# Genome-wide association studies reveal a rapidly evolving candidate avirulence effector in the Cercospora leaf spot pathogen *Cercospora beticola*

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**Summary**

The major resistance gene *BvCR4* recently bred into sugar beets provides a high level of resistance to Cercospora leaf spot caused by the fungal pathogen *Cercospora beticola*. The occurrence of pathogen strains virulent to *BvCR4* was studied using field trials in Switzerland. Virulence of a subset of these strains was evaluated in a field trial conducted under elevated artificial disease pressure. We created a new *C. beticola* reference genome and mapped whole genome sequences of 256 field-collected isolates. These were combined with virulence phenotypes to conduct three separate GWAS to identify candidate avirulence genes. We identified a locus associated with avirulence containing a single candidate avirulence effector gene named *AvrCR4*. All virulent isolates either lacked *AvrCR4* or had non-synonymous mutations within the gene. *AvrCR4* was present in all 74 isolates from non-*BvCR4* hybrids, whereas 33 of 89 isolates from *BvCR4* hybrids carried a deletion. We also mapped genomic data from 190 publicly available U.S. isolates to our new reference genome. The *AvrCR4* deletion was found in only one of 95 unique isolates from non-*BvCR4* hybrids in the U.S. *AvrCR4* presents a unique example of an avirulence effector in which virulent alleles have only recently emerged. Most likely these were selected out of standing genetic variation after deployment of *BvCR4*. Identification of *AvrCR4* will enable real-time screening of *C. beticola* populations for the emergence and spread of virulent isolates.

Keywords: avirulence effector, GWAS, pathogen evolution, R-gene durability

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35 **Introduction**

36 Plant pathogens pose significant risks to crop production and food security around the
37 world (Savary et al., 2019). Disease resistance genes (R-genes) provide economical and
38 ecologically friendly approaches to disease control that are important for the production of
39 many crops. Plant breeders often select for R-genes with major effects that form a critical
40 part of the plant's immune system. Major R-genes typically encode immune receptors, e.g.
41 receptor-like kinases (RLKs) or nucleotide-binding leucine-rich repeat proteins (NB-LRRs)
42 that detect pathogen proteins and trigger an immune response (Deng et al., 2020; Petit-
43 Houdenot and Fudal, 2017). In sugar beet (*Beta vulgaris ssp. vulgaris*), R-genes have
44 provided the only effective control for beet cyst nematode (*Heterodera schachtii*)
45 (Stevanato et al., 2015) and rhizomania (Beet necrotic yellow vein virus) (Capistrano-
46 Gossmann et al., 2017).

47

48 The effectiveness of R-genes can be reduced when virulent strains of the pathogen evolve.
49 This evolution can happen within a few years, with well-documented cases including 2-7
50 years for *Leptosphaeria maculans* on oilseed rape (Rouxel and Balesdent, 2017; Van De
51 Wouw et al., 2010); 3 years for *Zymoseptoria tritici* on wheat (Cowger et al., 2000), and 2-3
52 years for *Pyricularia oryzae* on rice (Kiyosawa, 1982). However, even when the pathogen
53 population contains virulent strains, R-genes can still prove useful, e.g. the *Rlm7* gene
54 encoding resistance to *L. maculans* remains useful more than 15 years after deployment
55 despite the identification of virulent isolates (Alnajar et al., 2022; Winter and Koopmann,
56 2016). Understanding the mechanisms governing the emergence of virulence is needed to
57 develop more effective strategies to mitigate the impact of pathogens in agro-ecosystems.

58

59 Plant-pathogen coevolution in natural ecosystems created the reservoir of R-genes that can
60 be integrated into domesticated crops via plant breeding programs. These R-genes typically
61 recognise pathogen molecules, often called effectors, that function to increase susceptibility
62 of the host to pathogen infection (Kamoun, 2007). If the recognition of an effector by an R-
63 protein leads to an immune response that severely limits pathogen infection, it is called an
64 avirulence effector. The genes encoding avirulence effectors typically encode small,
65 secreted, cysteine-rich proteins with limited homology to other characterized proteins
66 (Rouxel and Balesdent, 2017; Sonah et al., 2016). Many avirulence effector genes have now
67 been identified and cloned in several important plant pathosystems, including *L. maculans*
68 on oilseed rape (Rouxel and Balesdent, 2017), *P. oryzae* on rice (Fernandez, 2023), and *Z.*
69 *tritici* on wheat (Meile et al., 2018; Zhong et al., 2017).
70
71 Following deployment of an R-gene, the corresponding avirulence effector is under strong
72 directional selection and typically evolves rapidly to evade host recognition, creating
73 virulent pathogen strains that can infect plants carrying the R-gene. This evolution can
74 involve gene deletions, non-synonymous mutations, and alterations in gene transcription.
75 The genes encoding fungal effectors are often located in dynamic, TE-rich regions of the
76 genome that facilitate an accelerated evolution for these genes (Lo Presti et al., 2015). After
77 years of selection imposed by a widely deployed R-gene, the gene encoding the
78 corresponding avirulence effector typically displays very high levels of genetic diversity,
79 while the original wild-type sequence of the effector gene may have disappeared entirely or
80 be present at only very low frequencies (Brunner et al., 2007; Brunner and McDonald, 2018;
81 Dodds et al., 2006; Stephens et al., 2021).
82

83 Sugar beet provides around one-fifth of global sucrose production every year (Rangel et al.,
84 2020). Cercospora leaf spot (CLS), caused by the fungus *Cercospora beticola*, is the most
85 common and destructive disease of sugar beet. Sugar beet cultivars with improved
86 resistance to CLS have been under development for thirty years (Rangel et al., 2020). Wild
87 sea beet (*Beta vulgaris* subsp. *maritima*) is the most important source of resistant genes and
88 provided at least four quantitative trait loci (QTL) for resistance to CLS . It has proved
89 difficult to combine high resistance and high yield into the same CLS-resistant cultivar
90 (Setiawan et al., 2000; Taguchi et al., 2011). The most widely planted cultivars combine high
91 yield and moderate CLS resistance (Windels et al., 1998), but rely mainly on fungicides to
92 limit losses associated with CLS. Recently, Törjék et al (2022; 2020) identified an NBS-LRR
93 resistance gene named *BvCR4* that showed a high level of CLS resistance when bred into
94 sugar beets, enabling a unique combination of high CLS resistance and high yield in the
95 same variety. However, *BvCR4* might be vulnerable to the evolution of virulent *C. beticola*
96 strains if it were deployed in the absence of supporting quantitative background resistance
97 (Pilet-Nayel et al., 2017) and without supplemental plant protection measures.
98
99 The evolutionary potential of *C. beticola* is high because it has a mixed reproductive system,
100 exhibits extensive gene flow, and undergoes several cycles of reproduction during each
101 growing season. Field populations contain high levels of genetic diversity as expected for
102 pathogen populations that maintain a high effective population size (Vaghefi et al., 2017). *C.*
103 *beticola* has already shown the capacity to rapidly evolve resistance to several fungicides
104 representing different modes of action, including triazole, benzimidazole, difenoconazole,
105 organotin, sterol demethylation inhibitor (DMI), and quinone outside inhibitor (QoI) (Bolton
106 et al., 2012; Karaoglanidis et al., 2000). This evolution of fungicide resistance underlines the

107 need to make more effective use of CLS resistance, but also illustrates the potential for
108 pathogen adaptation following the deployment of R-genes.
109
110 We conducted a series of field, greenhouse, and lab experiments to assess the risks
111 associated with deployment of *BvCR4*. We monitored CLS epidemics in experimental hybrids
112 carrying *BvCR4* and isolated putative virulent strains of *C. beticola* from infected leaves. We
113 confirmed the virulence of many of these strains on *BvCR4* hybrids growing under field
114 conditions. We created a new reference genome sequence for *C. beticola*, analysed genome
115 sequences for 256 isolates and conducted three GWAS analyses to search for candidate
116 avirulence effectors. Each GWAS identified the same candidate avirulence effector (*AvrCR4*)
117 on chromosome 1. The *AvrCR4* gene contained several different mutations encoding
118 virulence and was deleted in many isolates coming from *BvCR4* hybrids.
119
120 **Results**
121 We monitored CLS epidemics at two sites in Switzerland planted with four hybrids: two
122 resistant hybrids carrying *BvCR4* and two susceptible hybrids without *BvCR4* (Table S1,
123 Figure S1). Plots planted to resistant *BvCR4* hybrids showed later onset of CLS and slower
124 epidemic progress compared to plots planted to susceptible hybrids. Total levels of CLS
125 infection in the resistant hybrids never reached the levels found in the susceptible hybrids.
126 Leaf death and subsequent regrowth was less prevalent in the resistant hybrids carrying
127 *BvCR4*. Leaves with CLS lesions were collected throughout the season. Similar experimental
128 plots were established in Germany, but diseased leaves were collected only at the end of
129 the season (Figure S1). We made pure cultures of *C. beticola* and sequenced these isolates
130 using Illumina short read sequencing (sample information see Table S2). Interestingly, one

131 of the genotypes observed only on resistant hybrids carrying *BvCR4* was frequent and
132 widely distributed at the Rudolfingen site, suggesting it was a virulent clone.
133
134 To assess the virulence of isolates we performed a field trial in 2022 in Northeim, Germany.
135 Four sugar beet hybrids (Table 1) were inoculated, and the disease was scored using a 9-
136 point rating scale (Table S3) at 32-, 40-, 47-, 56- and 64-days post inoculation (dpi) (Table
137 S4). Fifty-two unique isolates from the Swiss plots were phenotyped in the virulence trial, 49
138 from plots planted with *BvCR4* hybrids (18 isolates from Hybrid_R2, 31 isolates from
139 Hybrid_R3), and three were from susceptible hybrids (2 from Hybrid_S3, 1 from Hybrid_S4).
140 We also included 5 separate isolations of the putatively virulent clone from the Rudolfingen
141 site to test reproducibility of the phenotypes, but only one was used for GWAS analyses.
142 Four additional reference inocula with known virulence properties were used as controls.
143
144 Disease ratings increased over the growing season, peaking at 56 dpi (Figure 1). Disease
145 ratings at 32-56 dpi were highly correlated while the rating at 64 dpi was less correlated
146 with other time points (Figure S2). Using the 40-dpi rating on Hybrid_R2, isolates were
147 scored as virulent (>4) or avirulent (≤4), resulting in 25 of the 52 unique field-collected
148 isolates scored as virulent and 27 scored as avirulent. The three isolates from susceptible
149 hybrids were all avirulent. Next, we considered how dpi, hybrid, and virulence score (0/1)
150 explained variation in the disease rating using linear models. We found significant pairwise
151 interactions but no three-way interaction (Table S5). Disease ratings increased over time on
152 all hybrids, but the pattern differed between hybrids with and without *BvCR4* (Figure 1).
153 Isolates scored as virulent caused more disease at all timepoints on Hybrid_R3 carrying
154 *BvCR4* compared to the avirulent isolates (it was not statistically appropriate to do this

155 analysis on Hybrid_R2 because this was used to define virulent/avirulent). When tested on
156 hybrids without *BvCR4* (Hybrid_S3 and Hybrid_S4), virulent and avirulent isolates caused
157 similar levels of disease.
158
159 **Genome-wide association scans identified a single effector gene candidate**
160 We performed three GWAS analyses; two based on SNPs and one based on k-mers. For the
161 SNP-based GWAS, the sequence data was mapped to a high-quality reference genome that
162 we created using Nanopore long read sequencing. The reference genome was assembled
163 into 11 continuous sequences (hereafter called chromosomes). The genome showed a
164 completeness of 98.4% according to the BUSCO benchmark against Ascomycota single copy
165 orthologs, with 13,869 annotated genes (Table S6). We retained 358,365 high quality
166 variants for the SNP GWAS. The first GWAS used the quantitative virulence rating on the
167 resistant hybrid (Hybrid_R2) at 40 dpi as the phenotypic data (n=52), referred to hereafter
168 as the 'quantitative GWAS'. The resulting Manhattan plots showed a single distinctive, but
169 not statistically significant, LOD peak on chromosome 1 (Figure 2a). The second GWAS
170 coded the phenotype data as either virulent (Hybrid_R2 rating at 40 dpi > 4) or avirulent
171 (Hybrid_R2 rating at 40 dpi ≤ 4) and included 38 additional isolates that were sampled from
172 the susceptible hybrid Hybrid_S2 and scored as avirulent, giving a total sample size of 90.
173 This GWAS identified a significant LOD peak on chromosome 1 with an interval spanning
174 6730 bp (4087634-4094364 bp) (Figure 2b). This peak was in the same position as the peak
175 observed in the quantitative GWAS. The GWAS interval contained a single gene
176 (ID=Hevensen_hq-v2.G01679) that is 340 bp long (Figure 3). The gene is predicted to encode
177 an effector protein that is 89 amino acids (aa) long. The putative effector contains a signal
178 peptide of 16aa (predicted by TOPCONS, Tsirigos et al., 2015) and is cysteine-rich (6%). We

179 did not find a significant match using a BLAST search against the publicly available reference
180 genome, but a partial hit was found on Chr 7. A copy of the gene with 88% identity was
181 found in *Cercospora sojina* (strain RACE15) chromosome XII: 4346561-4346898.
182
183 The k-mer GWAS used the quantitative virulence rating on the resistant hybrid (Hybrid_R2)
184 at 40 dpi (n=47). Several significant peaks were identified (Figure 2c). A peak on
185 Chromosome 1 had significant k-mers located within the same interval identified in the
186 previous analyses. Other significant associations containing more than one k-mer were
187 found on Chromosomes 2 and 7. In both cases the k-mers with significant associations were
188 not tightly clustered, unlike the peak on Chr 1. So, we consider these to be false positives.
189 Significant singleton k-mers found on Chromosomes 1 (1644617bp) and Chromosome 8
190 (2223033bp) were also treated as false positives.
191
192 The effector gene candidate was in a gene-sparse, repeat-rich region of the genome that
193 exhibits signatures of Repeat Induced Point mutations (RIP) (Figure 3). To investigate
194 variation in the effector gene, we used genomic data from field collected isolates and
195 publicly available sequence data from the Short Read Archive (SRA). After mapping, quality
196 checking and removal of sequence duplicates and clones we retained 258 unique genomes
197 (163 sequenced by us, 95 from SRA) for further analyses. Of these 258 unique genotypes, 34
198 had the candidate effector deletion. Among isolates collected from Switzerland and
199 Germany, the gene was absent in over a third of isolates collected from resistant hybrids
200 carrying *BvCR4* (37%) and was present in all isolates collected from susceptible hybrids
201 (Table 2). In the publicly available genome sequences from the USA, the gene deletion was
202 observed only once out of 95 unique genotypes (1%).

203

204 **Sequence variation in the candidate effector gene**

205 Among the 224 isolates carrying the candidate effector gene, 195 (87%) had no variation in
206 the gene (158 from susceptible hybrids, 37 from resistant hybrids). Among the remaining 29
207 isolates, we identified 24 SNPs within the gene. No SNPs were found within the intron.
208 Haplotype network analysis revealed higher diversity in the isolates collected on the
209 resistant hybrids, compared to the susceptible hybrids (Figure 4a). The sequence diversity
210 (pi) was 10X higher in the isolates originating from the resistant hybrids (pi=0.0037)
211 compared to isolates coming from susceptible hybrids (pi = 0.00030) in Switzerland.
212 Translation of the two exons identified 12 different amino acid sequences, with most of the
213 variation in the amino acid sequence resulting in virulence at 40 dpi on Hybrid_R2 (Figure
214 4b).

215

216 To assess a potential fitness cost associated with loss of function of the putative effector, we
217 tested if the loss or inactivation of the effector influenced the virulence of an isolate
218 infecting resistant and susceptible hybrids in the 2022 field trial. We grouped isolates into
219 two categories: those encoding the reference wild type isoform of the putative effector, and
220 those encoding either deletions or alternative effector isoforms. We found no difference in
221 disease rating between isolates with the deleted isoform or with alternative isoforms (Table
222 S7). Across phenotyped isolates, we found that dpi, hybrid and effector gene isoform
223 explained variation in disease rating, with significant interactions between all pairs of
224 factors but no three-way interaction (Table S8). Considering each hybrid and dpi separately,
225 we found that the effector isoform had little effect on an isolate's virulence rating when
226 infecting the susceptible hybrids but had a significant effect on an isolate's virulence when

227 infecting the resistant hybrid carrying *BvCR4* (Figure 5). Thus, there was no evidence for a
228 reduction in fitness in strains lacking *AvrCR4* or encoding alternative isoforms of AvrCR4.
229 Rather it appears that strains carrying deletions or alternative isoforms exhibit higher
230 virulence on the susceptible hybrids (Figure 5, p-values ranging from 0.02-0.09, See Table
231 S8).
232
233 **Discussion**
234 The identification and use of *BvCR4* represents an important advance in breeding for
235 resistance to CLS, as it provides a high level of resistance to CLS without a reduction in sugar
236 yield. Given the long history of boom-and-bust cycles associated with the release of major-
237 effect R-genes, there was concern that *C. beticola* could evolve virulence against resistant
238 hybrids carrying *BvCR4*, the first reported major R-gene deployed against this pathogen.
239
240 The first evidence that virulent isolates could evolve was observed as clusters of CLS-
241 infected plants, hereafter called hotspots, in experimental fields planted with *BvCR4* hybrids
242 in Switzerland in 2019. Although these hotspots did not emerge until late in the growing
243 season, their occurrence suggested selection and local spread of *C. beticola* clones that
244 were virulent to *BvCR4*. The discovery of these hotspots triggered our study to assess the
245 emergence and spread of virulent strains in *C. beticola*.
246
247 Our GWAS used collections of *C. beticola* from plots planted with resistant *BvCR4* hybrids
248 and susceptible hybrids lacking *BvCR4* growing in the same field. A subset of these *C.*
249 *beticola* isolates were phenotyped under field conditions to test their virulence. The
250 quantitative GWAS using 52 phenotyped isolates identified a region on Chromosome 1

251 associated with virulence, but the LOD scores were not significant. To increase the power to
252 detect genes associated with virulence on *BvCR4*, we assumed that any allelic variants
253 encoding virulence to *BvCR4* would be present at a very low frequency (e.g., at mutation-
254 drift equilibrium) prior to the deployment of *BvCR4* hybrids. Hence, we scored virulence as a
255 binary trait and included an additional 38 *C. beticola* isolates from susceptible hybrids
256 growing in the same field scored as avirulent, although we lacked phenotypes for these
257 strains. This binary phenotype approach identified the same Chromosome 1 region, with a
258 significant LOD score of 9.3. A third GWAS based on k-mers, including only the phenotyped
259 strains, identified the same Chromosome 1 region, with a very high LOD score of 21.3, as
260 well as small intervals on Chromosomes 2 (LOD = 14.5) and 7 (LOD = 11.4).
261
262 A single predicted gene was found within the Chromosome 1 interval that was identified in
263 all three GWAS analyses. This gene shares many properties found in functionally validated
264 avirulence effectors in other plant pathogenic fungi, including: a) it encodes a small,
265 secreted, cysteine-rich protein; b) it displays a presence-absence polymorphism and the
266 absence allele associated with a gain in virulence occurs at a higher frequency on *BvCR4*
267 hybrids compared to susceptible hybrids; c) its exons encode non-synonymous substitutions
268 associated with virulence, but nucleotide diversity is lower in the introns and the signal
269 peptide sequence; d) it is located in a genomic region that is rich in transposable elements
270 and associated with RIP. Based on these properties, we named this candidate avirulence
271 effector gene *AvrCR4*.
272
273 The main difference between *AvrCR4* and other cloned fungal avirulence effectors is that
274 the overall level of DNA and amino acid diversity found in this study is lower than what has

275 been reported for other avirulence effectors (Brunner et al., 2007; Brunner and McDonald,
276 2018; Dodds et al., 2006; Schürch et al., 2004). This lower diversity may reflect the very
277 short period over which selection has been operating on this gene. In Germany, the first
278 commercial cultivation of *BvCR4* sugar beet hybrids occurred in 2021 and in Switzerland in
279 2022. Based on the patterns of diversity we observe in this gene and comparisons to other
280 well characterized avirulence effectors, we believe we have captured AvrCR4 at the very
281 onset of its evolution. Firstly, the same wild-type *AvrCR4* sequence is found at a high
282 frequency on both resistant and susceptible sugar beets growing on different continents
283 (Figure 4a). In contrast, in *Z. tritici* the *AvrStb6* gene is present in every isolate analyzed to
284 date and there is no predominant wild-type, despite the use of *Stb6* in European wheat
285 cultivars for at least 100 (Dutta et al., 2021; Stephens et al., 2021). Secondly, there was little
286 nucleotide diversity in *AvrCR4* in *C. beticola*. Amongst 224 isolates with AvrCR4 present, only
287 29 strains carried *AvrCR4* alleles that differed from the predominating wild type sequence.
288 Among those 29 strains, we identified 11 distinct DNA haplotypes carrying 24 polymorphic
289 sites encoding 13 AvrCR4 protein isoforms. No variation in the intron or signal peptide was
290 observed. In comparison, in a global sample of 383 *Z. tritici* strains, 52 haplotypes encoding
291 44 AvrStb6 protein isoforms were found, with several polymorphisms found in the signal
292 peptide (Stephens et al., 2021). In another study, evidence of diversifying selection acting on
293 codons in AvrStb6 was found, suggesting that diversity in this gene has been maintained via
294 a co-evolutionary arms between host and pathogen (Brunner and McDonald, 2018).
295
296 Finally, the *AvrCR4* presence/absence frequency is closely associated with the deployment
297 of *BvCR4*. *AvrCR4* was present in nearly all *C. beticola* isolates sampled from locations where
298 *BvCR4* has not yet been deployed (USA: 94/95). In experimental fields planted to both,

299 hybrids carrying and lacking *BvCR4*, it was present in all isolates collected from hybrids
300 lacking *BvCR4* and was often missing in isolates sampled from hybrids carrying *BvCR4*
301 (33/89).
302
303 We believe that *AvrCR4* is the first avirulence effector to be discovered at the very onset of
304 its evolution. This provides a unique opportunity to monitor in real time the changes in
305 effector amino acid sequence that enable an escape from recognition by the cognate NBS-
306 LRR protein. We also consider this to be a unique opportunity to search for compensatory
307 mutations that could ameliorate any reductions in pathogen fitness caused by changing the
308 function of the encoded wild-type avirulence effector protein.
309
310 Our results also provide important insights into how *C. beticola* can overcome resistant
311 cultivars and if there are potential fitness costs associated with virulence. Standing genetic
312 variation in *C. beticola* populations is the most likely source for the *AvrCR4* mutations
313 creating virulence, as the gene deletion was detected in populations not exposed to *BvCR4*
314 hybrids. Recent work has shown that populations of *Z. tritici* can generate and maintain vast
315 reservoirs of functional genetic diversity facilitating rapid adaption to changes in their local
316 environments (McDonald et al., 2022). Like *Z. tritici*, *C. beticola* has a mixed reproduction
317 system and high gene flow (Rangel et al., 2020; Vaghefi et al., 2017), suggesting that its
318 populations will also maintain high levels of standing genetic variation. Approximately half
319 of the isolates collected from *BvCR4* hybrids were avirulent on *BvCR4* hybrids in our field
320 phenotyping, illustrating that *BvCR4* hybrids can be colonized by *C. beticola* strains that are
321 not specifically adapted to *BvCR4*. We speculate that other abiotic or biotic stresses could
322 play a role. Although we did not test this specifically, we observed several instances of

323 plants infected by *Rhizoctonia solani* in Rudolfingen in 2020 that might have compromised
324 CLS resistance. Furthermore, experiments with mixed infections in *Z. tritici* showed that
325 virulent strains were able to suppress the immune system of plants carrying the *Stb6* R-
326 gene, enabling avirulent strains to infect the resistant plants (Bernasconi et al., 2023). We
327 hypothesize that similar mechanisms could be operating in the CLS pathosystem. Finally, our
328 study found little evidence of a fitness cost associated with the deletion or loss of function
329 of AvrCR4. Strains carrying deletions for *AvrCR4* showed a similar disease development on
330 sugar beet hybrids lacking *BvCR4*. This is in line with expectations of functional redundancy
331 in effectors (Rouxel and Balesdent, 2017) and with previous work in *Z. tritici* (Zhan and
332 McDonald, 2013). While we did not detect any fitness costs in the field environment, we are
333 currently conducting additional lab-based experiments to search for fitness costs associated
334 with loss of the *AvrCR4* function.
335
336 The identification of *AvrCR4* will benefit CLS management by enabling regular monitoring of
337 the frequency of virulent mutants in CLS populations. Monitoring can support region-
338 specific variety choice and management strategies aimed at reducing the overall CLS
339 population and the frequency of virulent strains. These management strategies will likely
340 utilize an integrated plant protection approach that combines precise timing of fungicide
341 applications with agronomic practices such as crop rotation and tillage to reduce the
342 buildup of inoculum in the soil (Harveson et al., 2009; Khan et al., 2008; Khan and Khan,
343 2010; Secor et al., 2010) (Khan et al., 2008; Khan and Khan, 2010; *Jacobsen and Franc, 2007;*
344 *Secor et al 2010*). Varieties that combine *BvCR4* with other sources of resistance can provide
345 a strong CLS background resistance that will further contribute to a reduction of the CLS
346 population and increase the durability of *BvCR4*. This latter approach was shown to be

347 effective in oilseed rape, where the adaptation time of *L. maculans* to the resistance gene
348 *Rlm6* was doubled by protecting this resistance with polygenic background resistance (Brun
349 et al., 2010; Delourme et al., 2014; Pilet-Nayel et al., 2017).
350
351 **Experimental Procedures**
352 **Collections of leaves infected by CLS**
353 In 2019 a field in Switzerland (Table S9) was planted with two experimental sugar beet
354 hybrids; Hybrid_R1 carrying *BvCR4* in a genetic background with low quantitative resistance,
355 and Hybrid_S1 carrying only a low level of quantitative resistance. Both hybrids were
356 examined in 2019 for evidence of CLS infections. Infected leaves were collected from
357 hotspots exhibiting CLS symptoms and stored for later isolation.
358
359 In 2020, four sugar beet experimental hybrids with different levels of resistance to CLS were
360 planted at two field sites in Switzerland separated by 60 km (Table S9); Hybrid_R2 with
361 *BvCr4* embedded in a background with low quantitative resistance, Hybrid_R3 with *BvCr4*
362 embedded in a background with moderate quantitative resistance, Hybrid_S2 with only low
363 quantitative resistance and Hybrid_S3 with moderate quantitative resistance (Table S1). In
364 Hendschiken, 12 rows of each hybrid were planted in adjacent plots and in Rudolfingen, 18
365 rows of each hybrid were planted in adjacent plots. Sugar beets were sown in March and
366 harvested in early November. The same four experimental hybrids were planted in similar
367 experimental plots at two field sites in Germany (Table S1 and S9). No fungicides were
368 applied at any of the field sites.
369

370 We used two sampling strategies, one temporal (Swiss sites only) and the other spatial
371 (Swiss and German sites), to obtain leaves infected by *C. beticola* (Figure S1). The temporal
372 sampling strategy was designed to identify infected plants in each plot and monitor how CLS
373 infection spreads from a solitary infected plant to neighboring plants to form a cluster of
374 CLS-infected plants, called a hotspot. We identified every newly infected plant in each plot
375 at weekly intervals from July 21$^{st}$ to September 24$^{th}$ 2020. Every infected plant was given an
376 alphanumeric code and its position was marked with a labeled flag to avoid resampling. One
377 young leaf with CLS lesions was taken from each infected plant. The excised leaves were
378 transported back to the laboratory for *C. beticola* isolation. A GPS location was recorded for
379 the first infected plant in each hotspot. A maximum of five plants was sampled from each
380 hotspot.
381
382 Transect sampling was conducted in all plots in Switzerland and Germany at the end of the
383 2020 season (13$^{th}$ and 15$^{th}$ October in Switzerland and 17$^{th}$ September in Germany). A total
384 of 7 transect lines each 3 m apart were made for each transect site, giving a total of 21
385 leaves per transect site (see Figure S1). Two transect sites were sampled for each hybrid,
386 yielding 42 leaf samples total for each hybrid in each plot at both sites in Switzerland and
387 Germany.
388
389 **Pathogen isolation and DNA extraction**
390 Leaf pieces bearing CLS lesions were cut from each leaf and surface-sterilized by placing
391 them in a 2% sodium hypochlorite solution for 3 min and then rinsing them twice in sterile
392 water. The leaf pieces were then placed in glass petri dishes on a sterile plastic screen net
393 on top of a piece of moist filter paper. The petri dishes were incubated at 24°C for 48-72

394 hours to induce sporulation. Spores from single sporulating lesions were gathered using
395 hand-made fine glass needles and transferred to fresh potato dextrose agar (PDA, Difco).
396 The plates were cultured under constant darkness at 24°C for 7-10 days. Any plates
397 contaminated by other fungi were discarded.
398
399 To obtain fresh mycelia for DNA extraction, we transferred hyphae from pure culture plates
400 to 100 mL flasks with 50 mL potato dextrose broth (PDB, Difco). DNA for 103 isolates was
401 extracted with a Kingfisher Flex robot (Thermo Fisher Scientific, U.S.A.) following the
402 recommended protocol for fungal samples (Fungus Sbeadex, LGC Genomics GmbH) and
403 DNA for the remaining 153 isolates was extracted using DNeasy Plant Mini Kits (Qiagen)
404 with a modified protocol; 3.5 µL of proteinase K was added to each sample at the lysis step.
405 DNA was quantified using the Qubit 2.0 Fluorometer (Themo Fisher Scientific, U.S.A.) with a
406 Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, U.S.A.) and Nanodrop (ThermoFisher
407 Scientific, U.S.A.). Species identity was confirmed using PCR (Knight and Pethybridge, 2020).
408
409 For the reference genome, *C. beticola* strain "Hevensen" was grown in liquid media by
410 transferring mycelium from a V8 agar petri dish (500 ml of V8 juice, 500 ml distilled water, 3
411 g of $CaCO_3$ and 15 g of agar) to 225 ml of CB medium (1 g/l casein, 1 g/l yeast extract, 10 g/l
412 D-Glucose, 10 mM NaCl, 0.02 mM $FeCl_3x6H_2O$, 0.1 mM $ZnSO_4x7H_2O$, 7.9 µM $CuSO_4x5H_2O$,
413 0.4 mM $MnSO_4xH_2O$, 0.9 mM $H_3BO_3$, 4.2 mM $Ca(NO_3)_2x4H_2O$, 1.5 mM $KH_2PO_4$, 1 mM
414 $MgSO_4x7H_2O$). The culture was incubated for 6 to 8 days at 24°C at 100 rpm. Mycelium was
415 ground to a fine powder in liquid nitrogen. DNA was extracted using the method described
416 by (Huang et al., 2018) with minor modifications. DNA was repaired and end-prepped using
417 NEBNext FFPE DNA repair mix (NEB) and the NEBNext Ultra II End Repair/dA-Tailing Module

418 (NEB), respectively. Small DNA fragments were removed from each sample using a Short
419 Read Eliminator reagent (Circulomics).
420
421 **Genome data acquisition and assembly**
422 The Hevensen reference isolate was prepped for Oxford Nanopore Technology (ONT)
423 sequencing using SQK-LSK109 library kit (ONT). Each library was loaded onto three FLO-
424 FLG001 (R9.4.1 pore) flongle cells (ONT) and run for 24 hours on MinION or GridIONx5
425 instruments (ONT). Raw data was base called using Guppy (version 4.0.15, default
426 parameters) and assembled with miniasm (version r179; default parameters). The resulting
427 draft assembly was polished with Racon (version 1.4.13, input long reads, default
428 parameters), Medaka (version 1.2.0, input long reads, default parameters) and Pilon
429 (version 1.23, input short reads, default parameters). Polished sequences were size-ordered
430 and named in consecutive order as chromosomes 1 through 11. Two short contigs were
431 annotated via Uniprot Blast and contained rDNA (CB_CbHevensen-1_hq-v2.rDNA) and
432 mtDNA (CB_CbHevensen-1_hq-v2.mitochondrial). Final sequences were structurally
433 annotated with Funannotate (version 1.8.3, RNA-seq evidence – NCBI PRJNA294383, protein
434 evidence - NCBI RefSeq Fungi 2021-05-06, otherwise default fungal parameters) and
435 CodingQuarry-PM (run_CQ-PM_mine.sh, version 2.0) (Table S6a). RepeatMakser (v4.0.6)
436 and RepeatModeler (v1.0.11) were used to identify repetitive elements in the genome (-
437 species Fungi) (Figure S3). Signatures of RIP across the genome were investigated using
438 RIPPER (van Wyk et al., 2019) (Table S6b).
439
440 DNA from the 256 field-collected isolates was sequenced using Illumina paired end (150 bp)
441 sequencing (sequence provider: Novogene). In addition, raw reads from 199 isolates were

442 downloaded from the SRA (https://www.ncbi.nlm.nih.gov/sra) using SRAtoolkit (v2.0) for
443 downstream analyses. We performed multiple analyses to check the quality of the isolate
444 sequence data, see Supplementary Information (SI) for details. Briefly, we used k-mer based
445 approaches to identify potential contaminants or mixed cultures by examining the
446 heterozygosity and genome coverage. We estimated heterozygosity and sequence coverage
447 using Smudgeplot (https://github.com/KamilSJaron/smudgeplot) and removed isolates with
448 high heterozygosity (>0.1%) or lower than expected genome coverage. We drew a k-mer
449 based distance tree with mashtree (Katz et al., 2019) and removed isolates that were
450 genetically distinct and potentially different species. Sequence data processing and variant
451 discovery are described in detail at https://github.com/jessstapley/CercosporaGWAS. After
452 trimming reads were mapped to the Hevensen reference genome using bwa-mem (v0.7.17).
453 Samples that did not map well to the reference genome (< 80% properly paired reads) were
454 removed. After mapping, quality checking and removal of sequence duplicates, we retained
455 422 isolate genomes (232 sequenced by us, 190 from SRA) for further analyses. See SI.1 and
456 SI.2 for details and Table S1 for information about each isolate, including why they were
457 removed from further analyses.
458
459 Variant calling was done using the GATK (v4.2.4.1) Germline Short Variant Discovery
460 pipeline, following their Best Practices recommendations
461 (https://gatk.broadinstitute.org/hc/en-us/sections/360007226651-Best-Practices-
462 Workflows). For the GWAS analyses we used only biallelic SNPs and we removed markers
463 with minor allele frequency <0.05. The SNP error rate based on isolates sequenced twice
464 (sequence duplicates, n=29) was 0.00125. Clones were identified using an IBS distance
465 matrix from LD pruned data (13,696 SNPS) (See SI.3 for details). Following clone correction,

466 we retained 163 unique isolates from Switzerland and Germany, and 95 unique isolates
467 from the U.S.
468
469 **Field phenotyping of virulence for strains used in the GWAS**
470 Sixty fungal inocula were tested in total. Fifty-two unique genotypes originating from Swiss
471 plots were tested, 49 from plots planted to *BvCR4* hybrids (18 isolates from Hybrid_R2, 31
472 isolates from Hybrid_R3) and three from the susceptible hybrids (2 from Hybrid_S3, 1 from
473 Hybrid_S4). One putatively virulent clone that was widespread in one of the experimental
474 fields was replicated five times, giving a total of 56 field-collected isolates. Four additional
475 reference inocula were used as controls with known virulence properties but were not
476 sequenced nor included in the GWAS analyses.
477
478 Inoculum of the *C. beticola* strains was produced at KWS in Einbeck, Germany by first
479 growing on V8 agar (see S1.4 for details). Next, groups of 30, 12-week old plants of
480 Hybrid_R2, were inoculated with each of the isolates used in the GWAS by spraying
481 suspensions of ~25,000 hyphal fragments/spores per ml onto each group of plants until
482 runoff. Inoculated plants were then covered with plastic sheets draped over metal frames
483 and incubated for 5 days without supplemental lighting at 25°C and kept at 100% RH. The
484 first symptoms were observed at 14 dpi for most isolates. Inoculum for each isolate was
485 harvested between 28 and 42 dpi by removing leaves that were fully covered with CLS
486 lesions. Leaves were dried, milled and mixed with semolina flour.
487
488 Virulence assessments were conducted in 2022 on a field site in Germany. To minimize the
489 risk of infection by local strains of *C. beticola*, the field had not been planted to sugar beet

490 for about 20 years (corn-wheat rotation), the nearest sugar beet field was 800 m distant,
491 and the experimental field site was surrounded by a 50 m border of maize. Seeds treated
492 with the fungicide Tachigaren 70WP (Hymexazole) and the insecticide Force 20CS
493 (Tefluthrine) from four sugar beet hybrids with varying levels of CLS resistance (Table 1)
494 were planted on 19$^{th}$ April 2022 in a randomized block design with 6-row plots that were
495 0.45 m wide and 5 m long. Each isolate was inoculated onto a block of plots that contained
496 all 4 hybrids. Weeds were controlled with three herbicide applications and aphids were
497 controlled with two insecticide applications during the growing season.
498
499 Plots were inoculated after row closure on 27$^{th}$ July 2022. Inoculation was performed by
500 spreading 60 g of leaf inoculum mix (10 g milled leaf inoculum + 50 g semolina flour) onto
501 one row (3$^{rd}$ or 4$^{th}$ row, 5 m length, approx. 25 plants) of each 6-row plot. Inoculation rows
502 were sprayed with water containing an adjuvant (Silwet 0.05%) prior to inoculation so that
503 most of the inoculum mix would stick to the leaf canopy. Following the inoculation, all plots
504 were watered twice a week for 6 weeks to facilitate infection and disease progress.
505 Approximately 4 l water/m$^2$ was applied each week with a tractor-mounted crop protection
506 sprayer. We recorded the disease rating at 32, 40, 47, 56 and 64 dpi. We analysed changes
507 in disease rating using linear models and least significant differences to make post hoc
508 comparisons (Bonferroni-adjusted) between mean rating at each time point. Disease rating
509 was bimodal at 40 dpi on the resistant hybrid Hybrid_R2 (Figure S4), providing a clear
510 distinction between virulent (rating >4 on KWS rating scale) and avirulent (rating ≤ 4) strains
511 and enabling us to code isolates as either virulent or avirulent for some of the GWAS
512 analyses.
513

514 **GWAS analyses**

515 SNP-based GWAS were performed using GAPIT (http://zzlab.net/GAPIT). We performed two
516 SNP-GWAS analyses using different phenotype data as described above. We used a Mixed
517 Linear Model (MLM), which accounts for relatedness of individuals, and included three
518 principal components into the model as covariates (random effects). The model fit was
519 assessed by inspecting the Q-Q plots (Figure S5). We repeated the analysis using
520 quantitative virulence ratings at other time points (Figure S6). A quantitative GWAS using
521 the Hybrid_R2 virulence rating at 47 dpi identified the same peak that was found using the
522 rating at 40 dpi.

523

524 We conducted a k-mer GWAS (see https://github.com/jessstapley/CercosporaGWAS for
525 details) as a recent analysis conducted in *Z. tritici* (Dutta et al., 2022) showed that these can
526 provide more power to detect associations. We used recommendations and scripts from
527 https://github.com/voichek/kmersGWAS, but used GAPIT to run the GWAS. In brief, KMC
528 was used to count k-mers with a length of 31 bp. The k-mer plink output file (.bed) was
529 imported into plink (v1.9, https://www.cog-genomics.org/plink/) and converted to a vcf file
530 (--recode vcf). The vcf file was split into 99 smaller files (containing 20,000 k-mers each),
531 then converted to a hapmap file using R::vcfR (https://github.com/knausb/vcfR). GAPIT
532 GWAS was then run on each of these smaller files. The k-mers were mapped to the
533 reference genome using bowtie2 (v2.3) and the results were concatenated into
534 chromosome files for plotting. The k-mer GWAS was performed using only 47 of the
535 phenotyped strains because we could not count k-mers in all 52 strains.

536

537 **Annotation of the GWAS interval**

538 Genes within the GWAS interval were identified using the reference genome annotation file
539 (gff3). The function of the putative effector was confirmed using EffectorP (v3.0)
540 (https://effectorp.csiro.au/ (Sperschneider and Dodds, 2022)). Additional annotation of the
541 GWAS interval was performed to identify possible transposable elements (TEs) using Repeat
542 Masker (v4.0.6) and One Code to Find them all (Bailly-Bechet et al., 2014).
543
544 SNP genotypes across the GWAS interval were obtained for every isolate using bcftools
545 (samtools v1.9). Individuals with missing genotype calls spanning the entire candidate gene
546 were recorded as 'gene deleted' for the candidate gene. Individuals with SNP genotype calls
547 within the gene were recorded as 'gene present'. We compared the frequency of gene
548 presence or absence between resistant and susceptible hybrids in Germany and Switzerland
549 using a binomial exact test.
550
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553 lab.
554
555 **Data availability statement**
556 All sequence data has been deposited on the NCBI SRA (SRA NUMBER). The genome is
557 available at DOI. All other data is available in the Supplementary Information.
558
559 **Conflict of Interest**
560 CC was funded by a research grant from KWS SAAT SE & CO. KGaA to the BAM lab at ETH
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731 **Table 1.** Sugar beet hybrids used for the virulence assessments.

| **Experimental Hybrids** | ***Cercospora* resistance level** |
|---|---|
| Hybrid_S4 | low quantitative resistance |
| Hybrid_S3 | moderate quantitative resistance |
| Hybrid_R2 | *BvCR4* with low quantitative background resistance |
| Hybrid_R3 | *BvCR4* with moderate quantitative background resistance |

732

733 **Table 2.** Frequency of unique isolates carrying a deletion of the candidate avirulence

734 effector gene. Frequency is the percentage of the total number of individuals analyzed. The

735 number of individuals with deletions and the total are shown in parentheses.

| Country | Sampling | Year | Resistant: Deletion freq. (n) | Susceptible: Deletion freq. (n) | Clone Corrected (Total) |
|---|---|---|---|---|---|
| Switzerland | Pilot | 2019 | 25% (1/4) | NA | 4 (7) |
| Switzerland | Temporal | 2020 | 32% (22/68) | 0% (0/42) | 110 (147) |
| Switzerland | Transect | 2020 | 57% (4/7) | 0% (0/15) | 22 (38) |
| Germany | Transect | 2020 | 60% (6/10) | 0% (0/17) | 27 (40) |
| USA | Public | 2016-17 | NA | 1.0% (1/95) | 95 (190) |
| Total | | | 37% (33/89) | 0.5% (1/169) | 258 (422) |

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739 **Figure 1.** Disease rating of isolates at five time points in the field trial. Each panel shows the
740 rating on one of the four hybrids (2 susceptible and 2 resistant). Red points are isolates
741 scored as virulent (rating >4 on Hybrid_R2 at 40 dpi) and black are avirulent (rating ≤4 on
742 Hybrid_R2 at 40 dpi), lines are loess-smoothed lines. Black letters at the top of the plots
743 indicate least significant differences between mean rating of all isolates at each time point.
744 Red text indicate difference in disease rating between virulent and avirulent for that hybrid
745 and time point (ns=nonsignificant, *p<0.05, **p<0.01, *** p<0.001). No analysis was
746 performed on Hybrid_K2 because data from this hybrid was used to score
747 virulence/avirulence.
748
749 **Figure 2.** Manhattan plots of GWAS. a) Manhattan plot from a genome-wide association
750 (GWAS) scan using 52 unique isolates with quantitative phenotypes at 40 dpi on Hybrid_R2.
751 b) Manhattan plot from GWAS using 90 unique isolates, with 52 isolates scored with binary
752 phenotypes (virulent or avirulent) and 38 isolates collected from Hybrid_S4 scored as
753 avirulent. c) Manhattan plot from a k-mer GWAS using 47 unique isolates with quantitative
754 phenotypes. Horizontal line is the genome-wide significance level. All SNPs and k-mers with
755 p-value >1.22e-01 were downsampled for plotting.
756
757 **Figure 3.** Manhattan plot of the significant GWAS interval (± 25 kb of flanking sequence) on
758 chromosome 1:4087634-4094364 bp (shaded green). Genes are shaded in blue, full length
759 transposable elements are shaded in grey and large RIP affected regions (LRAR) are shaded
760 in yellow. Inset: Gene and repeat density around the GWAS interval (including 300 kb of
761 flanking sequence). Gene (solid line) and repeat (dashed line) density shown in 10 kb
762 windows. The GWAS interval is shaded in green and the LRAR is shaded in yellow.

763

764 **Figure 4.** DNA and amino acid (aa) sequence variation in the candidate effector gene. a)
765 Haplotype networks for isolates collected in Switzerland from resistant and susceptible
766 hosts and in Renville USA. Ref = Identical to reference genome, 'aa' numbers correspond to
767 the translated effector sequence variation shown in (b). b) Shows the 13 (incl. Ref) different
768 amino acid sequences and the aa sequence for *Cercospora sojina*. In parentheses are the
769 numbers and their host or virulence status (if phenotyped): sus = collected on susceptible
770 hybrid, res = collected on resistant hybrid, virulence rating at 40 dpi on Hybrid_R2 vir >4
771 virulent, avr ≤4 avirulent. c) Effect of amino acid sequence variation (seen in b) and gene
772 deletion on virulence rating (40 dpi Hybrid_R2).

773

774 **Figure 5.** Field disease rating of isolates at five time points coloured according to the
775 effector gene variant (blue=same as reference, orange=gene absent or sequence codes for
776 different amino acids (inactivated)). Each panel shows the rating on one of four hybrids (2
777 susceptible and 2 resistant). Stars on top of the plots indicate significant differences
778 between gene variants at each time point (ns=nonsignificant, *p<0.05, **p<0.01, ***
779 p<0.001).

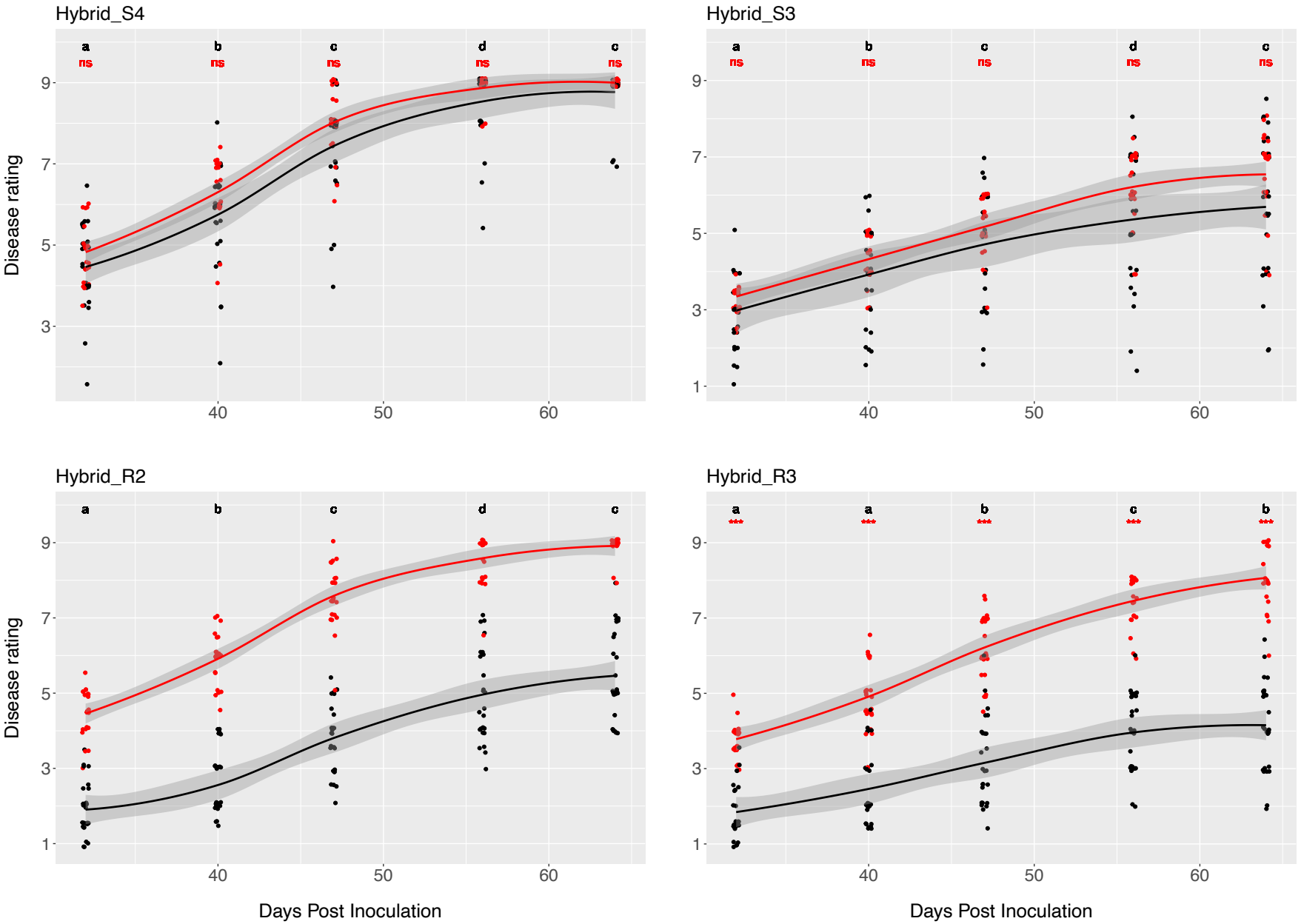


**Figure 1.** Disease rating of isolates at five different time points in the field trial. Each panel shows the rating on one of the four different hybrids (2 susceptible and 2 resistant). Red points are isolates scored as virulent (rating >4 on Hybrid_R2 at 40dpi) and black are avirulent (rating ≤4 on Hybrid_R2 at 40dpi), lines are loess-smoothed lines. Black letters at the top of the plots indicate least significant differences between mean rating of all isolates at each time point. Red text indicate difference in disease rating between virulent and avirulent for that hybrid and time point (ns=nonsignificant, *p<0.05, **p<0.01, *** p<0.001). No analysis was performed on Hybrid_K2 because data from this cultivar was used to score virulence/avirulence.

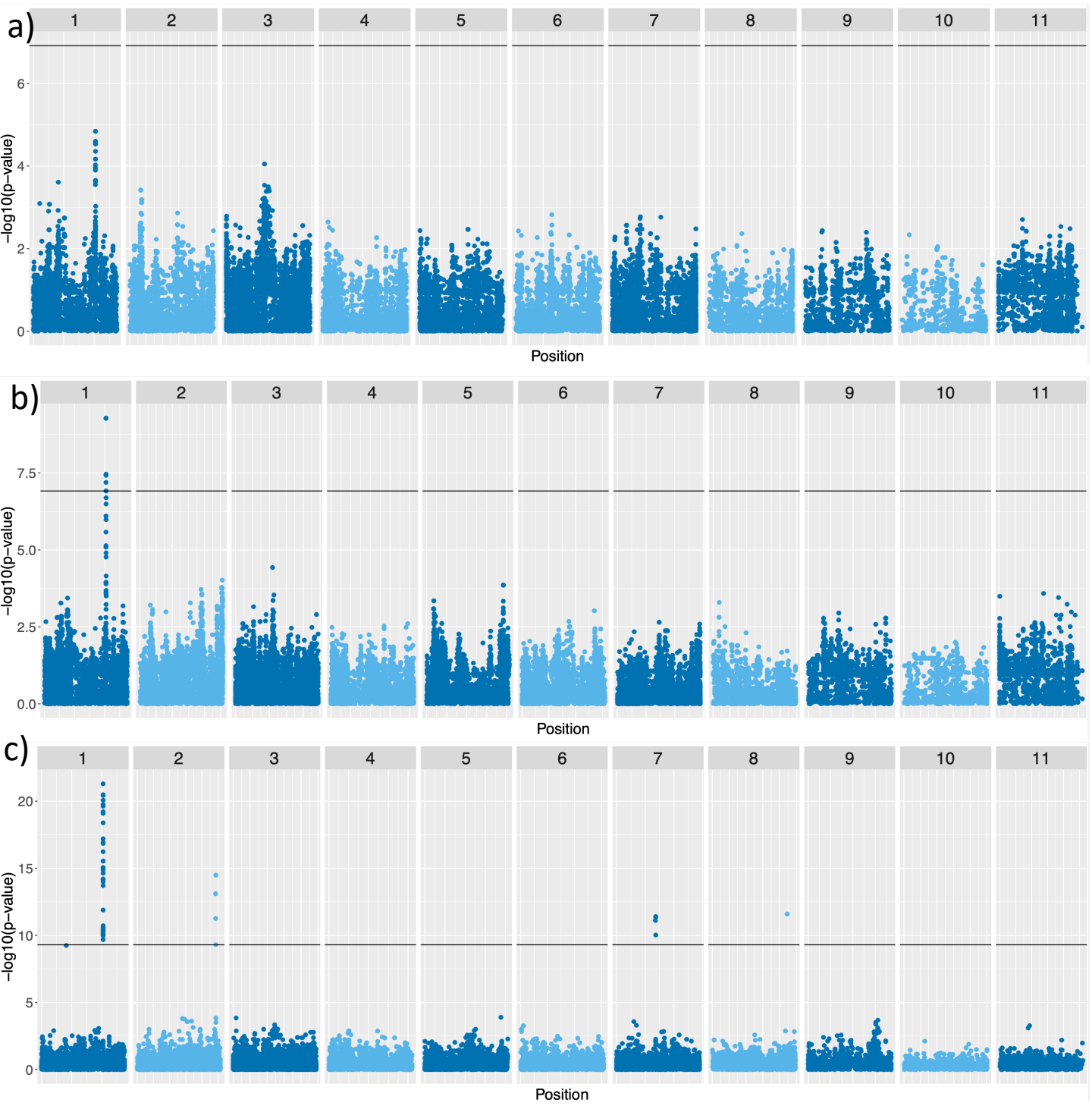


**Figure 2.** Manhattan plots of GWAS. a) Manhattan plot from a genome-wide association (GWAS) scan using 52 unique isolates with quantitative phenotypes at 40 dpi on Hybrid_R2. b) Manhattan plot from GWAS using 90 unique isolates, with 52 isolates scored with binary phenotypes (virulent or avirulent) and 38 isolates collected from Hybrid_S4 scored as avirulent. c) Manhattan plot from a k-mer GWAS using 47 unique isolates with quantitative phenotypes. Horizontal line is the genome-wide significance level. All SNPs and k-mers with p-value >1.22e-01 were downsampled for plotting.

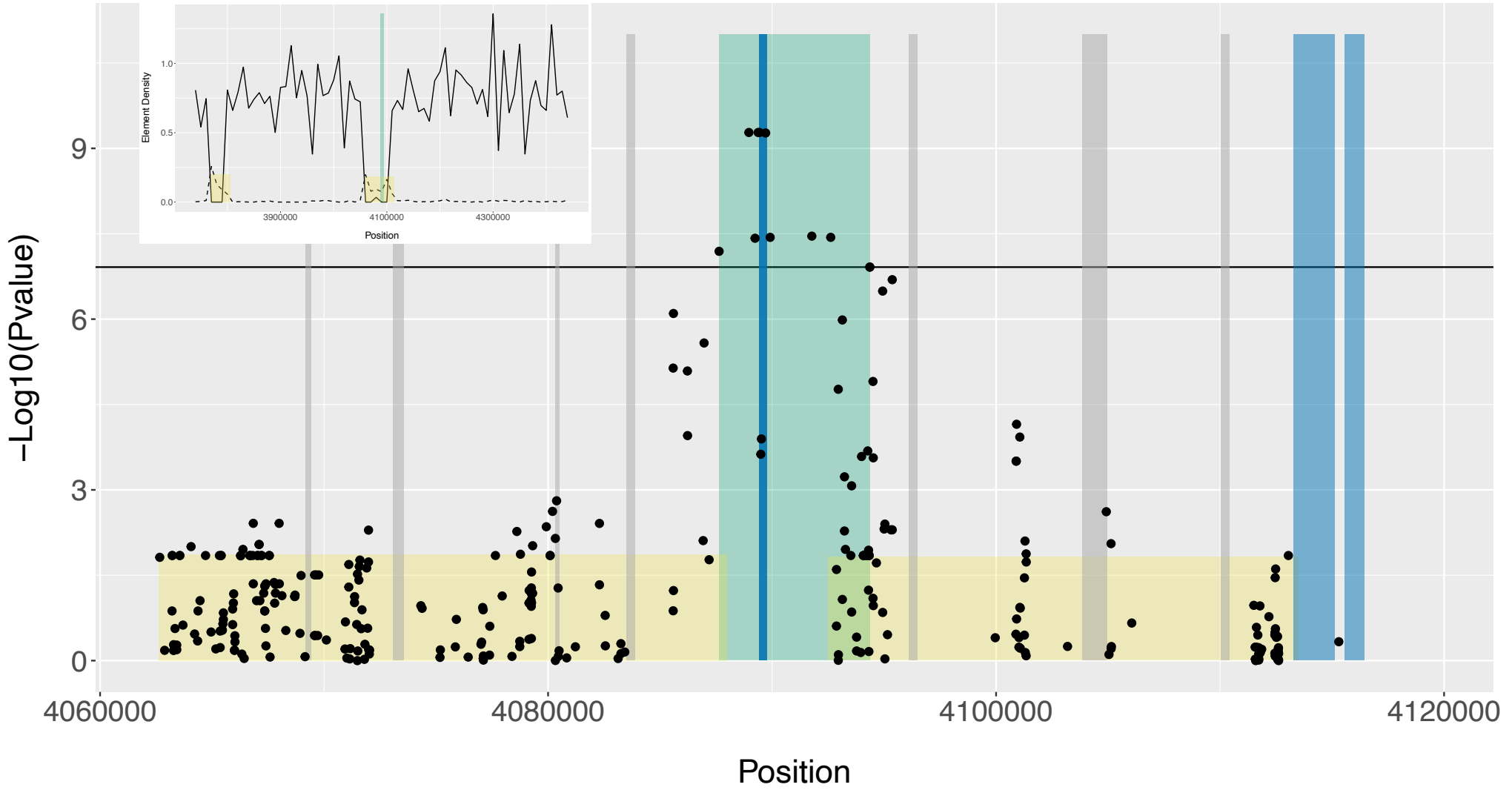


**Figure 3.** Manhattan plot of the significant GWAS interval (± 25 kb of flanking sequence) on chromosome 1:4087634-4094364 bp (shaded green). Genes are shaded in blue, full length transposable elements are shaded in grey and large RIP affected regions (LRAR) are shaded in yellow. **Inset:** Gene and repeat density around the GWAS interval (including 300 kb of flanking sequence). Gene (solid line) and repeat (dashed line) density shown in 10 kb windows. The GWAS interval is shaded in green and the LRAR is shaded in yellow.

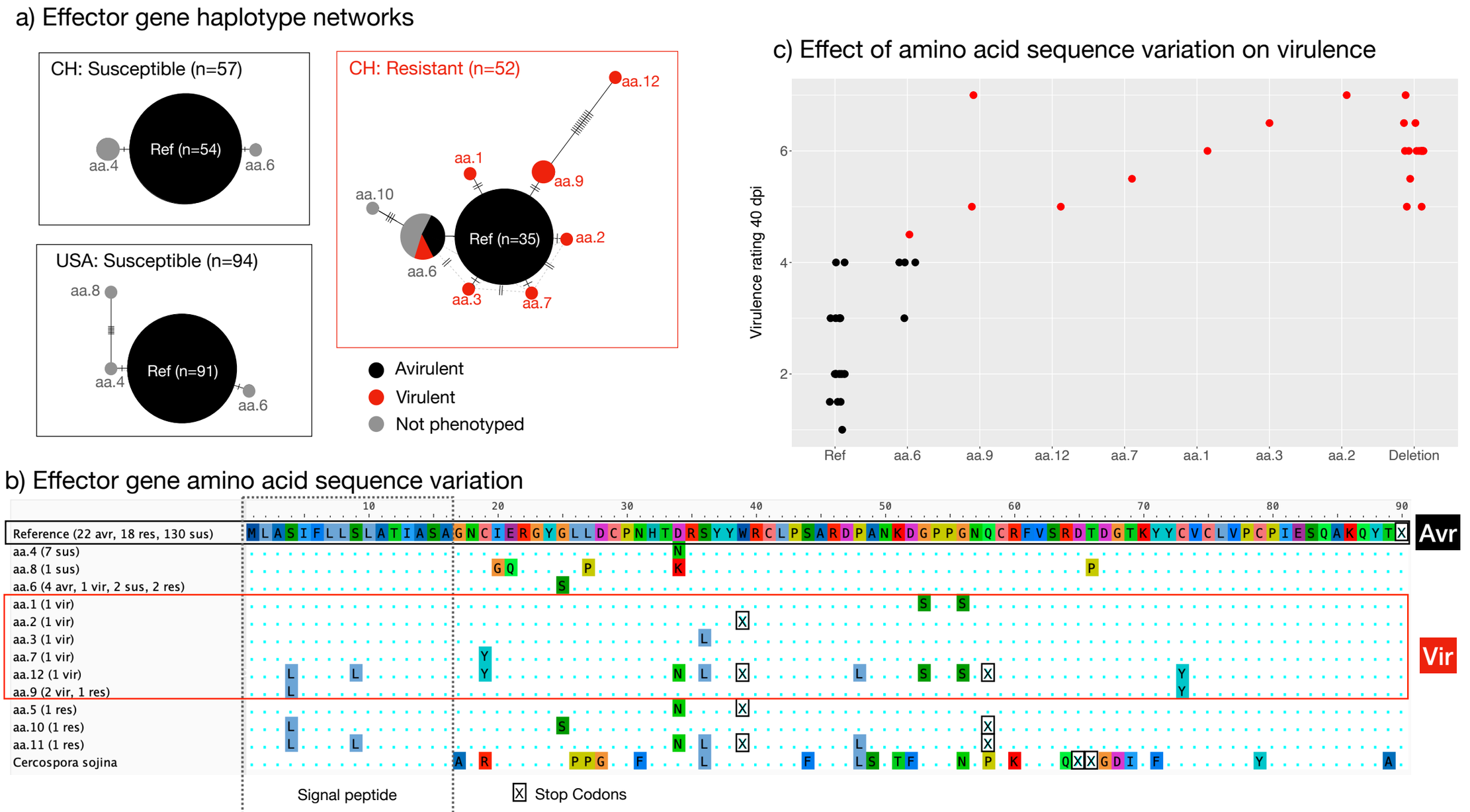


**Figure 4.** DNA and amino acid (aa) sequence variation in the candidate effector gene. a) Haplotype networks for isolates collected in Switzerland from resistant and susceptible hosts and in Renville USA. Ref = Identical to reference genome, 'aa' numbers correspond to the translated effector sequence variation shown in (b). b) Shows the 13 (incl. Ref) different amino acid sequences and the aa sequence for *Cercospora sojina*. In parentheses are the numbers and their host or virulence status (if phenotyped): sus = collected on susceptible hybrid, res = collected on resistant hybrid, virulence rating based on rating at 40 dpi on Hybrid_R2 (vir >4 virulent, avr ≤4 avirulent). c) Effect of amino acid sequence variation (seen in b) and gene deletion on virulence rating (40 dpi Hybrid_R2).

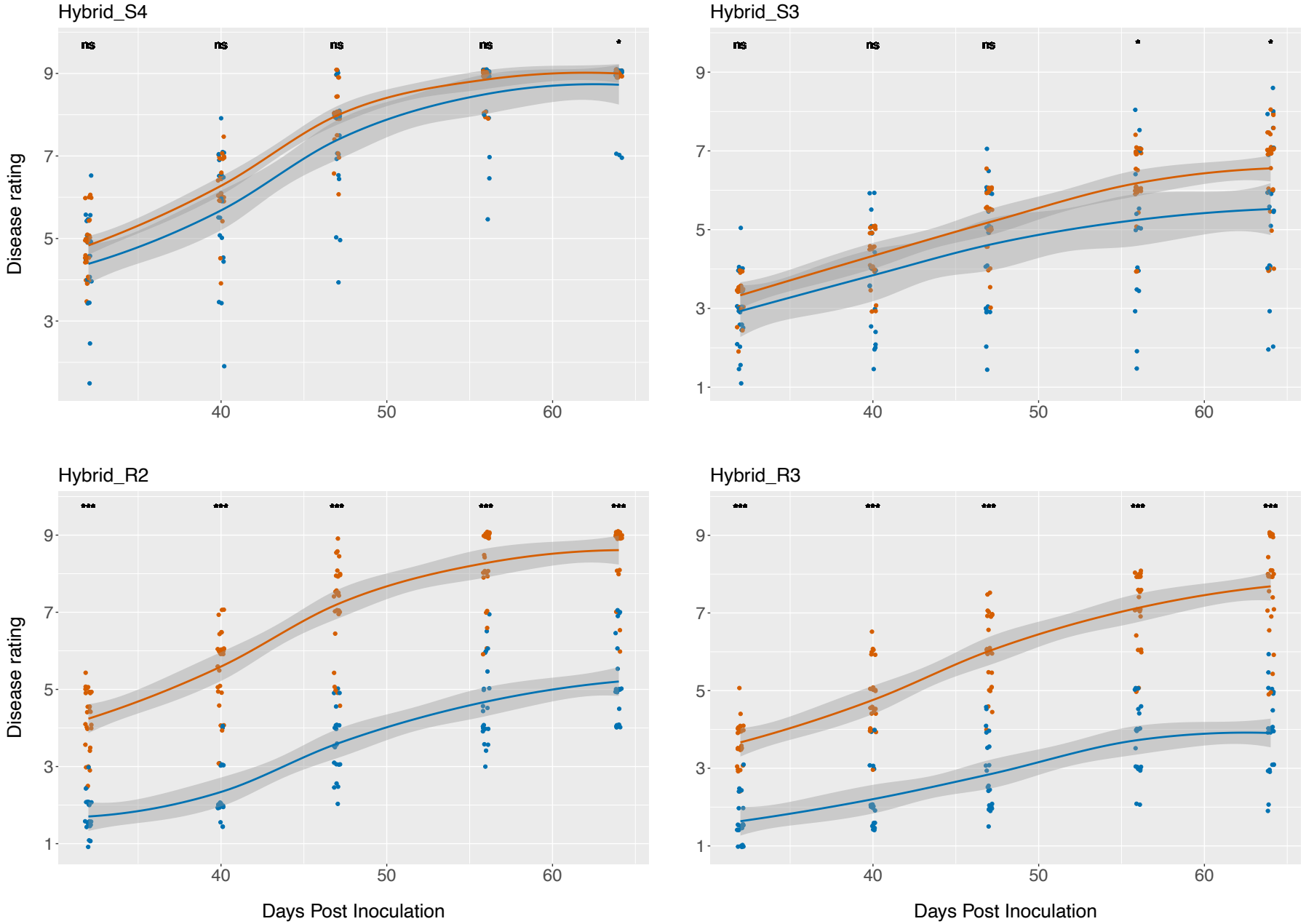


Figure 5. Disease rating of isolates at five different time points in the field trial coloured according to the effector gene copy (blue=same as reference, orange=gene absent or sequence codes for different amino acids (inactivated)). Each panel shows the rating on one of the four different hybrids (2 susceptible and 2 resistant). Stars at the top of the plots indicate significant differences between the gene variants at each time point (ns=nonsignificant, *p<0.05, **p<0.01, *** p<0.001).