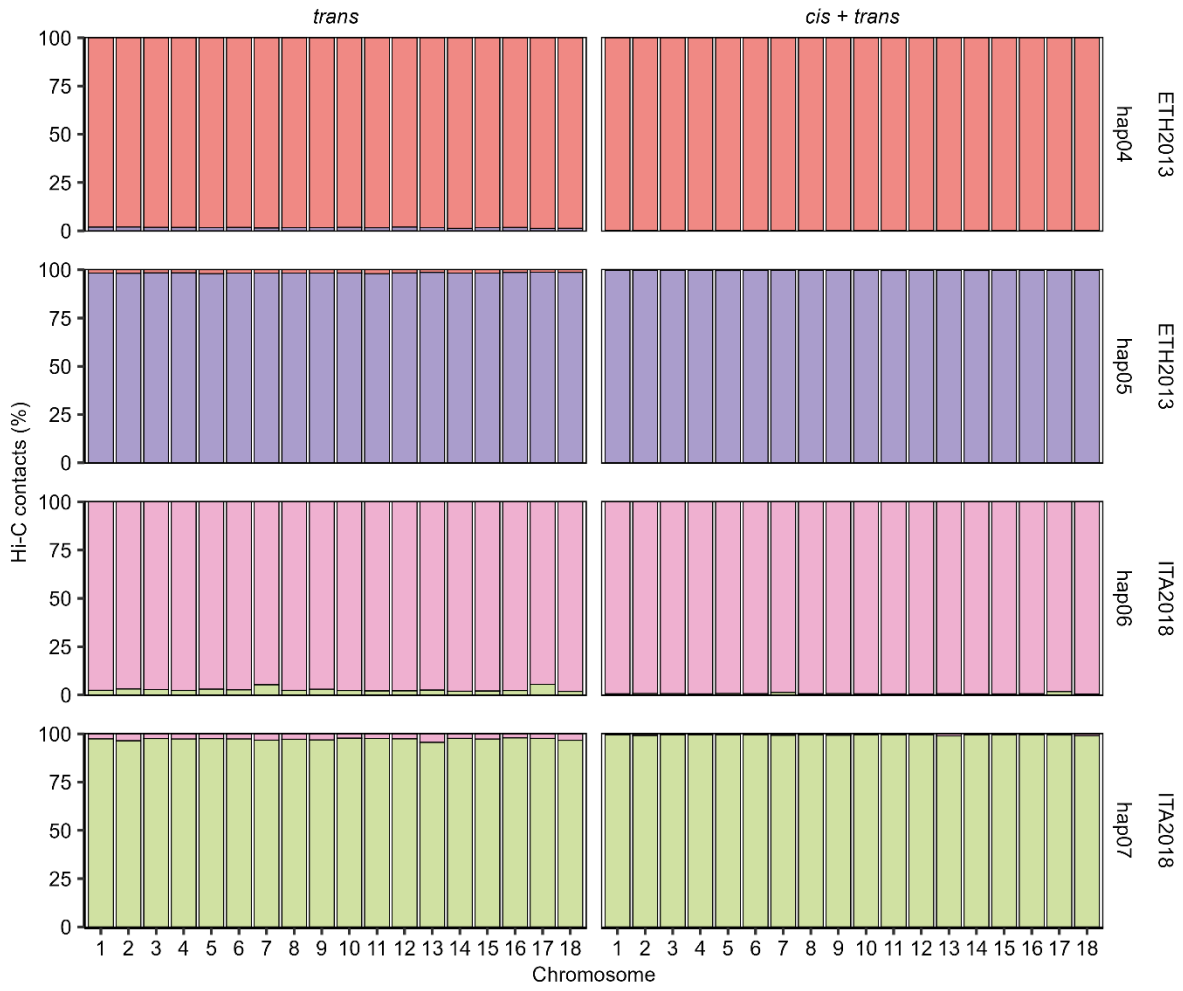


Supplementary Figure 1. Virulence phenotypes for ETH2013-1 and ITA2018-1. Infection types of single-gene transgenic wheat lines (wild-type Fielder with stem rust resistance genes *Sr13*, *Sr22*, *Sr27, Sr33*, *Sr35* and *Sr50*) and *Sr62*-containing wheat introgression line Zahir 1644 for *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 and ITA2018-1. Infection types for the susceptible wheat cultivar McNair and wild-type (WT) Fielder are given at the left for comparison.

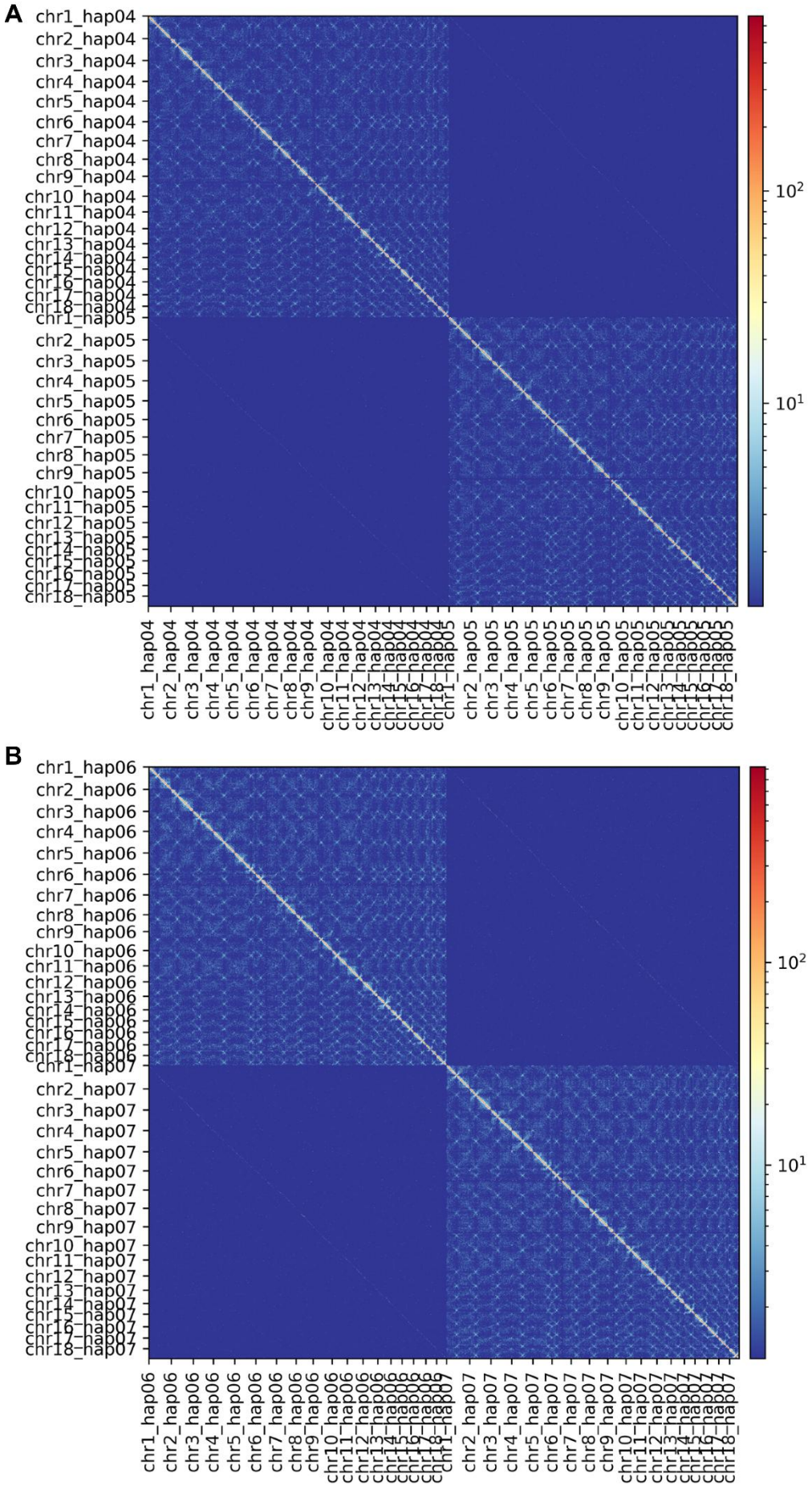


Supplementary Figure 2. Phylogenetic tree of wheat stem rust isolates. Maximum likelihood phylogenetic tree of 146 *Puccinia graminis* f. sp. *tritici* isolates against the compete Pgt21-0 genome (393,568 SNPs). Bootstrap values above 80% (500 cycles) are shown as white circles at branch midpoints. Tree scale is average substitutions per site. Coloured rectangles represent *b* locus alleles. Country abbreviations: BRA – Brazil; CZ – Czech Republic; DK – Denmark; EGY – Egypt; ET, ETH – Ethiopia; FR – France; DE, GER – Germany; H, HUN – Hungary; IE – Ireland; IND – India; IR – Iran; IS, ISR – Israel; IT, ITA – Italy; KEN – Kenya; M – Mexico; MAR – Morocco; MDG – Madagascar; PK, PAK – Pakistan; SA – South Africa; SE – Sweden; SYR – Syria;

TUR – Turkey; Ug – Uganda; UK – United Kingdom; UR - Uruguay; US – USA; ZIM - Zimbabwe. USA state abbreviations: ID – Idaho; KS – Kansas; LA – Louisiana; MN - Minnesota; MO – Missouri; PA – Pennsylvania; SD – South Dakota; WA – Washington; WI – Wisconsin.



Supplementary Figure 3. Chromatin contact frequencies on chromosomes of ETH2013-1 and ITA2018-1. Percentage Hi-C *trans* contact and *cis* + *trans* contact to chromosomes within a nucleus for *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 (hap04, hap05) and ITA2018-1 (hap06, hap07). The MAPQ cutoff used for HicPro was 10. Chromosome contact frequency data is provided in **Supplementary Data 4**.


A
chr1_hap04
chr2_hap04
chr3_hap04
chr4_hap04
chr5_hap04
chr6_hap04
chr7_hap04
chr8_hap04
chr9_hap04
chr10_hap04
chr11_hap04
chr12_hap04
chr13_hap04
chr14_hap04
chr15_hap04
chr16_hap04
chr17_hap04
chr18_hap04
chr1_hap05
chr2_hap05
chr3_hap05
chr4_hap05
chr5_hap05
chr6_hap05
chr7_hap05
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chr11_hap05
chr12_hap05
chr13_hap05
chr14_hap05
chr15_hap05
chr16_hap05
chr17_hap05
chr18_hap05
10^2
10^1
B
chr1_hap06
chr2_hap06
chr3_hap06
chr4_hap06
chr5_hap06
chr6_hap06
chr7_hap06
chr8_hap06
chr9_hap06
chr10_hap06
chr11_hap06
chr12_hap06
chr13_hap06
chr14_hap06
chr15_hap06
chr16_hap06
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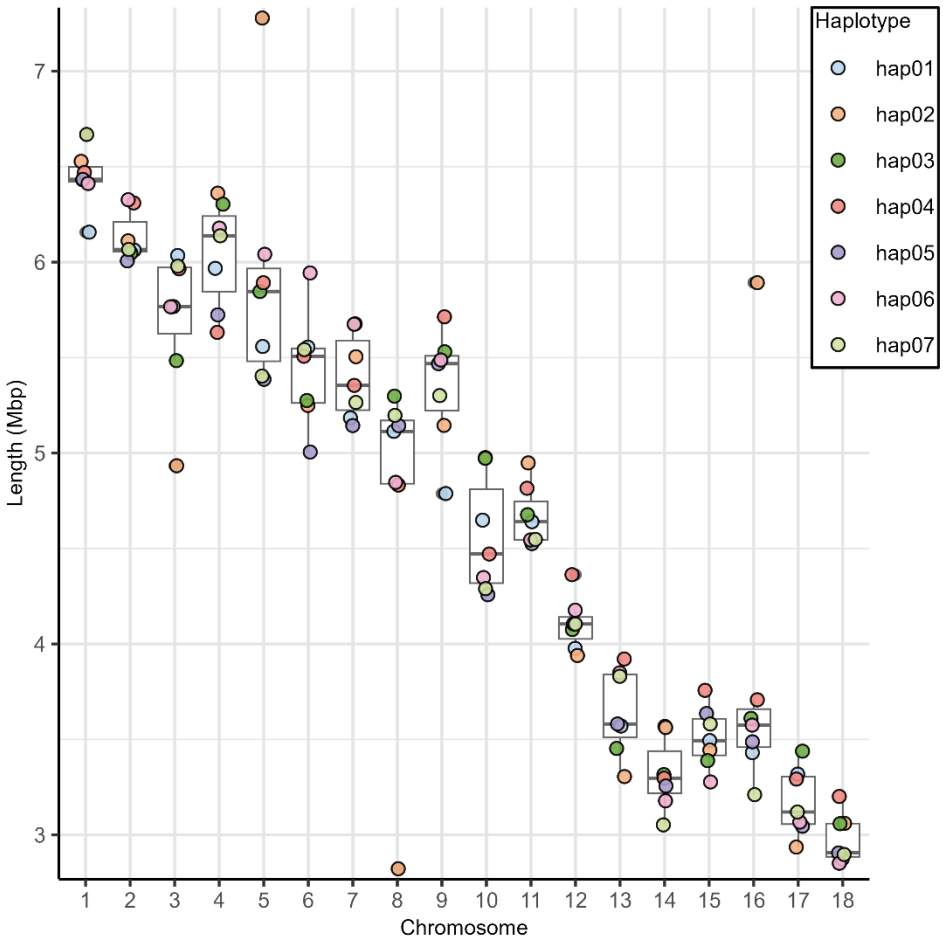
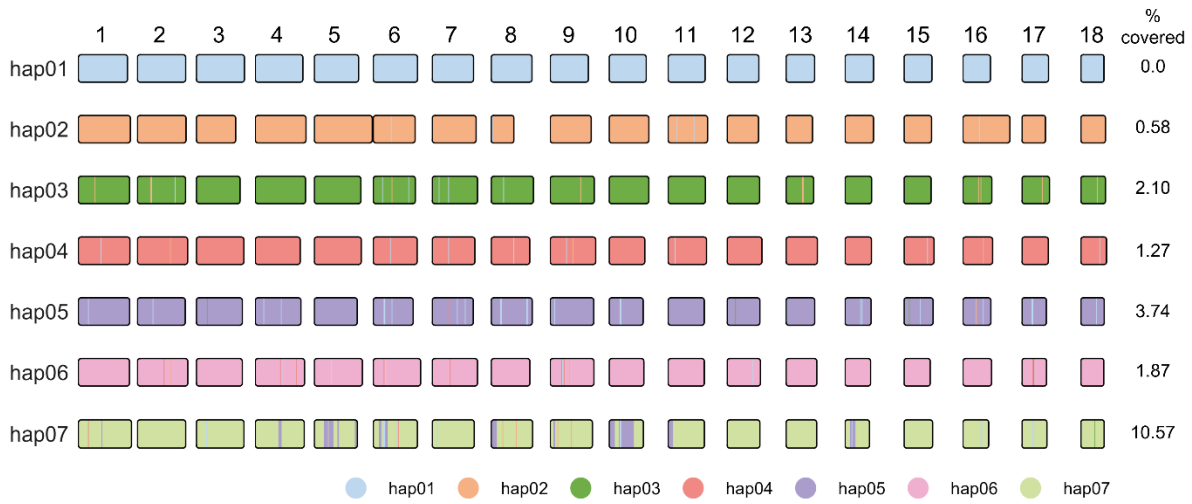
chr18_hap06
chr1_hap07
chr2_hap07
chr3_hap07
chr4_hap07
chr5_hap07
chr6_hap07
chr7_hap07
chr8_hap07
chr9_hap07
chr10_hap07
chr11_hap07
chr12_hap07
chr13_hap07
chr14_hap07
chr15_hap07
chr16_hap07
chr17_hap07
chr18_hap07
10^2
10^1

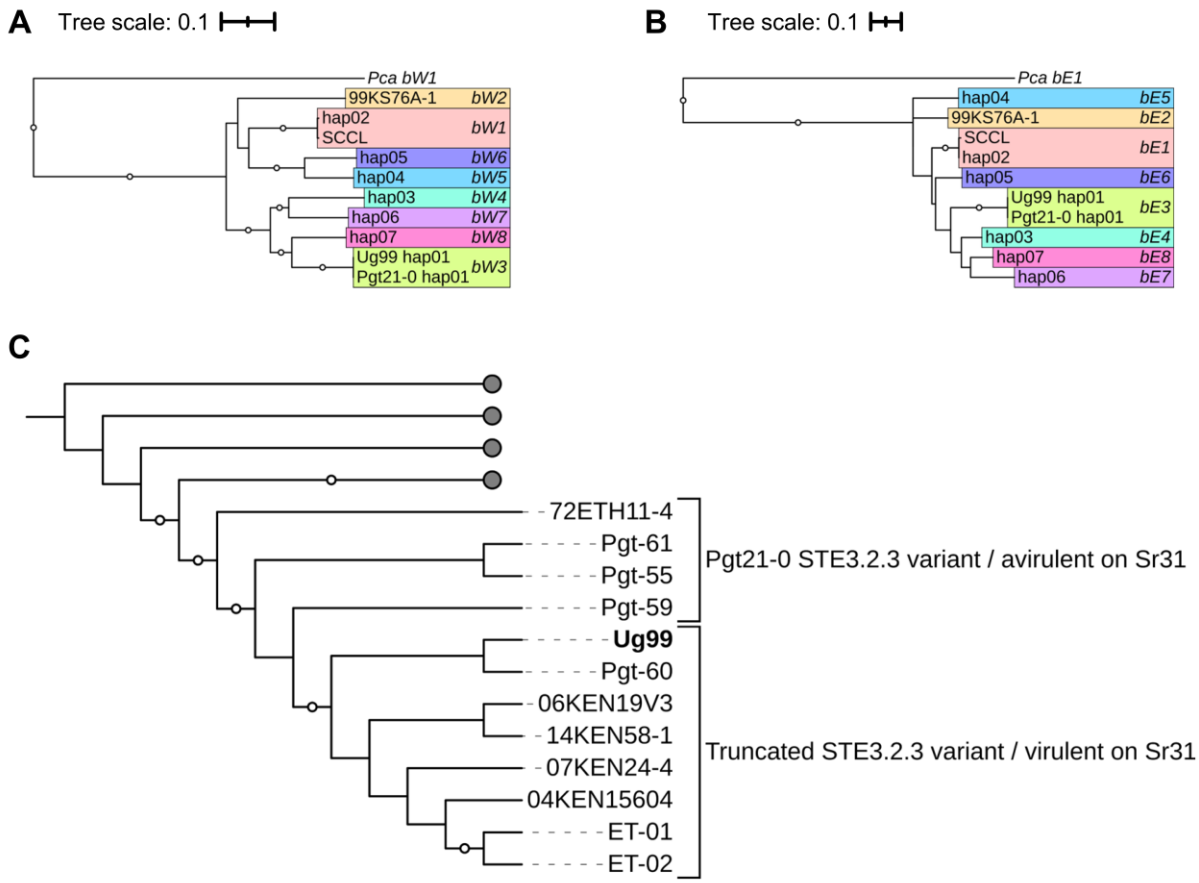
Supplementary Figure 4. Chromatin contact maps for chromosomes of ETH2013-1 and ITA2018-1. Hi-C contact maps (100kbp resolution) for chromosomes in *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 **(A)** and ITA2018-1 **(B)**. Centromeres are visible as white crosses or “bowties” that spread outwards. Colour scale indicates contact frequency from high (red) to low (blue).



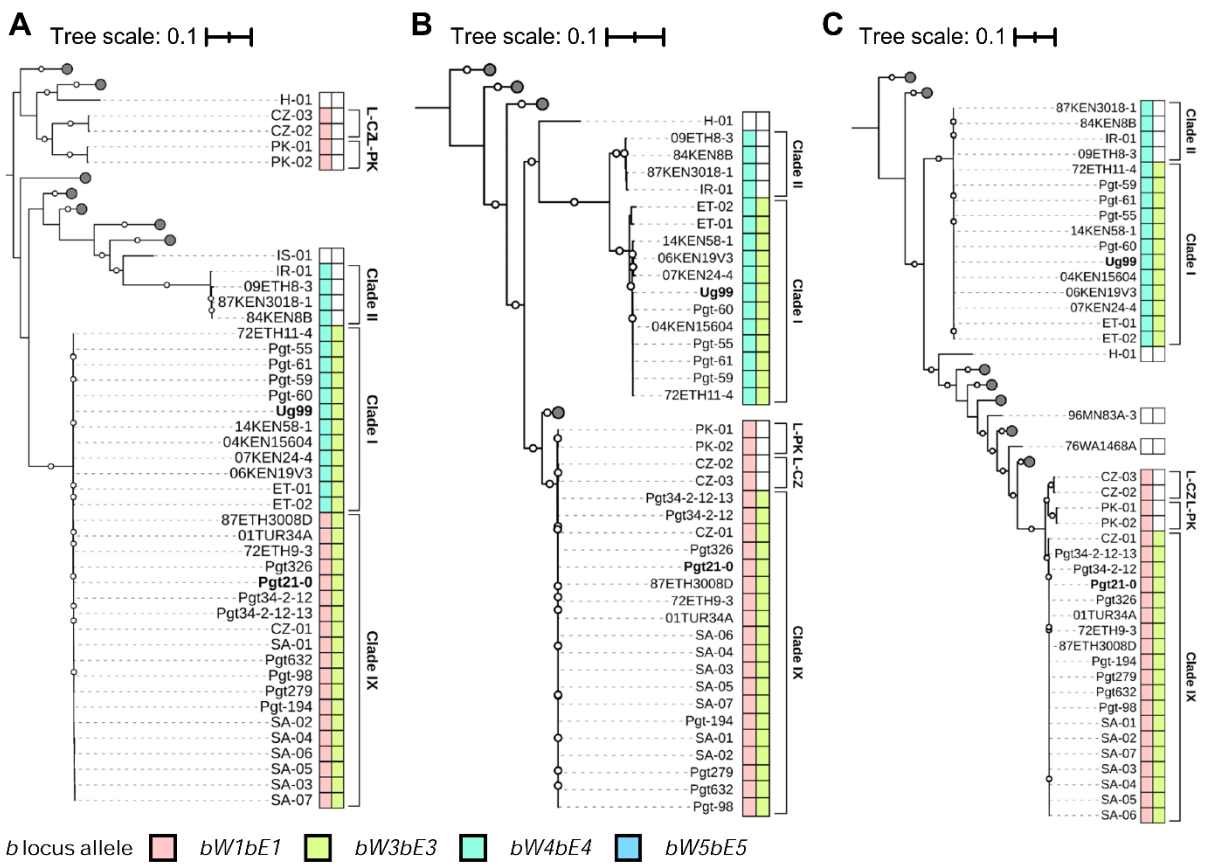
Supplementary Figure 5. Chromosome length distributions for wheat stem rust haplotypes. Boxplots of chromosome lengths for haplotypes of *Puccinia graminis* f. sp. *tritici* isolates Pgt21-0 (hap01, hap02), Ug99 (hap03), ETH2013-1 (hap04, hap05), and ITA2018-1 (hap06, hap07). Box boundaries represent the first and third quartile and the center line represents the median length. Colours represent different haplotypes as indicated in the inset. Note that the size outliers for chromosomes 3, 5, 8 and 16 in hap02 are the result of large translocations in this haplotype.



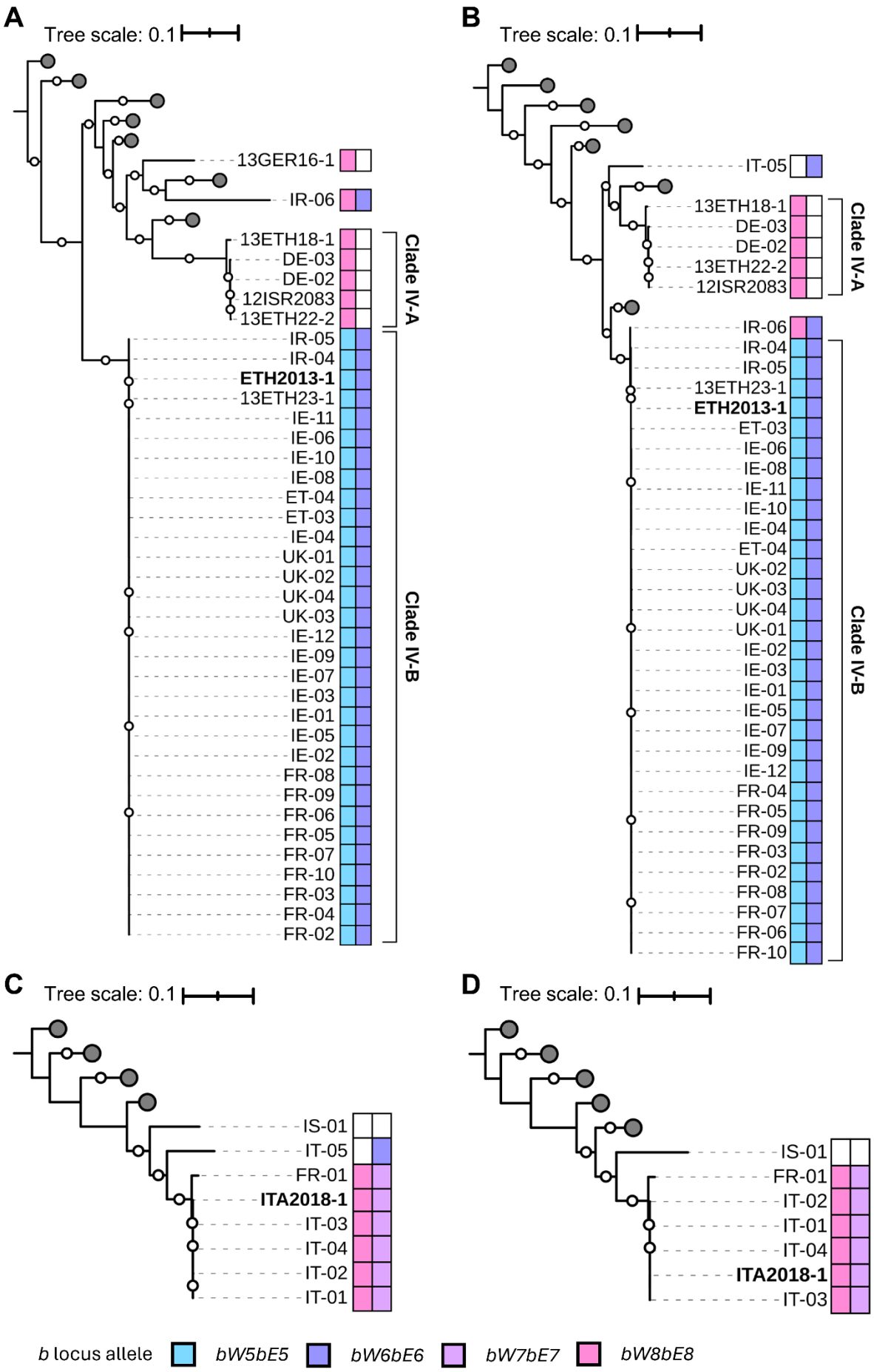
Supplementary Figure 6. Recombination blocks identified for wheat stem rust haplotypes. Sequences shared among seven *Puccinia graminis* f. sp. *tritici* haplotypes. Shared sequences are assigned hierarchically from top to bottom (order: hap01, hap02, hap03, hap04, hap05, hap06, hap07). Chromosome fill indicates assignment of sequences. Values to the right of chromosomes indicate the percentage of each haplotype's sequence that is shared with haplotypes above it in the hierarchy (i.e. percent of hap02 shared with hap01, percent of hap03 shared with hap01 and hap02).



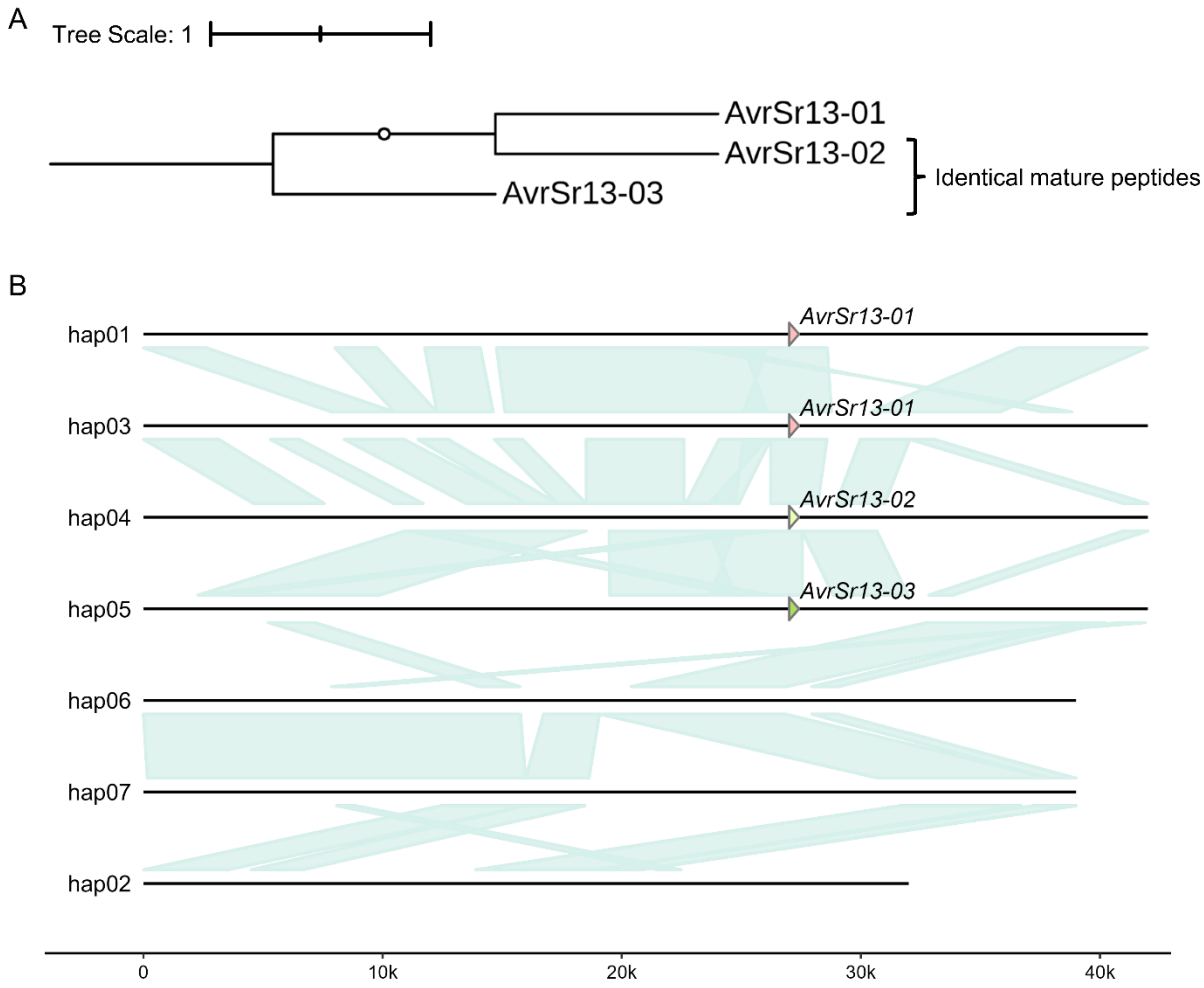
Supplementary Figure 7. Protein trees for mating locus alleles. (A) Maximum likelihood phylogenetic tree of eight *Puccinia graminis* f. sp. *tritici* (*Pgt*) *bW* allele protein sequences. Tree is rooted on the *P. coronata* f. sp. *avenae* (*Pca*) *bW1* allele. **(B)** ML tree of eight *Pgt bE* allele protein sequences. Tree is rooted on the *Pca bE1* allele. **(C)** Maximum Likelihood phylogenetic tree of 146 *Pgt* isolates using hap01 from Ug99 as the reference (218,530 SNPs), visualized as a dendrogram to illustrate phylogenetic distribution of *STE3.2.3* variants in Clade I, which also aligns with the distribution of virulence on *Sr31* in Clade I. Bootstrap values above 80% (100 cycles for panels A and B, 500 cycles for panel C) are shown as white circles at branch midpoints.



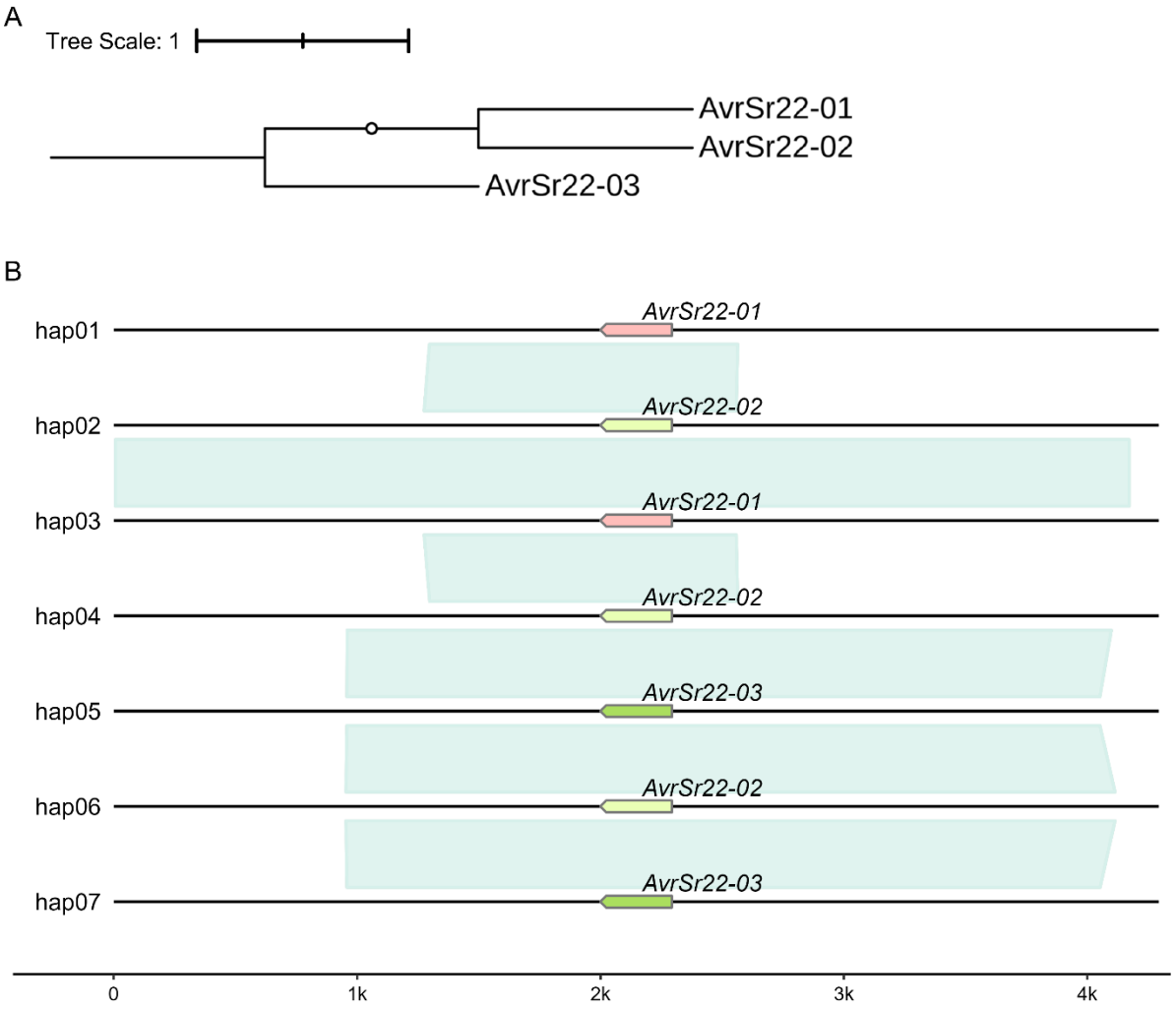
Supplementary Figure 8. Haplotype specific (hap01 to hap03) phylogenetic trees of selected wheat stem rust isolates. Maximum Likelihood phylogenetic trees for 146 *Puccinia graminis* f. sp. *tritici* isolates using references **(A)** hap01 (Ug99, 218,530 SNPs), **(B)** hap02 (Pgt21-0, 208,959 SNPs), and **(C)** hap03 (Ug99, 203,083 SNPs). Bootstrap values above 80% (500 cycles) are shown as white circles at branch midpoints. Tree scale is average substitutions per site. Coloured rectangles represent *b* locus alleles. Country abbreviations: CZ – Czech Republic; ET, ETH – Ethiopia; H – Hungary; IS – Israel; IR – Iran; KEN – Kenya; PK – Pakistan; SA – South Africa; TUR – Turkey; Ug – Uganda. USA state abbreviations: MN – Minnesota; WA – Washington.


A
Tree scale: 0.1
13GER16-1
IR-06
13ETH18-1
DE-03
DE-02
12ISR2083
13ETH22-2
Clade IV-A
IR-05
IR-04
ETH2013-1
13ETH23-1
IE-11
IE-06
IE-10
IE-08
ET-04
ET-03
IE-04
UK-01
UK-02
UK-04
UK-03
IE-12
IE-09
IE-07
IE-03
IE-01
IE-05
IE-02
FR-08
FR-09
FR-06
FR-05
FR-07
FR-10
FR-03
FR-04
FR-02
Clade IV-B
B
Tree scale: 0.1
IT-05
13ETH18-1
DE-03
DE-02
13ETH22-2
12ISR2083
Clade IV-A
IR-06
IR-04
IR-05
13ETH23-1
ETH2013-1
ET-03
IE-06
IE-08
IE-11
IE-10
IE-04
ET-04
UK-02
UK-03
UK-04
UK-01
IE-02
IE-03
IE-01
IE-05
IE-07
IE-09
IE-12
FR-04
FR-05
FR-09
FR-03
FR-02
FR-08
FR-07
FR-06
FR-10
Clade IV-B
C
Tree scale: 0.1
IS-01
IT-05
FR-01
ITA2018-1
IT-03
IT-04
IT-02
IT-01
D
Tree scale: 0.1
IS-01
FR-01
IT-02
IT-01
IT-04
ITA2018-1
IT-03
b locus allele
bW5bE5
bW6bE6
bW7bE7
bW8bE8

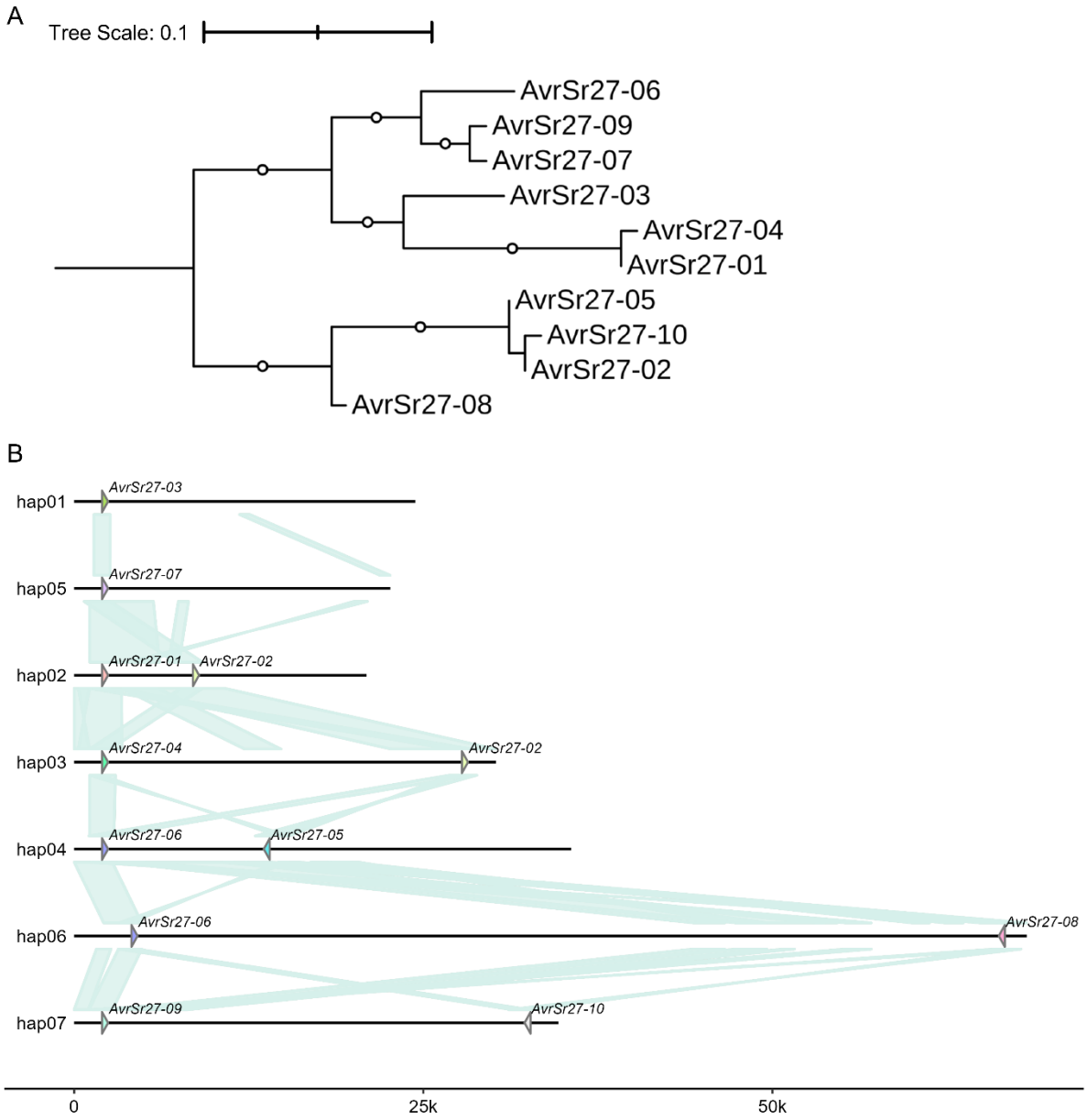
Supplementary Figure 9. Haplotype specific (hap04 to hap07) phylogenetic trees of selected wheat stem rust isolates. Maximum likelihood trees of 146 *Puccinia graminis* f. sp. *tritici* isolates against haplotypes **(A)** hap04 (222,364 SNPs), **(B)** hap05 (241,024 SNPs), **(C)** hap06 (220,258 SNPs), and **(D)** hap07 (226,566 SNPs). Bootstrap values above 80% are shown as white circles at branch midpoints. Tree scale is average substitutions per site. Coloured rectangles represent *b* locus alleles. Country abbreviations: DE, GER – Germany; ET, ETH – Ethiopia; FR – France; IE – Ireland; IR – Iran; IS, ISR – Israel; IT, ITA – Italy; UK – United Kingdom.



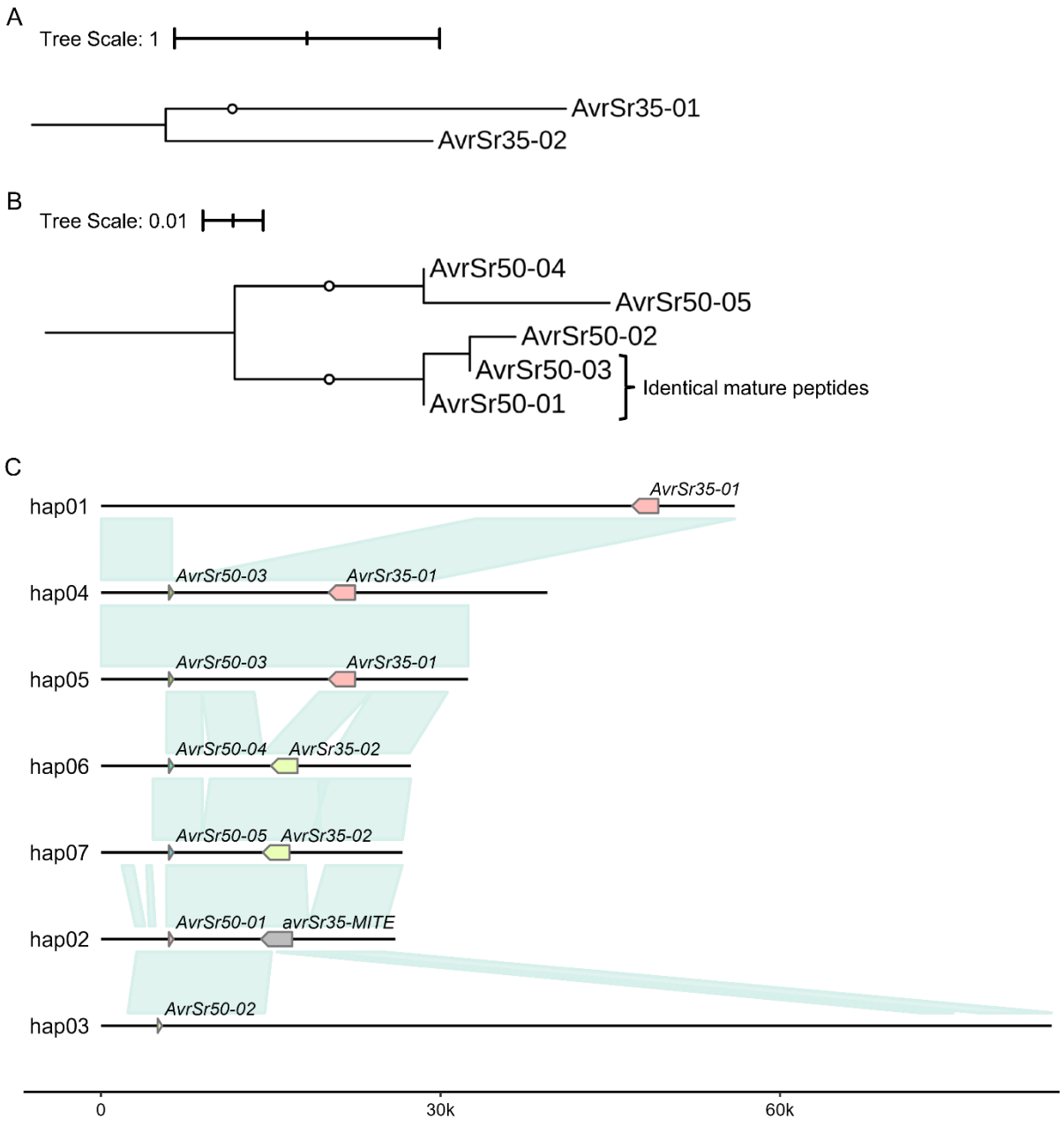
Supplementary Figure 10. Haplotype structure of the *AvrSr13* locus. (A) Maximum Likelihood phylogenetic tree of AvrSr13 protein variants from *Puccinia graminis* f. sp. *tritici* isolates Pgt21-0, Ug99, ETH2013-1, and ITA2018-1. Scale indicates mean substitutions per site. Open circles at branch midpoints indicate ≥ 80% bootstrap support (100 cycles). **(B)** Synteny plot of the *AvrSr13* region on chromosome 1 from seven *Pgt* haplotypes. Green filled rectangles between haplotypes indicate syntenous sequences. *X*-axis values reflect the scale of the aligned regions. Absolute start and end positions for genes in each haplotype can be found in **Supplementary Data 12**. Gene fill colour represents different alleles of *AvrSr13*.



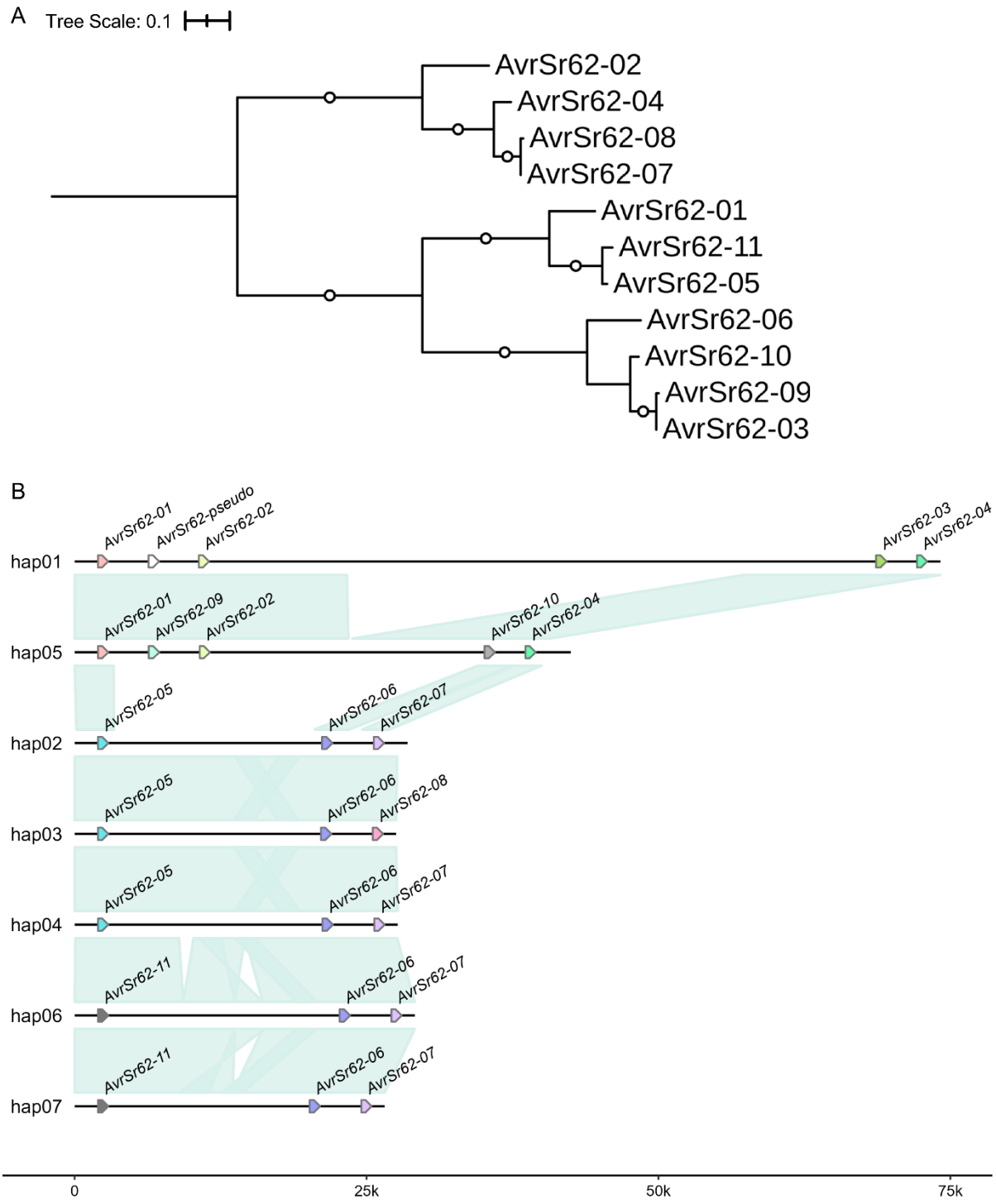
Supplementary Figure 11. Haplotype structure of the *AvrSr22* locus. (A) Maximum Likelihood phylogenetic tree of AvrSr22 protein variants from *Puccinia graminis* f. sp. *tritici* isolates Pgt21-0, Ug99, ETH2013-1, and ITA2018-1. Scale indicates mean substitutions per site. Open circles at branch midpoints indicate ≥ 80% bootstrap support (100 cycles). **(B)** Synteny plot of the *AvrSr22* region on chromosome 16 from seven *Pgt* haplotypes. Green filled rectangles between haplotypes indicate syntenous sequences. *X*-axis values reflect the scale of the aligned regions. Absolute start and end positions for genes in each haplotype can be found in **Supplementary Data 12**. Gene fill colour represents different alleles of *AvrSr22*.



Supplementary Figure 12. Haplotype structure of the *AvrSr27* locus. (A) Maximum Likelihood phylogenetic tree of AvrSr27 protein variants from *Puccinia graminis* f. sp. *tritici* isolates Pgt21-0, Ug99, ETH2013-1, and ITA2018-1. Scale indicates mean substitutions per site. Open circles at branch midpoints indicate ≥ 80% bootstrap support (100 cycles). **(B)** Synteny plot of the *AvrSr27* region on chromosome 2 from seven *Pgt* haplotypes. Green filled rectangles between haplotypes indicate syntenous sequences. *X*-axis values reflect the scale of the aligned regions. Absolute start and end positions for genes in each haplotype can be found in **Supplementary Data 12**. Gene fill colour represents different alleles of *AvrSr27*.

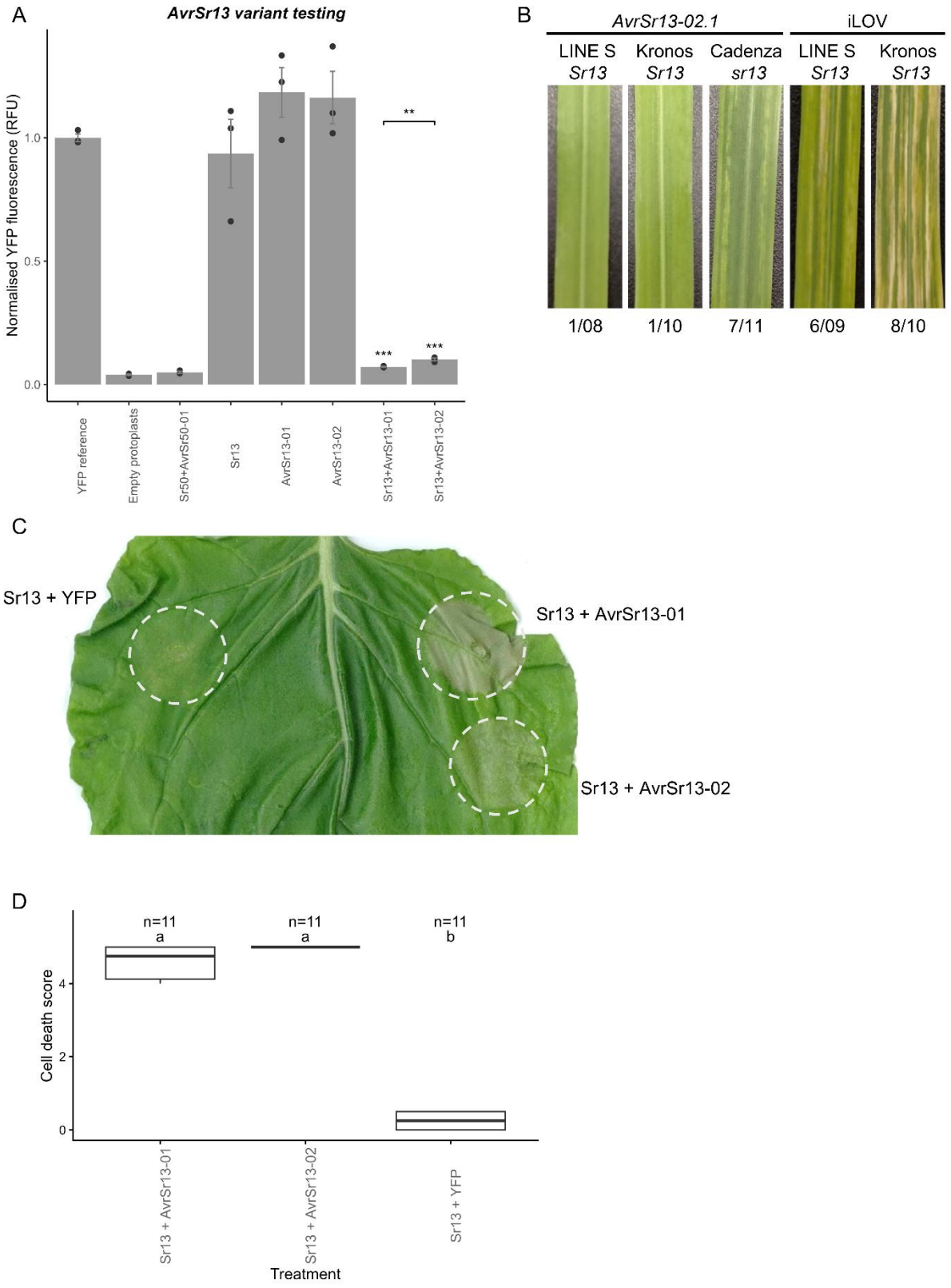


Supplementary Figure 13. Haplotype structure of the *AvrSr50* and *AvrSr35* loci. Maximum Likelihood phylogenetic trees of **(A)** AvrSr35 and **(B)** AvrSr50 protein variants from *Puccinia graminis* f. sp. *tritici* isolates Pgt21-0, Ug99, ETH2013-1, and ITA2018-1. Scale indicates mean substitutions per site. Open circles at branch midpoints indicate ≥ 80% bootstrap support (100 cycles). **(C)** Synteny plot of the *AvrSr35*/*AvrSr50* region on chromosome 14 from seven *Pgt* haplotypes. Green filled rectangles between haplotypes indicate syntenous sequences. *X*-axis values reflect the scale of the aligned regions. Absolute start and end positions for genes in each haplotype can be found in **Supplementary Data 12**. Gene fill colour represents different alleles of *AvrSr50* and *AvrSr35*.



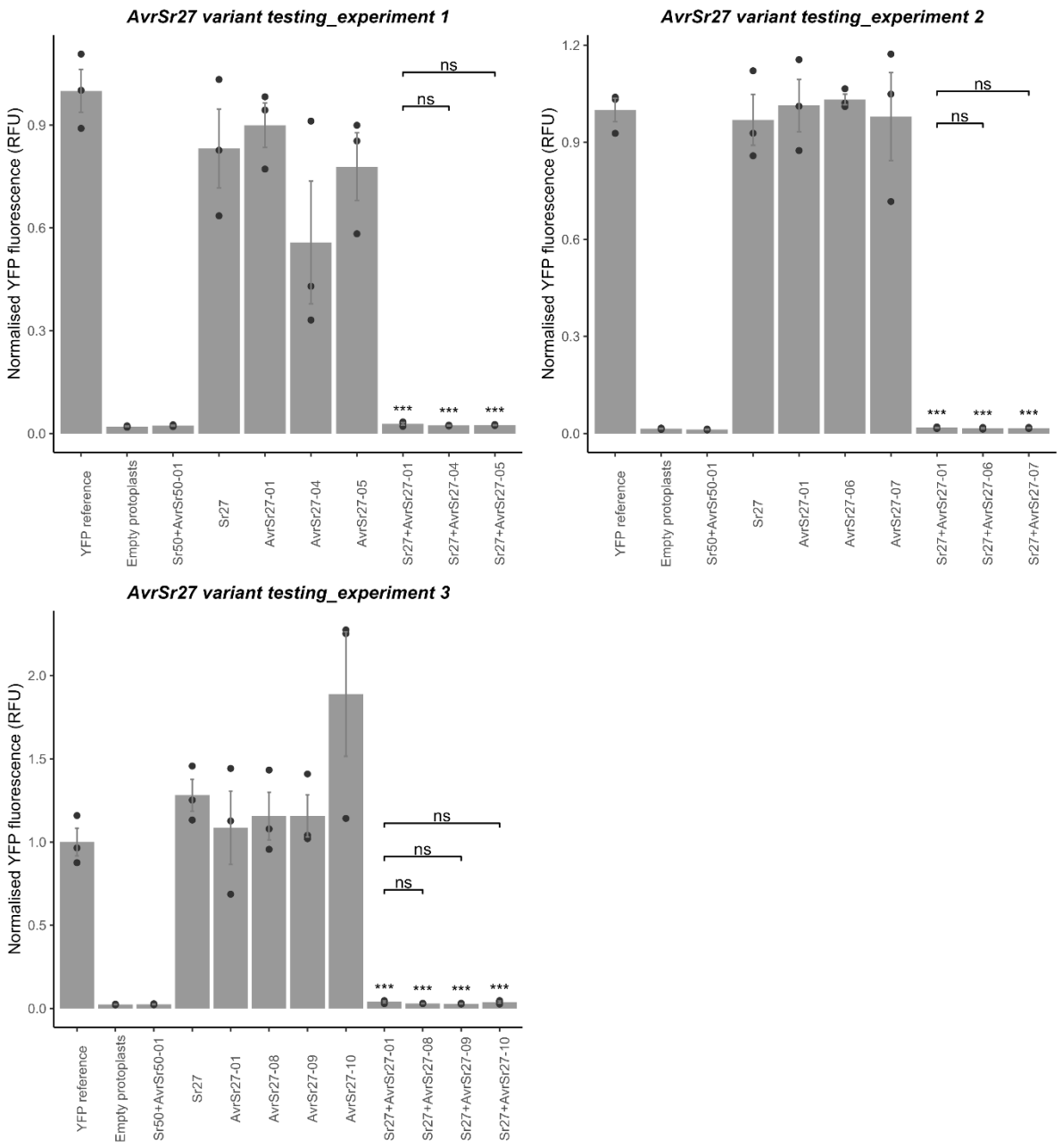
Supplementary Figure 14. Haplotype structure of the *AvrSr62* locus. (A) Maximum Likelihood tree of AvrSr62 protein variants from *Puccinia graminis* f. sp. *tritici* isolates Pgt21-0, Ug99, ETH2013-1, and ITA2018-1. Scale indicates mean substitutions per site. Open circles at branch midpoints indicate ≥ 80% bootstrap support (100 cycles). **(B)** Synteny plot of the *AvrSr62* region on chromosome 5 from seven *Pgt* haplotypes. Green filled rectangles between haplotypes indicate syntenous

sequences. *X*-axis values reflect the scale of the aligned regions. Absolute start and end positions for genes in each haplotype can be found in **Supplementary Data 12**. Gene fill colour represents different alleles of *AvrSr62*.



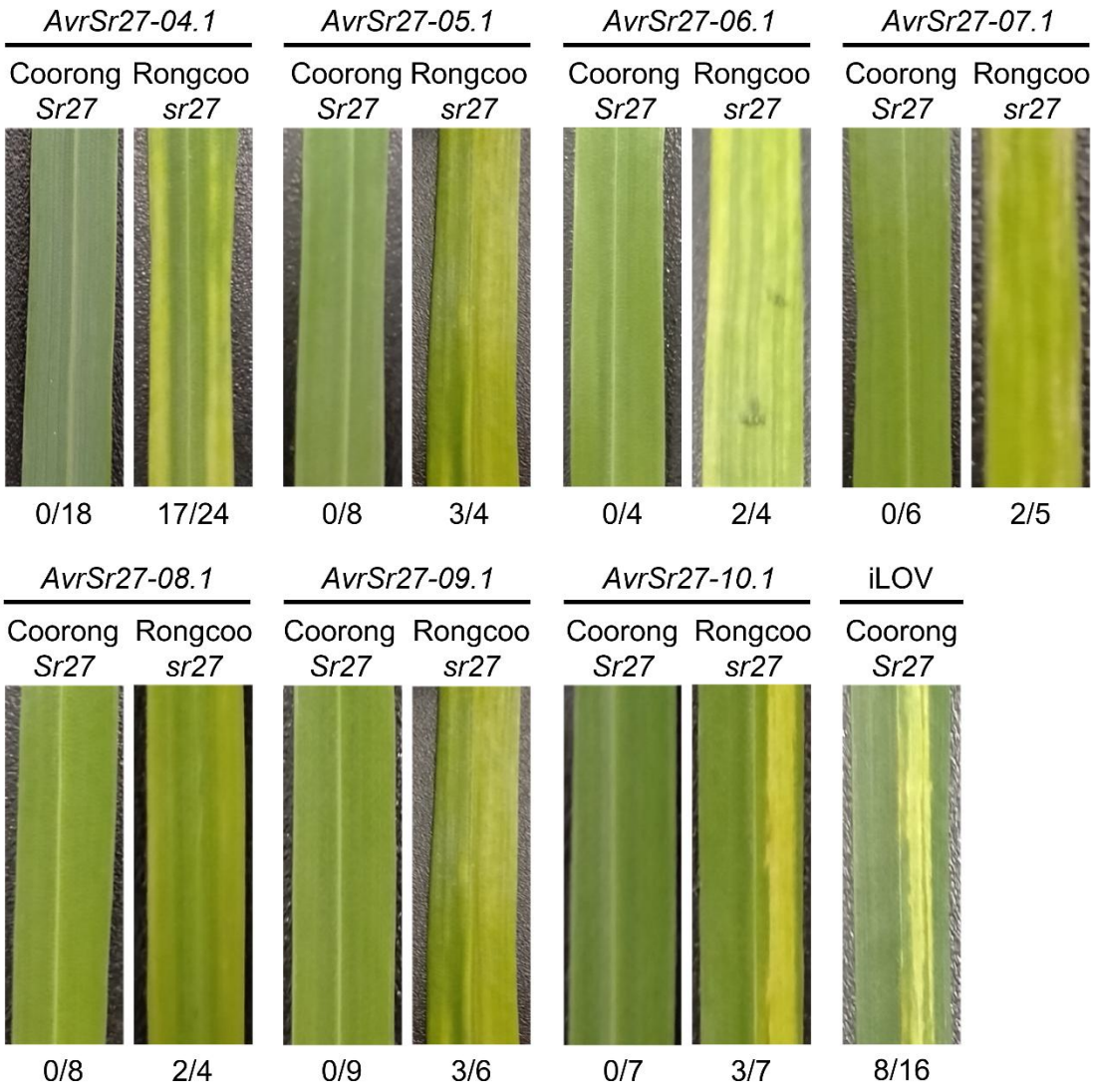
Supplementary Figure 15. Functional characterization of novel alleles of *AvrSr13* in *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 and ITA2018-1. **(A)** Transient expression in protoplasts of the wheat cultivar Fielder. The *x*-axis shows

genes used for transformation while the *y*-axis shows fluorescence values normalized to the YFP reference (RFU). Grey columns show mean values from three biological replicates. Error bars indicate the standard error. Black dots represent the result for each replicate of a given treatment. Two-tailed t-tests were performed for pairwise comparisons between specific treatments, '***' indicates significance at 0.001 level (*P*-value = 4.905e-7, 6.912e-7), '**' indicates significance at 0.01 level (*P*-value = 6.659e-3). A *t*-test significance value directly above a column indicates a comparison with the YFP reference. Raw data, detailed analysis and t-test *P*-values are provided in Source data file. **(B)** Infection of wheat cultivars LINE S (*Sr13*), Kronos (*Sr13*) and Cadenza (*sr13*) with Barley Stripe Mosaic Virus (BSMV) expressing *AvrSr13-02* or the negative control iLOV. Fractions under leaf pictures indicate the number of plants exhibiting BSMV symptoms over the total number of inoculated plants. **(C)** Co-expression assay in *Nicotiana benthamiana*. Dotted lines mark out the area infiltrated with an *Agrobacterium tumefaciens* suspension. **(D)** Cell death score in all infiltrated *N. benthamiana* leaves represented as a box and whiskers plot with the number (n) of replicates for each treatment indicated in the graph. The *x*-axis represents the different treatments, the *y*-axis represents the cell death score out of 5 (0=no cell death, 5=cell death over the whole inoculated area). Letters indicate samples that do not differ significantly (Welch's ANOVA followed by Dunnett's T3 multiple comparisons test, pairwise *P*-values in Source Data File).

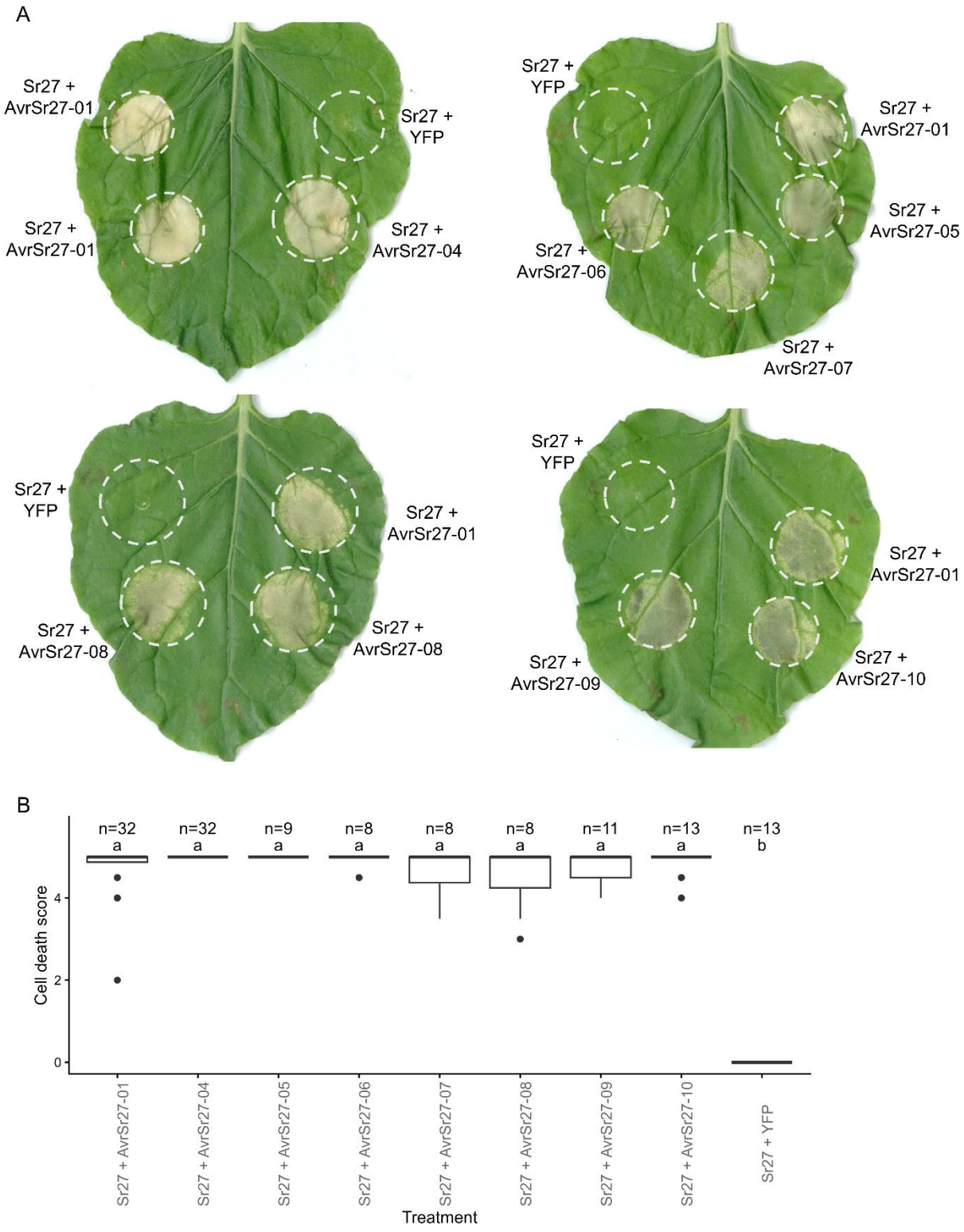


Supplementary Figure 16. Functional characterization of novel alleles of *AvrSr27* in *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 and ITA2018-1 via transient expression in protoplasts of the wheat cultivar Fielder. The *x*-axis shows genes used for transformation while the *y*-axis shows fluorescence values normalized to the YFP reference (RFU). Grey columns show mean values from three biological replicates. Error bars indicate the standard error. Black dots represent the result for each replicate of a given treatment. Two-tailed t-tests were performed for pairwise comparisons between specific treatments, '***' indicates significance at 0.001 level (experiment 1: *P*-values = 1.016e-4, 9.894e-5, 9.93e-5,

experiment 2: *P*-values = 1.106e-5, 1.1e-5, 1.101e-5, experiment 3: *P*-values = 3.28e-4, 3.12e-4, 3.12e-4, 3.26e-4), ‘ns’ non-significant. A *t*-test significance value directly above a column indicates a comparison with the YFP reference. Raw data, detailed analysis and t-test *P*-values are provided in Source data file.

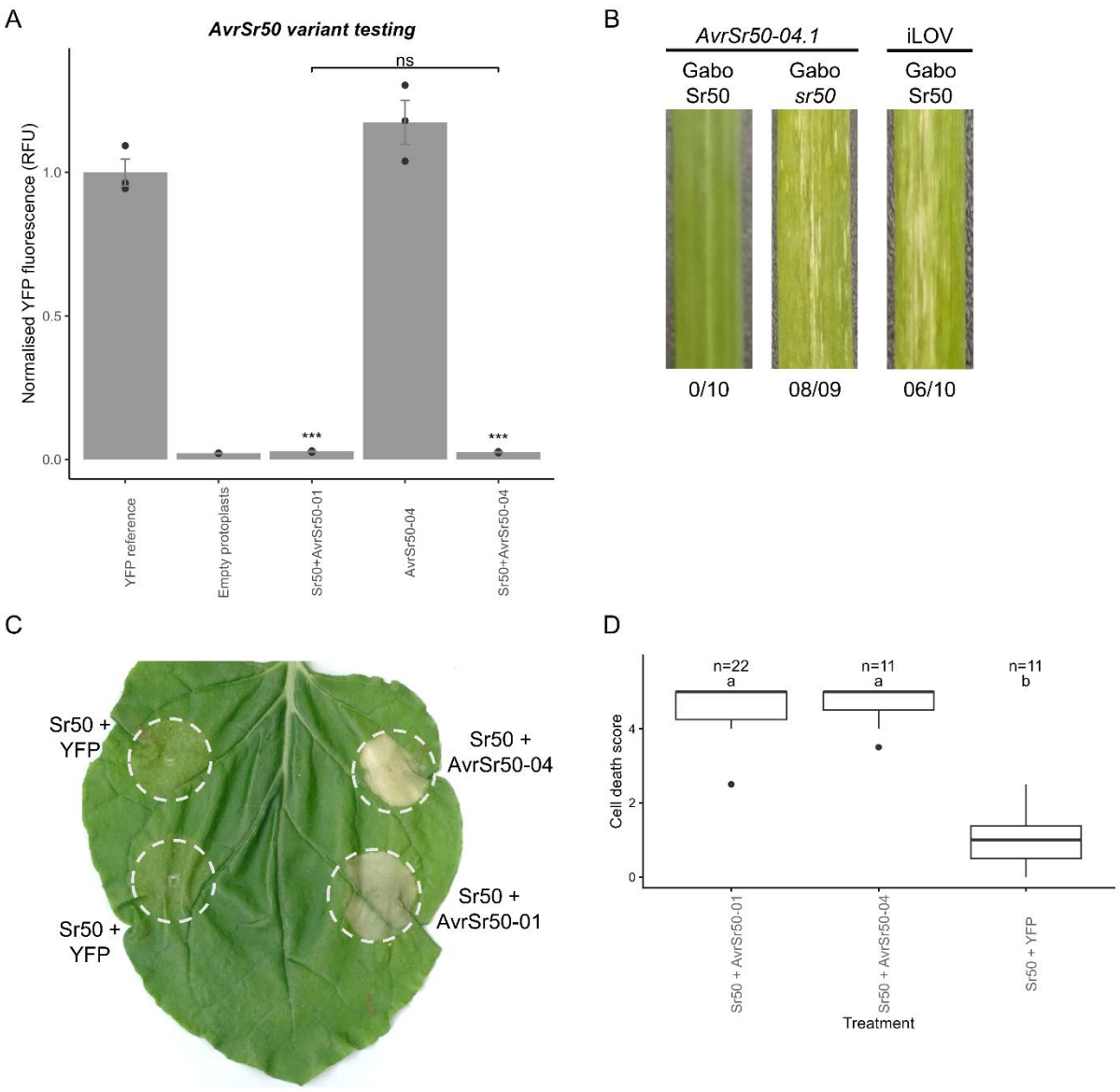


Supplementary Figure 17. Functional characterization of novel alleles of *AvrSr27* in the *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 and ITA2018-1 by VOX. Infection of wheat cultivars Coorong (*Sr27*) and Rongcoo (*sr27*) with BSMV expressing *AvrSr27* variants or the negative control iLOV. Fractions under leaf pictures indicate the number of plants exhibiting Barley Stripe Mosaic Virus (BSMV) symptoms over the total number of inoculated plants.



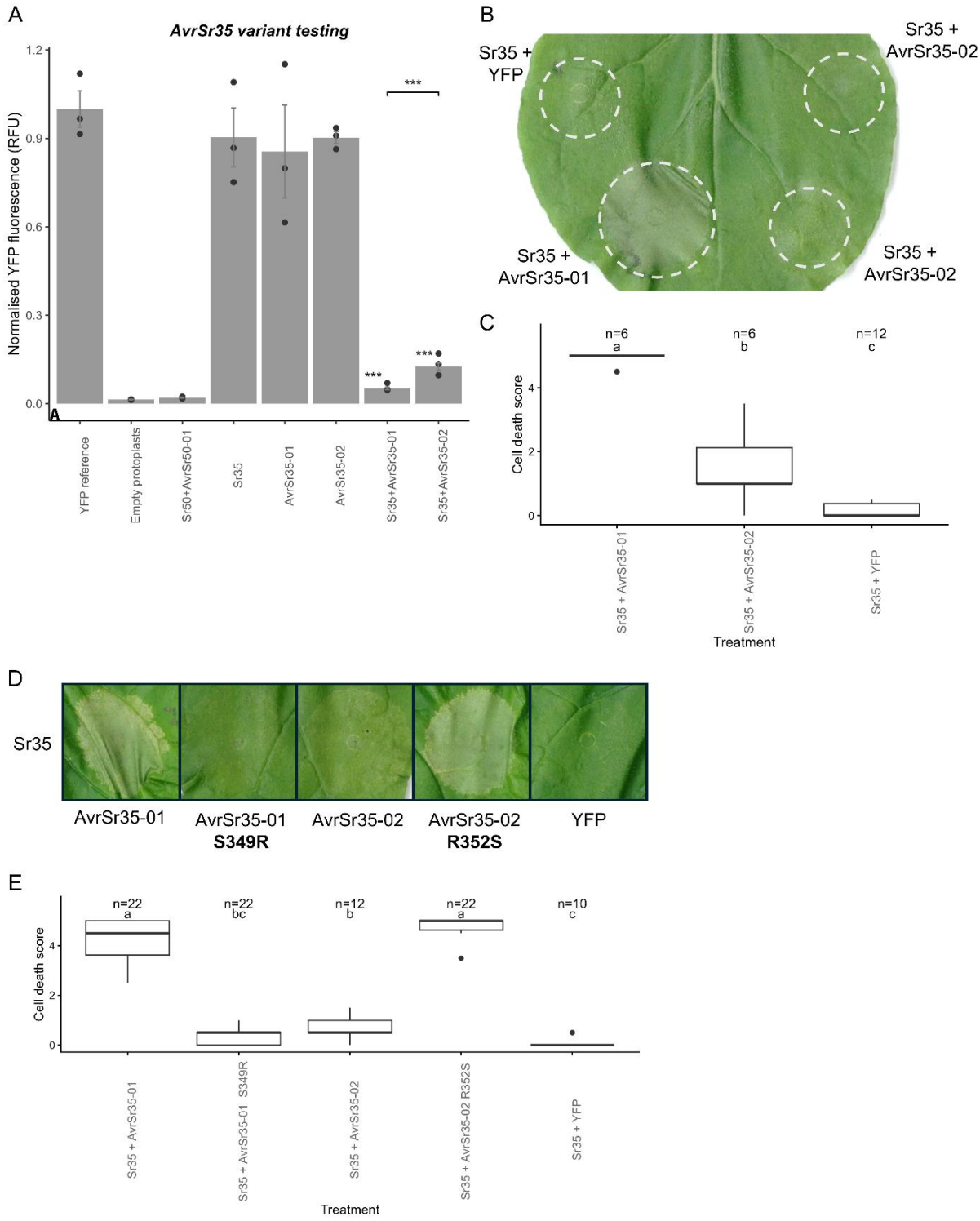
Supplementary Figure 18. Functional characterization of novel alleles of *AvrSr27* in *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 and ITA2018-1. **(A)** Co-expression assay in *Nicotiana benthamiana*. Dotted lines mark out the area infiltrated with an *Agrobacterium tumefaciens* suspension. **(B)** Cell death score in all infiltrated *N. benthamiana* leaves represented as a box and whiskers plot with the

number (n) of replicates for each treatment indicated in the graph. The *x*-axis represents the different treatments, the *y*-axis represents the cell death score out of 5 (0=no cell death, 5=cell death over the whole inoculated area). Letters indicate samples that do not differ significantly (Welch's ANOVA followed by Dunnett's T3 multiple comparisons test, pairwise *P*-values in Source Data File).



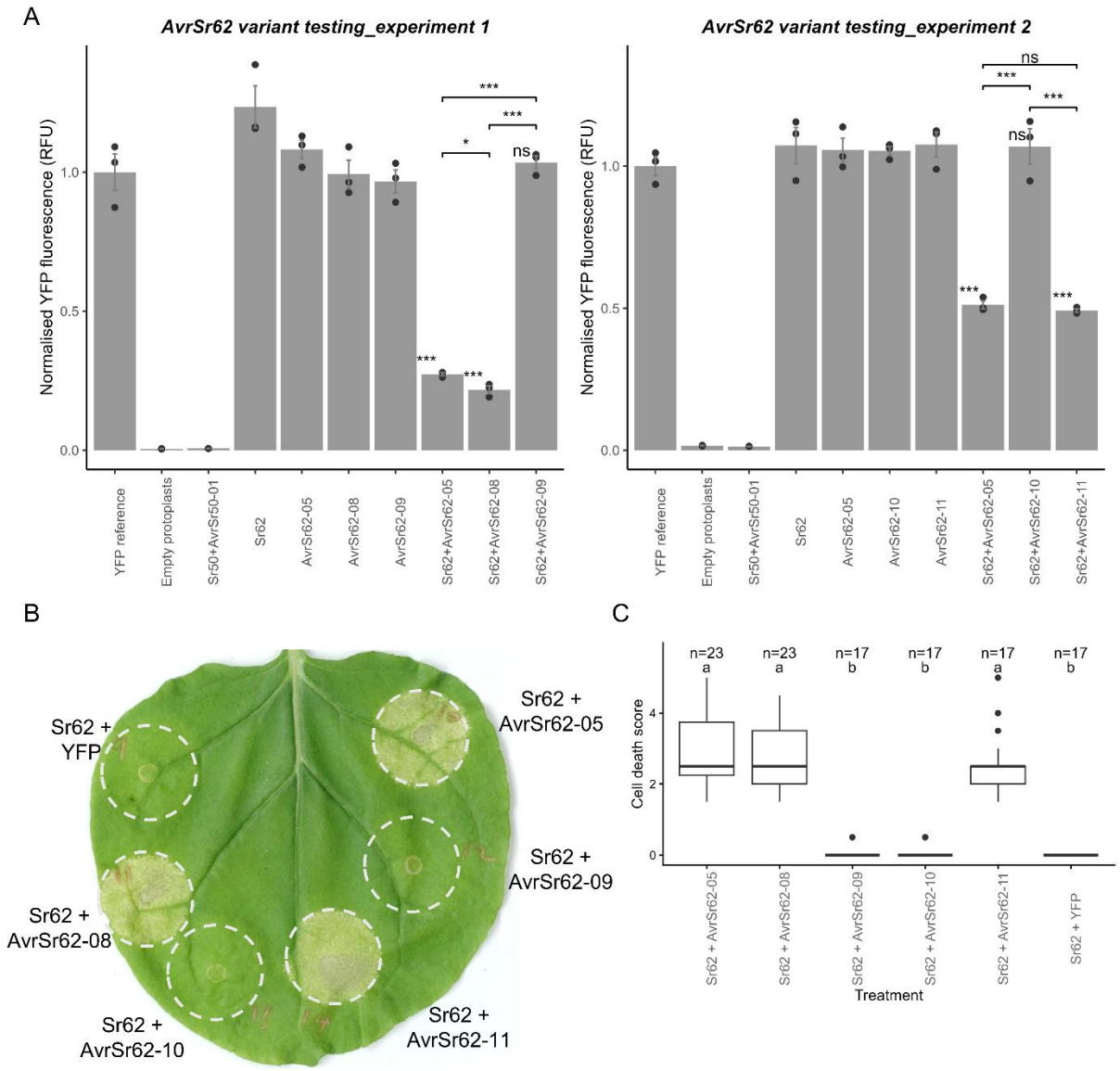
Supplementary Figure 19. Functional characterization of novel alleles of *AvrSr50* in *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 and ITA2018-1. **(A)** Transient expression in protoplasts of the wheat cultivar Fielder. The *x*-axis shows genes used for transformation while the *y*-axis shows fluorescence values normalized to the YFP reference (RFU). Grey columns show mean values from three biological replicates. Error bars indicate the standard error. Black dots represent the result for each replicate of a given treatment. Two-tailed t-tests were performed for pairwise comparisons between specific treatments, '***' indicates significance at 0.001 level (*P*-values = 3.183e-5, 3.151e-5), 'ns' non-significant. A *t*-test significance value directly above a column indicates a comparison with the YFP reference. Raw data, detailed analysis and t-test *P*-values

are provided in Source data file. **(B)** Infection of wheat cultivars Gabo (*Sr50*) and Gabo (*sr50*) by Barley Stripe Mosaic Virus (BSMV) expressing *AvrSr50-04* or the negative control iLOV. Fractions under leaf pictures indicate the number of plants exhibiting BSMV symptoms over the total number of inoculated plants. **(C)** Co-expression assay in *Nicotiana benthamiana*. Dotted lines mark out the area infiltrated with an *Agrobacterium tumefaciens* suspension. **(D)** Cell death score in all infiltrated *N. benthamiana* leaves represented as a box and whiskers plot with the number (n) of replicates for each treatment indicated in the graph. The *x*-axis represents the different treatments, the *y*-axis represents the cell death score out of 5 (0=no cell death, 5=cell death over the whole inoculated area). Letters indicate samples that do not differ significantly (Welch's ANOVA followed by Dunnett's T3 multiple comparisons test, pairwise *P*-values in Source Data File)



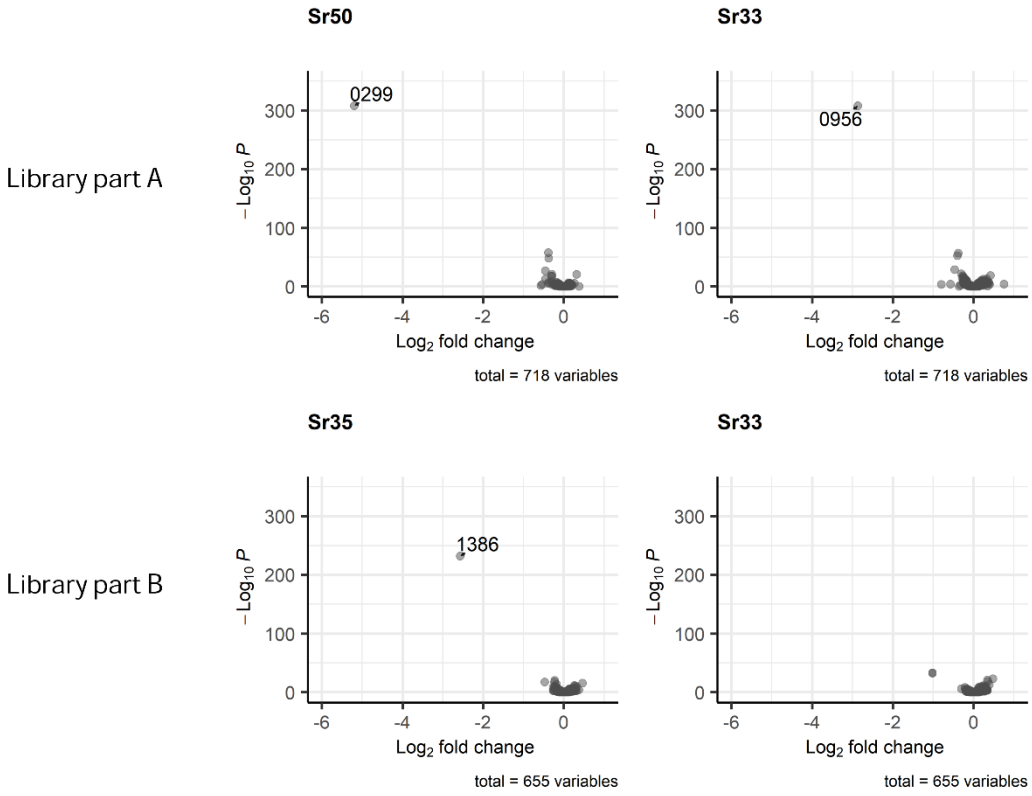
Supplementary Figure 20. Functional characterization of novel alleles of *AvrSr35* in *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 and ITA2018-1. (A) Transient expression in protoplasts of the wheat cultivar Fielder. The *x*-axis shows genes used for transformation while the *y*-axis shows fluorescence values normalized to the YFP reference (RFU). Grey columns show mean values from three biological replicates (YFP reference, Empty protoplasts, Sr50+AvrSr50-01, Sr35,

AvrSr35-01, AvrSr35-02) or five biological replicates (Sr35+AvrSr35-01, Sr35+AvrSr35-02). Error bars indicate the standard error. Black dots represent the result for each replicate of a given treatment. Two-tailed t-tests were performed for pairwise comparisons between specific treatments, '***' indicates significance at 0.001 level (*P*-values = 6.893e-4, 7.885e-7, 1.813e-6). A *t*-test significance value directly above a column indicates a comparison with the YFP reference. Raw data, detailed analysis and t-test *P*-values are provided in Source data file. **(B)** Co-expression assay in *Nicotiana benthamiana*. Dotted lines mark out the area infiltrated with an *Agrobacterium tumefaciens* suspension. **(C)** Cell death score in all infiltrated *N. benthamiana* leaves represented as a box and whiskers plot with the number (n) of replicates for each treatment indicated in the graph. The *x*-axis represents the different treatments, the *y*-axis represents the cell death score out of 5 (0=no cell death, 5=cell death over the whole inoculated area). Letters indicate samples that do not differ significantly (Welch's ANOVA followed by Dunnett's T3 multiple comparisons test, pairwise *P*-values in Source Data File). **(D)** Co-expression assay in *N. benthamiana.* Each infiltration site received a plasmid expressing Sr35 along with a plasmid expressing the protein indicated under the box. S349R and R352S indicate the mutations that were tested in AvrSr35-01 and –02. **(E)** Cell death score in all infiltrated *N. Benthamiana* leaves represented as a box and whiskers plot with the number (n) of replicates for each treatment indicated in the graph. The *x*-axis represents the different treatments, the *y*-axis represents the cell death score out of 5 (0=no cell death, 5=cell death over the whole inoculated area). Letters indicate samples that do not differ significantly (Welch's ANOVA followed by Dunnett's T3 multiple comparisons test, pairwise *P*-values in Source Data File).

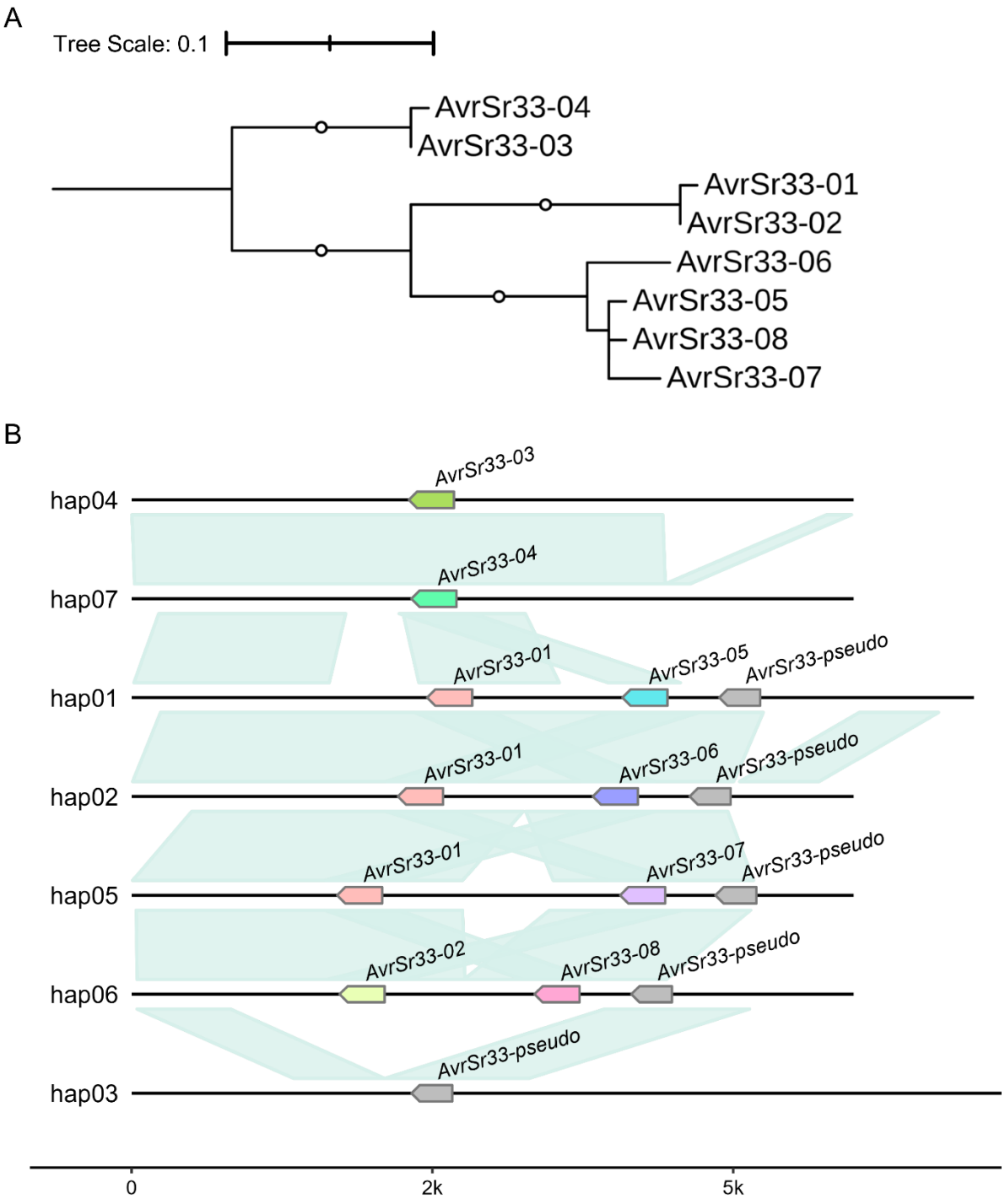


Supplementary Figure 21. Functional characterization of novel alleles of *AvrSr62* in *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 and ITA2018-1. **(A)** Transient expression in protoplasts of the wheat cultivar Fielder. The *x*-axis shows genes used for transformation while the *y*-axis shows fluorescence values normalized to the YFP reference (RFU). Grey columns show mean values from three biological replicates. Error bars indicate the standard error. Black dots represent the result for each replicate of a given treatment. Two-tailed t-tests were performed for pairwise comparisons between specific treatments, '***' indicates significance at 0.001 level (experiment 1: *P*-values = 6.075e-6, 7.246e-6, 3.76e-4, 3.009e-4, experiment 2: *P*-values = 9.63e-4, 7.83e-4, 1.67e-4, 1.12e-4), '*' indicates significance at 0.05 level (*P*-value = 1.889e-2), 'ns' non-significant. A *t*-test significance value directly above a column indicates a comparison with the YFP

reference. Raw data, detailed analysis and t-test *P*-values are provided in Source data file. **(B)** Co-expression assay in *Nicotiana benthamiana*. Dotted lines mark out the area infiltrated with an *Agrobacterium tumefaciens* suspension. Plasmids $Sr62^{TK}$-3HA and $Sr62^{NLR}$-3HA were used together as, unlike wheat cv. Fielder, *N. benthamiana* does not carry $Sr62^{NLR}$ endogenously**.** They are represented in the figure as only *Sr62* for simplicity. **(C)** Cell death score in all infiltrated *N. benthamiana* leaves represented as a box and whiskers plot with the number (n) of replicates for each treatment indicated in the graph. The *x*-axis represents the different treatments, the *y*-axis represents the cell death score out of 5 (0=no cell death, 5=cell death over the whole inoculated area). Letters indicate samples that do not differ significantly (Welch's ANOVA followed by Dunnett's T3 multiple comparisons test, pairwise *P*-values in Source Data File).

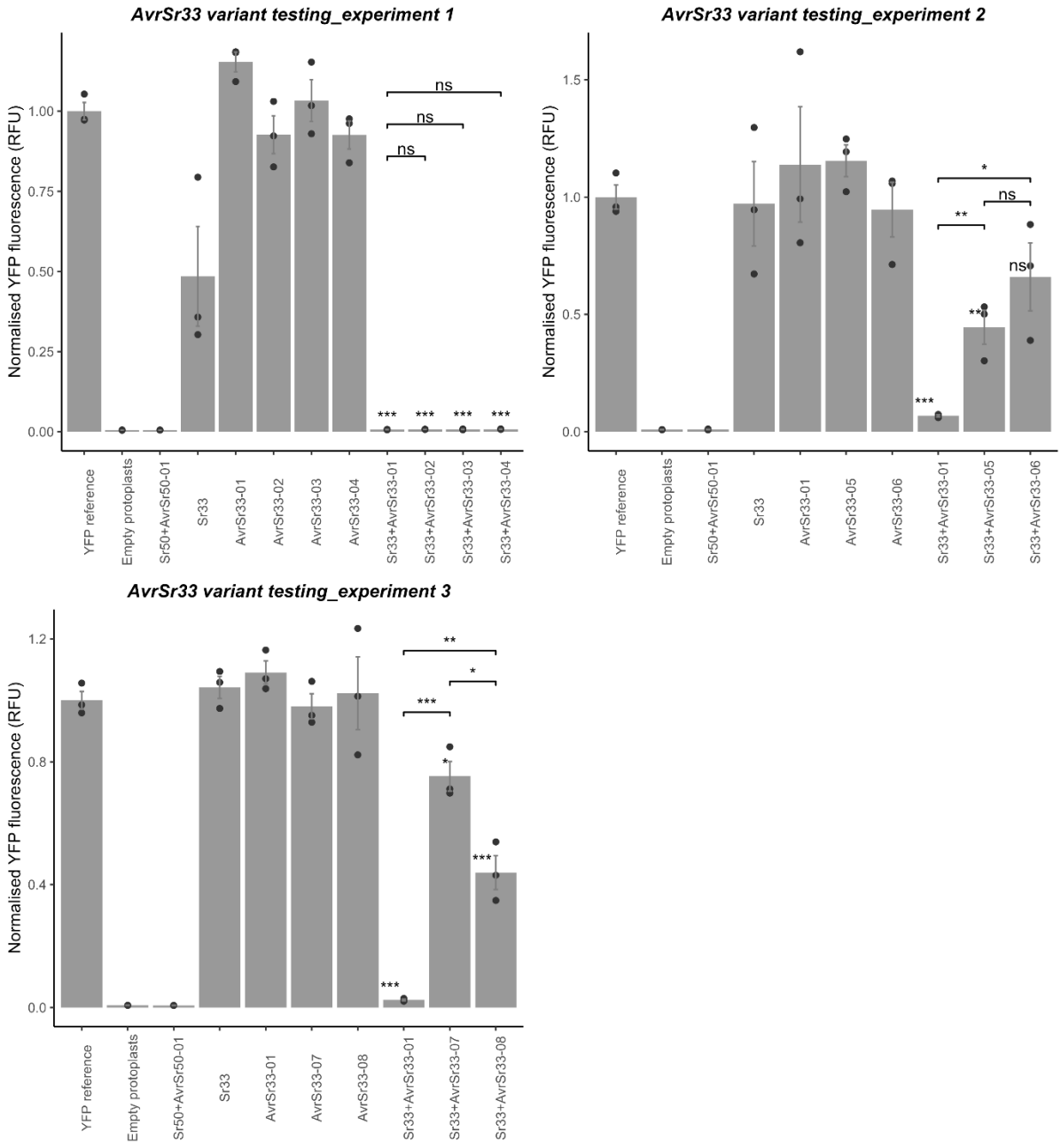


Supplementary Figure 22. Identification of *AvrSr33*. Differential gene expression analysis of a pooled effector library for *Puccinia graminis* f. sp. *tritici.* The library included a total of 1,373 predicted effectors divided into two pools, part A and part B, which were transformed separately into wheat protoplasts along with the *R* genes *Sr33*, *Sr50* (part A), *Sr35* (part B) or an empty vector. The volcano plots show differential expression (*x*-axis) versus adjusted *P*-value (*y*-axis) for each effector construct (dots) in the presence of an *R* gene compared to an empty vector. The effector gene construct that showed significantly reduced expression is labelled with its corresponding number in a given library (part A or B).



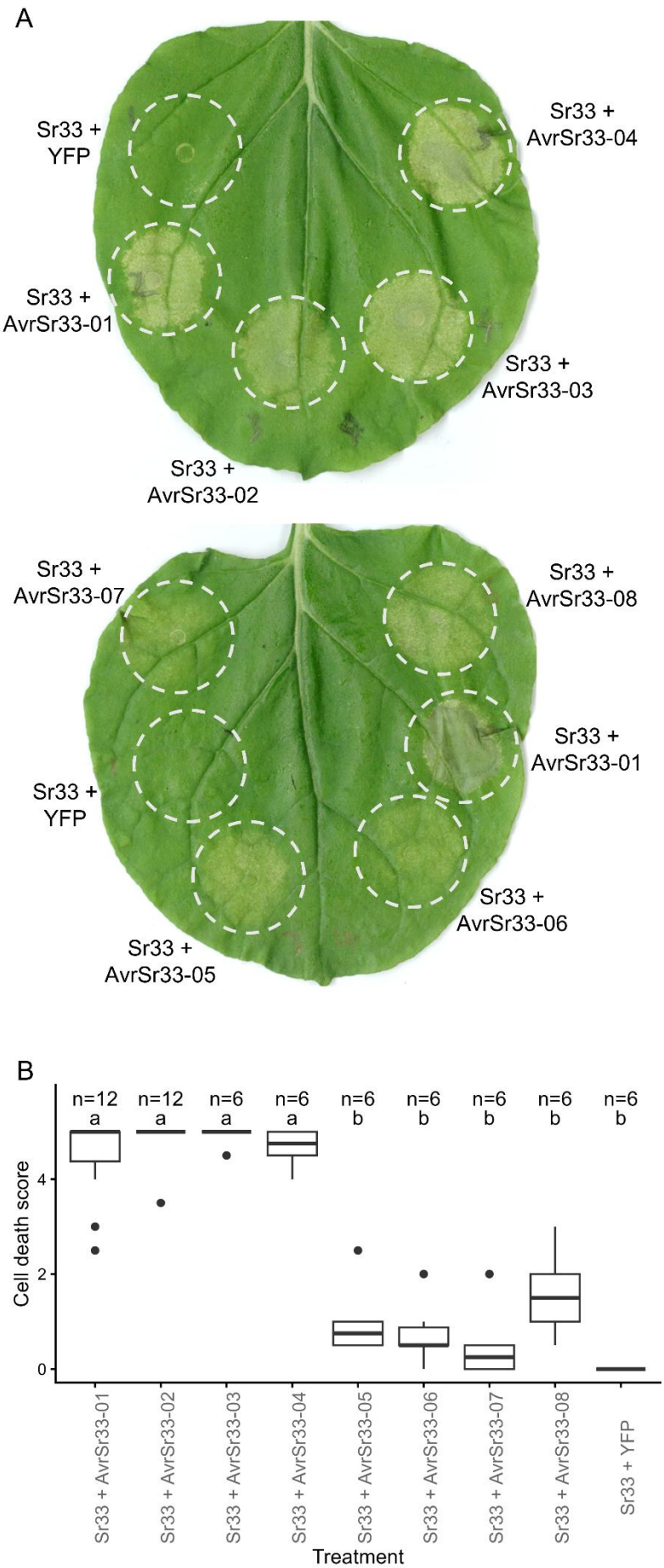
Supplementary Figure 23. Haplotype structure of the *AvrSr33* locus. (A) Maximum Likelihood phylogenetic tree of AvrSr33 protein variants from *Puccinia graminis* f. sp. *tritici* isolates Pgt21-0, Ug99, ETH2013-1, and ITA2018-1. Scale indicates mean substitutions per site. Open circles at branch midpoints indicate ≥ 80% bootstrap support (100 cycles). **(B)** Synteny plot of the *AvrSr33* region on chromosome 17 from seven *Pgt* haplotypes. Green filled rectangles between haplotypes (y-axis) indicate syntenous sequences. *X*-axis values reflect the scale of the aligned regions.

Absolute start and end positions for genes in each haplotype can be found in **Supplementary Data 12**. Gene fill colour represents different alleles of *AvrSr33*. *AvrSr33* pseudogene sequences are different in all haplotypes.

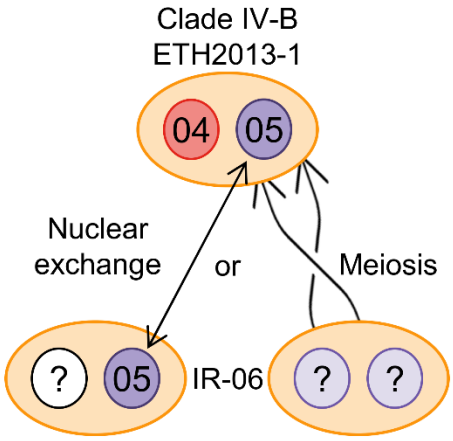


Supplementary Figure 24. Functional characterization of novel alleles of *AvrSr33* in *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 and ITA2018-1 via transient expression in protoplasts of the wheat cultivar Fielder. The *x*-axis shows genes used for transformation while the *y*-axis shows fluorescence values normalized to the YFP reference (RFU). Grey columns show mean values from three biological replicates. Error bars indicate the standard error. Black dots represent the result for each replicate of a given treatment. Two-tailed t-tests were performed for pairwise comparisons between specific treatments, '***' indicates significance at 0.001 level (experiment 1: *P*-values = 3.158e-6, 3.164e-6, 3.170e-

6, 3.171e-6, experiment 2: *P*-values = 5.674e-5, experiment 3: *P*-values = 1.135e-4, 4.646e-6, 8.495e-4), '**' indicates significance at 0.01 level (experiment 2: *P*-values = 6.313e-3, 3.318e-3, experiment 3: *P*-value = 1.698e-3), '*' indicates significance at 0.05 level (experiment 2: *P*-value = 1.489e-2, experiment 3: *P*-values = 1.301e-2, 1.171e-2), 'ns' non-significant. A *t*-test significance value directly above a column indicates a comparison with the YFP reference. Raw data, detailed analysis and t-test *P*-values are provided in Source data file.


A
Sr33 + YFP
Sr33 + AvrSr33-04
Sr33 + AvrSr33-01
Sr33 + AvrSr33-03
Sr33 + AvrSr33-02
Sr33 + AvrSr33-07
Sr33 + AvrSr33-08
Sr33 + AvrSr33-01
Sr33 + YFP
Sr33 + AvrSr33-06
Sr33 + AvrSr33-05
B
n=12 a
n=12 a
n=6 a
n=6 a
n=6 b
n=6 b
n=6 b
n=6 b
n=6 b
Cell death score
0
2
4
Sr33 + AvrSr33-01
Sr33 + AvrSr33-02
Sr33 + AvrSr33-03
Sr33 + AvrSr33-04
Sr33 + AvrSr33-05
Sr33 + AvrSr33-06
Sr33 + AvrSr33-07
Sr33 + AvrSr33-08
Sr33 + YFP
Treatment

Supplementary Figure 25. Functional characterization of novel alleles of *AvrSr33* in *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 and ITA2018-1. **(A)** Co-expression assay in *Nicotiana benthamiana*. Dotted lines mark out the area infiltrated with an *Agrobacterium tumefaciens* suspension. **(B)** Cell death score in all infiltrated *N. benthamiana* leaves represented as a box and whiskers plot with the number (n) of replicates for each treatment indicated in the graph. The *x*-axis represents the different treatments, the *y*-axis represents the cell death score out of 5 (0=no cell death, 5=cell death over the whole inoculated area). Letters indicate samples that do not differ significantly (Welch's ANOVA followed by Dunnett's T3 multiple comparisons test, pairwise *P*-values in Source Data File).



Supplementary Figure 26. Possible relationships between *Puccinia graminis* f. sp. *tritici* isolates ETH2013-1 and IR-06. These isolates could either share an identical haplotype (nuclear exchange) or be related as a parent (IR-06) and an F1 product of meiosis (hap05 from ETH2013-1).