Rapid breakdown of *Cf-6*-mediated immunity in tomato through loss or mutation of the *Avr6* effector gene in *Fulvia fulva*

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Word counts

Total: 8738

Summary: 249

Introduction: 954

Materials and Methods: 1906

Results: 2566

Discussion: 2151

Acknowledgements: 102

Figure legends: 810

Number of figures/tables/supporting Information:

Number of figures (colour): 8 (8)

Number of tables: 0

Supporting information: 18 = Figure S1–S9, Notes S1, Tables S1–S8

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Summary

Genetic resistance mediated by Cf cell-surface receptors is a cornerstone of tomato breeding against leaf mold disease caused by the fungal pathogen *Fulvia fulva*. After widespread deployment of the *Cf-9* resistance locus, breeders increasingly relied on *Cf-6*, yet *Cf-6*-mediated resistance has already been overcome in multiple regions. The molecular basis of this breakdown, and the identity of the matching avirulence (Avr) effector, have remained unresolved. Here, we use comparative genomics and functional genetics to identify the *F. fulva* effector Avr6 as the previously described apoplastic protein Ecp5. Sequencing of *Cf-6*-breaking strains revealed either deletion of the *Ecp5* locus or non-synonymous mutations in its coding sequence. Using transient expression, targeted gene knockout, and complementation assays, we show that *Ecp5* is both necessary and sufficient for Cf-6-mediated recognition and defence activation. We then use Avr6-triggered cell death as a phenotypic marker in a bulked segregant analysis combined with Comparative Subsequence Sets analysis (CoSSa) to map *Cf-6* to a ~2 Mb interval on the short arm of chromosome 12 that overlaps the previously described *Cf-Ecp5.12* locus. Sequencing of *Avr6* in a broad strain collection reveals a predominantly conserved allele under apparent purifying selection, with recent independent loss-of-function variants in Europe and South America that confer virulence on *Cf-6* plants. Together, these findings establish the *Avr6*–*Cf-6* gene-for-gene pair, explain the erosion of *Cf-6*-mediated resistance, and illustrate how effector loss or mutation provides a rapid route for *F. fulva* adaptation, with important implications for designing more durable resistance strategies against tomato leaf mold.

Keywords

Fulvia fulva, *Cladosporium fulvum*, Tomato leaf mold disease, *Solanum lycopersicum* (tomato), *Avr6* avirulence effector gene, *Cf-6* resistance gene, Breakdown of genetic resistance.

84

85 **Introduction**

86 *Fulvia fulva*, previously known as *Cladosporium fulvum*, is a Dothideomycete fungal pathogen that
87 causes leaf mold disease of tomato (*Solanum lycopersicum*) under humid greenhouse conditions
88 (Cooke, 1883; Thomma et al., 2005; Videira et al., 2017). It has no known sexual stage, although there
89 is evidence of cryptic sexual reproduction (de la Rosa & Schol et al., 2024; Gül et al., 2022;
90 Stergiopoulos, Groenewald, et al., 2007). Introgression of genetic host resistance from wild tomato
91 relatives, mediated by cell-surface anchored, receptor-like proteins (RLPs) that recognize specific
92 apoplastic Avirulence (Avr) effector proteins of *F. fulva* (Dixon et al., 1998; Dixon et al., 1996; Jones et
93 al., 1994; Takken et al., 1999; Thomas et al., 1997), is an important breeding strategy against this
94 disease. These RLPs, encoded by *Cf* (resistance to *C. fulvum*) genes, typically reside in clusters
95 throughout the tomato genome and mediate immune responses like the hypersensitive response (HR),
96 which is associated with a burst of reactive oxygen species (ROS) (Gust & Felix, 2014; Huang & Joosten,
97 2024; Kang & Yeom, 2018), the accumulation of pathogenesis-related (PR) proteins (Joosten & de Wit,
98 1989; Wubben et al., 1993), and the release of antimicrobial phytoalexins (de Wit & Flach, 1979). Our
99 understanding of these RLPs and their corresponding Avr effectors, together with extensive genetic and
100 molecular characterization of both partners, has positioned the *F. fulva*–tomato interaction as a long-
101 standing model for cell-surface receptor-mediated immunity and effector adaptation (de Wit, 2016;
102 Mesarich et al., 2023; Thomma et al., 2005).

103 Upon widespread deployment of *Cf* resistance genes in tomato cultivars, strong selection pressure is
104 exerted on *F. fulva* to circumvent Avr recognition to regain virulence. This has so far been shown to be
105 achieved through mutations in, or complete loss of the corresponding *Avr* gene (de la Rosa & Schol et
106 al., 2024; Iida et al., 2015; Joosten et al., 1994; Mesarich et al., 2023; Stergiopoulos, de Kock, et al.,
107 2007). To date, all known *F. fulva Avr* genes are highly expressed during infection and encode small
108 proteins that are secreted into the tomato leaf apoplast (Mesarich et al., 2023). These Avr effectors are
109 cysteine-rich and, in some cases, can evade recognition by their corresponding Cf receptor through
110 mutation of a single cysteine residue, which disrupts an associated disulfide bond (Joosten et al., 1997;
111 Joosten et al., 1994). Most Avr effectors of *F. fulva* show little homology to other proteins and lack
112 known functional domains (Mesarich et al., 2018). The only exceptions are Avr4 (chitin-binding
113 domain) and Avr2 (protease inhibitor), which protect against PR chitinases and proteases, respectively
114 (Rooney et al., 2005; Van Esse et al., 2007). Recently, a combined transcriptomics and proteomics
115 approach was used to show that *F. fulva* secretes at least 75 small proteins, named extracellular
116 proteins (Ecps), into the apoplast during infection, of which many are expected to be yet-identified Avr

effectors (Mesarich et al., 2018). In support of this, 10 Ecps were found to elicit an HR upon expression in various wild tomato accessions (Mesarich et al., 2018).

We recently demonstrated that *F. fulva* overcame the widely deployed *Cf-9* resistance locus by sequential loss of *Avr9* and loss or mutation of *Avr9B* (de la Rosa & Schol et al., 2024). Both of these *Avr* genes encode small apoplastic proteins and are recognized by the products of two RLP-encoding genes at the *Milky Way* (MW) locus on the short arm of chromosome 1, *Cf-9C* and *Cf-9B*, respectively (Jones et al., 1994; Parniske et al., 1997). This prompted breeders to deploy another resistance gene, *Cf-6*, which was tentatively mapped to the short arm of chromosome 12 in the breeding line Ontario 7818 and reported to originate from *S. pimpinellifolium* PI211839 (Kanwar et al., 1980). More recently, the International Seed Federation (ISF) updated the *F. fulva*–tomato differential sets used to define virulence and resistance spectra, now including five additional *F. fulva* races (9, 2.9, 4.9, 4.5.9 and 2.6.9) and designating Ontario 7818 as the differential line for *Cf-6* (Sangster, 2022). The inclusion of a race 2.6.9 strain (breaking *Cf-2*-, *Cf-6*- and *Cf-9*-mediated resistance) demonstrated that *Cf-6* provides race-specific resistance. Since then, *Cf-6*-breaking strains have been detected in multiple European countries, including Germany (Meyer & Gärber, 2021) and the Bretagne region in France, where several race 2.6.9 strains were collected between 2017 and 2021. These observations reveal that the more recently deployed *Cf-6* locus has already been overcome, with direct consequences for resistance breeding strategies.

To determine the genetic and molecular basis of *Cf-6* resistance breakdown, we set out to identify Avr6, the effector recognized by *Cf-6*-bearing tomato plants, using a comparative genomics approach. Previous efforts to identify *F. fulva Avr* genes such as *Avr5* and *Avr9B* by comparative transcriptomics and genomics compared a single resistance-breaking strain with a single non-breaking strain (de la Rosa & Schol et al., 2024; Mesarich et al., 2014). Here, we perform whole-genome sequencing and assembly of 15 *F. fulva* race 2, 2.9 and 2.6.9 strains, allowing us to systematically scan polymorphisms in small secreted protein-encoding genes, most of which had previously been characterized as Ecps (Laugé et al., 2000; Mesarich et al., 2018; Van den Ackerveken et al., 1993). We then validate the Avr6–Cf-6 interaction through PVX-mediated *in planta* expression, gene knockout, and complementation assays. Using Avr6-triggered hypersensitive cell death as a phenotypic marker, we apply bulked segregant analysis (BSA) coupled to a Comparative Subsequence Sets analysis (CoSSa) pipeline (Prodhomme et al., 2019) to map *Cf-6* in Ontario 7818. Finally, we link Avr6 recognition to resistance to *F. fulva* in segregating populations. In doing so, we identify *Avr6*, map *Cf-6* to the short arm of chromosome 12, and provide a mechanistic explanation for the breakdown of *Cf-6*-mediated resistance, while highlighting broader challenges for achieving durable resistance to tomato leaf mold.

150

151 **Materials and Methods**

152 **Fungal and plant material**

153 Information on the *F. fulva* strains used in this study can be found in **Table S1**. Novel French strains
154 (sample IDs starting with FR_) were collected by VEGENOV and their virulence spectrum was
155 determined by inoculation onto a tomato differential set carrying different *Cf* genes (including *Cf-6*),
156 according to the ISF (Sangster, 2022). Argentinean *F. fulva* strains were previously collected and
157 characterized by Medina et al. (2015) and Lucentini et al. (2021). Their virulence spectrum was
158 determined by the authors using a tomato differential set (excluding *Cf-6*) and, in this study we assessed
159 their virulence on Ontario 7818 (CGN15839; carrying *Cf-6*) and Moneymaker (MM) plants (CGN14330).
160 Transgenic *F. fulva Avr6* knockout strains in the 0WU background were previously generated by Ökmen
161 (2013). Strain 0WU (race 0) (de Wit et al., 2012) was used as a control strain in the inoculation studies
162 and strain IPO 2679 (de la Rosa & Schol et al., 2024) was included as it produces Avr6^{Cys30Tyr}.

163 For BSA-sequencing, an F2 population of 133 individuals derived from a cross between Ontario 7818
164 (*Cf-6*) and MM-*Cf-0* was utilized. *S. lycopersicum* Ontario 7818 (*Cf-6*), breeding line G1.1161 (*Cf-Ecp5*)
165 (Haanstra et al., 2000) and MM-*Cf-9* (CGN15338) were utilized for PVX-mediated expression of Avr6,
166 Avr9 and Avr9B. Detailed information regarding CGN accessions as provided by the Center of Genetic
167 Resources Netherlands (CGN) in Wageningen, The Netherlands, can be found in **Table S2**.

168 **DNA isolation, whole-genome sequencing and assembly**

169 *F. fulva* strains were grown on potato dextrose agar (PDA) at 25°C for 10–14 days, after which spores
170 and mycelium were harvested for genomic DNA (gDNA) extraction by scraping with a sterile scalpel
171 blade. gDNA was extracted from *F. fulva* strains FR_2_1, FR_2_2, FR_269_1, FR_269_2, FR_269_3,
172 FR_269_4, FR_269_5, FR_29_1, FR_29_2, FR_29_3, FR_29_4, FR_29_5, FR_29_6, FR_29_7 and
173 NZ_269_1 using a cetyltrimethylammonium bromide (CTAB) DNA protocol (Murray & Thompson, 1980)
174 and purified using a Genomic DNA Clean & Concentrator (Zymo Research). gDNA quality and
175 concentration were measured using the Nanodrop™ One (Thermo Scientific) and Qubit 2.0
176 Fluorometer (Thermo-Scientific). One hundred and fifty bp paired-end (PE150)-sequencing was
177 performed by Novogene (https://www.novogene.com) on an Illumina NovaSeq6000 sequencing
178 platform. Sequencing reads were trimmed with Trimmomatic v0.39 (Bolger et al., 2014) with
179 parameters set to SLIDINGWINDOW:4:20, MINLEN:100 using Truseq2 adapter sequences, and quality
180 of trimmed reads was checked with FASTQC (v0.12.1) (Andrews, 2019). Trimmed reads were assembled
181 with Spades v4.0.0 (Bankevich et al., 2012) and contigs < 1 kb were removed post assembly. Assembly

182 quality was assessed with Quast (v5.3.0) (Gurevich et al., 2013) and BUSCO (v5.7.1, genome-mode,
183 with dothideomycetes_odb10 as reference BUSCO set and metaEUK as gene predictor) (Manni et al.,
184 2021) (**Table S3**).

185 For *F. fulva* strains CF301, CF-Nangol, Can_38, 2_5, IPO_2459_60787, IPO_248911, IPO_249, Turk_1a,
186 IMI_Argent_358077, 2_5_9 and 18-A6, gDNA was extracted using an SDS-based method (Penouilh-
187 Suzette et al., 2020) and quality and concentration measured as above. gDNA sequencing libraries were
188 prepared with the Invitrogen Collibri ES DNA Library Prep Kit for Illumina Systems (Thermo Fisher
189 Scientific) according to the manufacturer's instructions (protocol MAN001845) and sequenced (PE150)
190 on an Illumina NovaSeq 6000 at the UC Davis Genome Center. Sequencing read quality was assessed
191 using FastQC (v0.11.9) (Andrews, 2010) and fastp (v0.23.2) (Chen et al., 2018). Assemblies were
192 generated using the shovill pipeline (v1.1) (https://github.com/tseemann/shovill), which merged
193 overlapping PE reads with FLASH (v1.2.11) (Magoč & Salzberg 2011) and assembled the reads with
194 SPAdes (v3.15.5) (Bankevich et al., 2012).

195 gDNA was extracted from leaves of 13-day old MM-*Cf-0*, Ontario 7818 (*Cf-6*) and Ontario 7818 x MM-
196 *Cf*-0 F2 plants (grown under greenhouse conditions) using a cetyltrimethylammonium bromide (CTAB)
197 DNA protocol (Murray & Thompson, 1980) and purified using a Genomic DNA Clean & Concentrator
198 (Zymo Research). For BSA-sequencing, 200 ng of gDNA per F2 plant was pooled into the according R-
199 or NR-pool and then purified, with gDNA quality and concentration measured as above. All 150 bp PE-
200 sequencing of (pooled) tomato gDNA was performed by BGI (https://www.bgi.com) on a DNBseq
201 sequencing platform.

202 **Compiling a dataset of public genomes and inference of phylogeny**

203 Publicly available genome sequences were downloaded from GenBank using the query '*Fulvia fulva*'
204 (**Table S4**). The genome of *Dothistroma septosporum* NZE10 (GCA_000340195.1) was downloaded
205 separately. We used BUSCO as described above to identify single-copy BUSCO genes in these
206 assemblies and those generated in this study. For each BUSCO query gene, we created a fasta file with
207 corresponding DNA sequences from each strain (33 *F. fulva* + 1 *D. septosporum*) if it was present as
208 single-copy in all assemblies using a custom Python script. Next, we used Muscle (v 5.3.linux64) (Edgar,
209 2022) with default settings to infer a multiple sequence alignment for each fasta and trimmed
210 alignments with TrimAl (v1.5.rev0) (Capella-Gutiérrez et al., 2009) with settings optimized for maximum
211 likelihood inference (-automated1). Trimmed alignments were then concatenated with a second
212 custom Python script into a single alignment with 6917 parsimony informative sites. We used IQtree
213 (Minh et al., 2020) with ModelFinder (Kalyaanamoorthy et al., 2017) and UFBoot (-m MFP -B 1000 -
214 bnni -alrt 1000) (Hoang et al., 2018) to identify an optimal substitution model (GTR+F+I+R10) and infer

215 a consensus maximum likelihood phylogeny from this concatenated alignment with 1000 bootstrap
216 replicates. For the figures, the outgroup (*D. septosporum* NZE10) and isolate CBS131901 (short-read
217 assembly of 0WU) were removed. All custom scripts can be downloaded from
218 https://git.wur.nl/like.fokkens/clado_avr6.

219 **Identification of *MAT* loci and sequence comparison of known and predicted effector genes**

220 To infer mating types of *F. fulva* strains, we used megablast (v 2.13.0+) (Morgulis et al., 2008) with
221 default settings to align *Mat1-1* and *Mat1-2* sequences (accessions DQ659350.2 and DQ659351.2
222 respectively). For identification of effector genes in the assemblies, we used a custom python script
223 implementing bedtools getfasta (v2.30.0) (Quinlan & Hall, 2010) and a list of predicted effector genes
224 from Zaccaron et al. (2022) to obtain a fasta file with sequences from the assembly of the Race 5
225 reference strain (GCA_020509005.2) including introns. We then used megaBLAST (v 2.13.0+) with –
226 default settings to search for homologs of each of these genes. To obtain a similarity score for each
227 strain/assembly and each effector gene, relative to the Race 5 reference, we divided the maximum
228 Smith-Waterman score of the effector gene against the assembly with its maximum score against the
229 assembly of Race 5. We used a custom Python script to parse the BLAST output table and calculate
230 these scores. For known *Avr* genes we generated MSAs to identify distinct genotypes. To confirm
231 presence/absence of predicted effectors and to determine the size of the deletion resulting in loss of
232 *Avr6*, we used Bowtie2 (v2.5.4, with -I 100 -X 1000) (Langmead & Salzberg, 2012) to align reads to the
233 assembly of the Race 5 strain, and checked the regions where effectors were predicted to be absent in
234 IGV (Robinson et al., 2011). For some strains, certain effectors were not present in the assembly, while
235 we did find reads mapped to the gene in the Race 5 genome, which is indicated in **Figure S1**. We used
236 custom R scripts to plot the phylogeny with different *Avr* genotypes (**Figure 1**), and with a heatmap
237 with effector gene sequence similarity (**Figure S1**).

238 **Bioinformatics pipeline for BSA-sequencing by means of Comparative Subsequence Sets analysis**
239 **(CoSSA)**

240 A Comparative Subsequence Sets Analysis (CoSSA) bioinformatics pipeline was applied to find the
241 resistance-specific haplotype (Prodhomme et al., 2019). Sequence reads were trimmed and sequencing
242 adaptors were removed using fastp (version 0.23.4) (Chen, 2023) (**Table S5**). *K*-mer Counter (KMC) and
243 KMC tools (version 3.2.4) were used to produce *k*-mer (*k*=31) tables from the trimmed reads, and *k*-
244 mer table subtraction, intersection and coverage-based filtering (Kokot et al., 2017). GenomeScope 2.0
245 (Ranallo-Benavidez et al., 2020) was used to visualize the *k*-mer coverage profiles. The NR-bulk specific
246 *k*-mer table was subtracted from the R-bulk specific table, and a *k*-mer table of the susceptible parent

247 (MM-*Cf-0*) was subtracted from the resulting R-bulk specific *k*-mers, and intersected by a *k*-mer table
248 of the resistant parent (Ontario 7818). *K*-mer sequences with coverage outside a range of 26 to 43 were
249 removed to reduce error-to-noise ratio. Cutoff values are calculated as 25% margins of the expected
250 coverage of the *R* haplotype. Filtered *k*-mers were mapped to the SL4.00 tomato reference genome
251 (Hosmani et al., 2019) using BWA aln (version 0.7.18) (Li & Durbin, 2009) and plotted based on genomic
252 location and coverage. Used scripts reflect the workflow of (Prodhomme et al., 2019) and are available
253 on Github (https://github.com/PBR/CoSSA). Structural predictions of Cf-6 candidate proteins were
254 performed using predicted protein sequences from the ITAG4.0 annotation of the Heinz 1706 SL4.0
255 genome using InterPro (Paysan-Lafosse et al., 2023).

256 **Homolog searches and AlphaFold2 structural predictions**

257 The Basic Local Alignment Search Tool for nucleotide (BLASTn), translated nucleotide (tBLASTn), and
258 protein (BLASTp) were used to identify *Avr6-/*Avr6-like sequences in the National Centre for
259 Biotechnology Information (NCBI) and Joint Genome Institute (JGI) Mycocosm sequence databases.
260 (Laugé et al., 2000) Signal peptides were predicted using SignalP v4.1 (Nielsen, 2017). The ColabFold
261 (Mirdita et al., 2022) implementation of AlphaFold2 (Jumper et al., 2021) was used to predict the
262 tertiary structure of Avr6 (without its endogenous signal peptide) and was assisted through use of a
263 custom MSA generated with Avr6-like proteins, using Geneious v9.1.8 (Kearse et al., 2012). Foldseek
264 (Van Kempen et al., 2024) was used to identify structural homologs of Avr6 in filamentous fungi in the
265 RCSB Protein Data Bank. The predicted tertiary structure of Avr6, along with those of proteins identified
266 using Foldseek, were visualized, rendered, and aligned (CEalign plugin) using PyMOL (DeLano, 2002).

267 ***F. fulva* disease assays**

268 *F. fulva* strains were inoculated from glycerol stocks on PDA plates supplemented with 1.5% extra
269 technical agar and 33 mg/L chloramphenicol and grown for approximately 2–3 weeks at room
270 temperature in the dark. Then, conidia were harvested and suspended in tap water to a concentration
271 of approximately $1x10^6$ conidia/mL. This suspension was then spray-inoculated on the abaxial side of
272 3- to 4-week-old tomato plants which were subsequently kept under high (90–100%) humidity in a
273 plastic tent for 3 days, after which the tent was opened. Plants were maintained in a greenhouse
274 compartment set to 21–23°C. Disease symptoms were scored at 14 days post-inoculation (dpi).

275 **PVX-mediated expression assays**

276 Systemic PVX-based expression of Avr effectors was performed by means of agroinfiltration of the
277 cotyledons of 10-day (for the validation of Avr6) or 16-day (for BSA-sequencing of *Cf-6*) old tomato
278 seedlings, as previously described by Mesarich et al. (2018) and de la Rosa & Schol et al. (2024). The

279 *Agrobacterium* strain GV3101 carrying *pSfinx::Ecp5* was previously generated by Laugé et al. (2000) and
280 the strain carrying *pSfinx::Avr9* by Hammond-Kosack et al. (1995). Symptoms were scored at 10 dpi.

281 **_F. fulva_ gene complementation**

282 Complementation of strains with the wild-type *Avr6* gene was performed by means of *Agrobacterium*-
283 mediated transformation using the same method and construct as described in (de la Rosa & Schol et
284 al., 2024).

285 **_Avr6_ allelic variation analysis**

286 *Avr6* was amplified from the genomic DNA of 90 *F. fulva* strains using primers Avr6 Fw2 (5'-
287 CTGACATAGCATTACACGAGCAG-3') and Avr6 Rev (5'-CGCGTCGCCTGATAGATTTG-3) by PCR, as previously
288 described for the *Avr9B* gene (de la Rosa & Schol et al., 2024). Amplicons were purified using ExoSAP-
289 IT™ PCR Product Cleanup Reagent (Thermo Scientific) and sequenced by Macrogen™ Europe
290 (https://www.macrogeneurope.com) by means of Sanger Sequencing using an ABI 3730xl DNA analyzer
291 (Applied Biosystems). Consensus sequences were generated and imported in Benchling (Biology
292 Software, https://benchling.com) for end-trimming and sequence alignment to the *Ecp5* coding
293 sequence (CLAFUR5_03423) from the *F. fulva* strain Race 5 reference genome (Zaccaron et al., 2022).

294

295 **Results**

296 **Deletion or mutation of _Extracellular protein 5_ (_Ecp5_) is associated with _Cf-6_-breaking _F. fulva_ strains**

297 As a starting point for identifying *Avr6*, we obtained 14 French *F. fulva* strains that were sampled from
298 diseased tomato plants in the French region of Brittany between 2017 and 2021, of which five were
299 reported to break *Cf-6,* and a strain from New Zealand that was reported to break *Cf-6* in 2012. We
300 confirmed that six of these strains indeed break *Cf-6*-mediated resistance with a disease assay on MM-
301 *Cf-0* and Ontario 7818 (*Cf-6*) tomato plants (**Figure S2**). To further expand the allelic diversity of
302 candidate effectors, we also obtained 11 additional *F. fulva* strains from diverse locations around the
303 world (Stergiopoulos et al., 2007). Subsequently, all 26 *F. fulva* strains were subjected to Illumina
304 sequencing to generate whole-genome assemblies (**Table S3**) and we compiled these with publicly
305 available *F. fulva* assemblies (**Table S4**) into a dataset of 32 genomes. Then, we inferred a phylogeny
306 based on a concatenated alignment of 3484 conserved, single-copy orthologs and found that the
307 strains fall into four distinct clades, with most (11) clustering into a single, closely related clade (**Figure**
308 **1**). Then, we inferred mating types for all strains in the dataset and found that 11 had *Mat1-1* (36.4%)
309 and 21 had *Mat1-2* (63.6%) at their mating type (MAT) locus. Further inspection of the distribution of

genotypes for known *Avr* genes *Avr2*, *Avr4*, *Avr4E*, *Avr5*, *Avr9* and *Avr9B* revealed that predicted mutations in Avr2 (p.del54-78, p.Tyr41fs*34, p.Lys23fs*42), Avr4 (p.Cys109*) and Avr4E (p.Phe82Leu and p.Met93Thr) occur in both mating types, which suggests occurrence of recombination and thus sexual reproduction within *F. fulva* populations.

Next, we queried these genomes to identify candidate genes for *Avr6*, i.e. putative effector genes that have non-synonymous mutations or are (partially) absent in all race 2.6.9 strains but not in others. Hypothesizing that *Avr6* should be an small apoplastic protein, we used BLAST to compare the sequences of a set of known and predicted *Ecp* genes from the *F. fulva* race 5 reference strain (Mesarich et al., 2018; Zaccaron et al., 2022) to our dataset, and searched for those that were absent or exclusively had one or more mutations in the race 2.6.9 strains (**Figure S1**). As previously reported/predicted, the *Ecp* genes, although displaying some minor sequence variation, are present in most or all strains (Mesarich et al., 2018; Stergiopoulos, de Kock, et al., 2007), with *Ecp24-2* and *Ecp5* being notable exceptions. Strikingly, we found that *Ecp5* (Laugé et al., 2000) was deleted in four of the six *Cf-6*-breaking strains (**Figure 1**, **Figure S1**). In these strains, we observed a deletion of approximately 18 kb on chromosome 2, resulting in the loss of *Ecp5* (*CLAFUR5_03423*) and three adjacent genes, i.e. *CLAFUR5_20157*, *CLAFUR5_03424*, and *CLAFUR5_03425* (**Figure S3**). However, these three genes are predicted to code for uncharacterized, non-secreted proteins and are therefore unlikely candidates for *Avr6*. The deletion of the 18 kb locus was likely mediated by transposable elements (TEs) that, as previously shown, surround *Ecp5* (de Wit et al., 2012). In the other two *Cf-6*-breaking strains, non-synonymous mutations in *Ecp5*, resulting in amino acid substitutions p.Tyr88His and p.Gly53Val, were observed. Strain IPO 2679, which was used for the identification of *Avr9B* (de la Rosa & Schol et al., 2024), was the only other strain showing sequence variation for *Ecp5*, resulting in a p.Cys30Tyr substitution. In all other strains, a wild-type *Ecp5* allele was present. For *Ecp24-2*, we did not observe a clear correlation with the ability to overcome *Cf-6* (**Figure S1**) and it was therefore omitted as a candidate for *Avr6*. Together, these results suggest that *Ecp5* is *Avr6*.

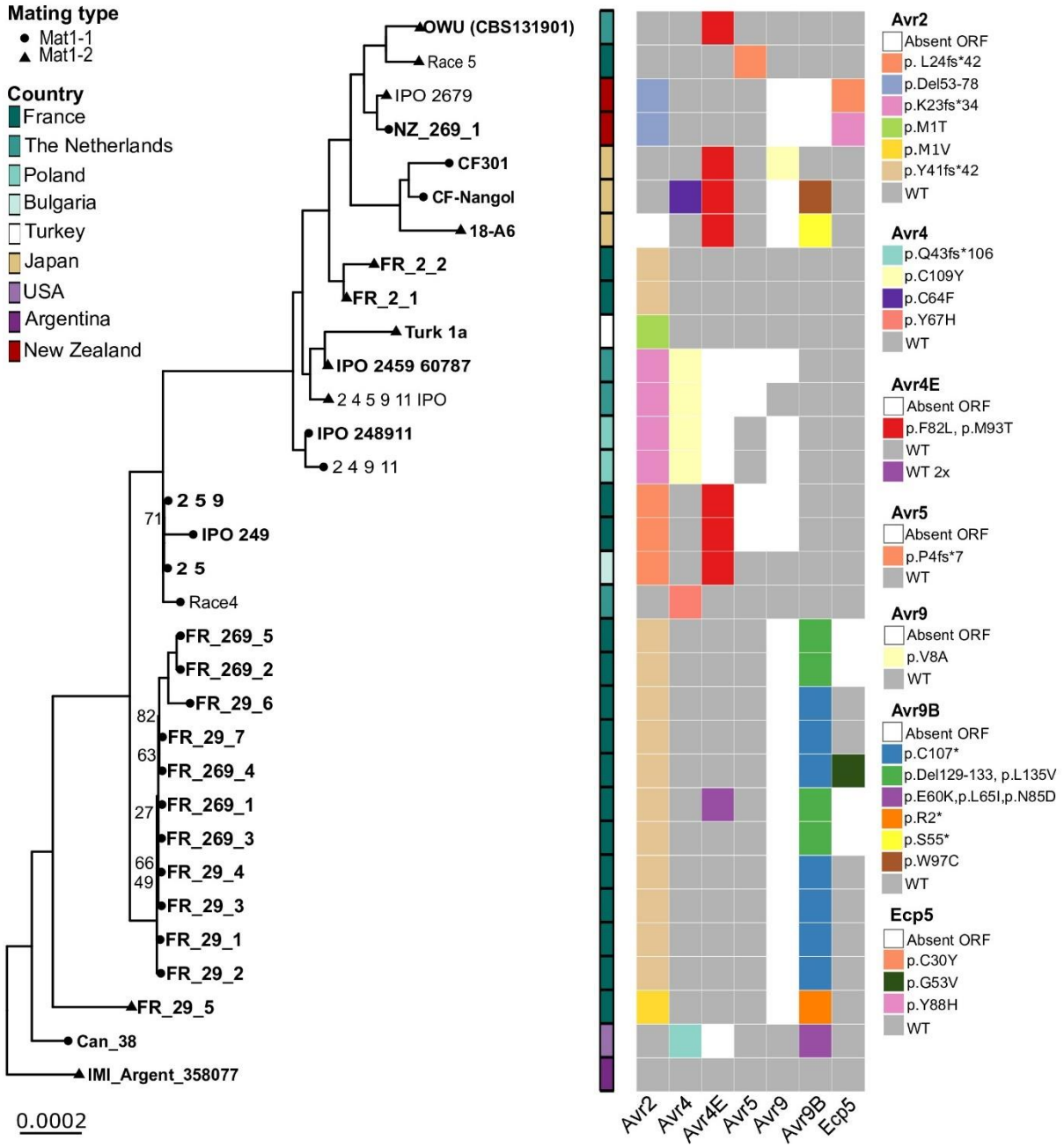


Figure 1: Phylogeny and predicted amino acid sequence comparisons of known Avrs, and Ecp5, across a panel of 32 *F. fulva* genomes.

Maximum-likelihood phylogeny inferred from a concatenated alignment of 3484 BUSCO genes, rooted with *Dothistroma septosporum* as outgroup (not shown in the tree). Only branches with bootstrap values < 95 are shown in the tree. Sequences generated for this study are shown in bold. Mating types are indicated with symbols at the tips of the tree (circles and triangles), and the country of origin is indicated with colored rectangles. Distinct predicted amino acid sequence mutations in known Avr proteins Avr2, Avr4, Avr4E, Avr5, Avr9 and Avr9B, and in Ecp5 are indicated with squares in different colors in the right panel, with wild-type (WT) sequences indicated in grey, and absence of an open

345 reading frame (ORF) indicated in white. p., protein sequence numbering; del, deletion; fs, frame shift;
346 *, stop codon.

347 **Molecular validation confirms that Ecp5 is Avr6**

348 To confirm that Ecp5 is Avr6, we systemically expressed it in *Cf-6* tomato (Ontario 7818) and *S.*
349 *lycopersicum* G1.1161, the line carrying the *Cf-Ecp5* locus that was mapped to the short arm of
350 chromosome 1 (Haanstra et al., 2000). Here, potato virus X (PVX)-based expression was employed,
351 whereby plants are transiently transformed with a recombinant version of PVX containing *Ecp5*
352 (PVX::*Ecp5*) using *Agrobacterium tumefaciens* (Haanstra et al., 2000; Laugé et al., 2000). As controls,
353 *Ecp5* was also expressed in MM-*Cf-0* plants that are universally susceptible to *F. fulva* and in MM-*Cf-9*
354 plants carrying the *Cf-9* locus. Concomitant with the systemic movement of the recombinant virus in
355 the plant, Ecp5 is produced systemically. Should Ecp5 be recognized by Cf-6, a systemic, visible HR,
356 resulting in chlorosis, necrosis, stunting, or death of the plant is expected to occur (Haanstra et al.,
357 2000; Laugé et al., 2000).

358 An HR developed in Ontario 7818 and G1.1161 plants, but not in MM-*Cf-0* and MM-*Cf-9* plants,
359 supporting that Ecp5 is Avr6 (**Figure 2**). To further validate that *Cf-6*-mediated resistance is solely based
360 on Ecp5 recognition, we inoculated MM-*Cf-0* and Ontario 7818 plants with the wild-type race 0 *F. fulva*
361 strain 0WU that produces Ecp5 (de Wit et al., 2012), and two independent *Ecp5* knockout strains in this
362 background, generated previously by Ökmen (2013). As expected, the wild-type 0WU strain was
363 virulent on MM-*Cf-0* plants, but avirulent on Ontario 7818 plants, whereas the two *Ecp5* knockout
364 strains both had gained virulence on Ontario 7818 plants, with symptoms visually indistinguishable
365 from those caused by the wild-type 0WU strain on *Cf-0* plants (**Figure 3**). This confirms that only Ecp5
366 is recognized by *Cf-6* tomato plants to mediate resistance to *F. fulva.* From here on, we will refer to
367 Ecp5 as Avr6.

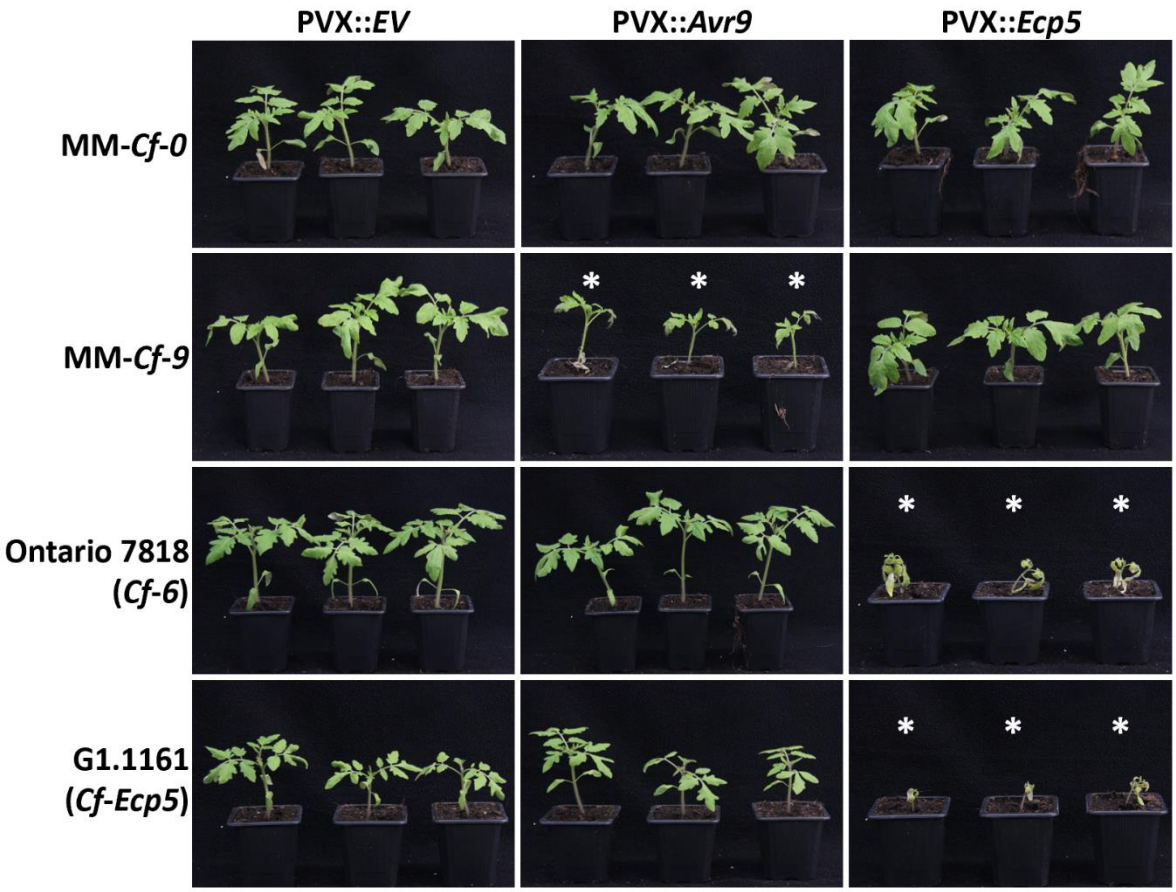


Figure 2: Ecp5 elicits a systemic HR in young tomato plants carrying *Cf-6* and *Cf-Ecp5*. Ecp5 was systemically expressed in seedlings of MM-*Cf-0* (no *Cf* genes), MM-*Cf-9* (*Cf-9B* and *Cf-9C*), Ontario 7818 (*Cf-6*), and G1.1161 (*Cf-Ecp5*), using the PVX-based expression system, with three replicates per tomato line. The recombinant virus PVX::*EV* (empty vector) was used as a negative control, while the recombinant virus PVX::*Avr9* was used as positive control. All recombinant viruses were delivered by *A. tumefaciens*-mediated transient transformation assays, through infiltration of the cotyledons of 10-day-old seedlings. Photographs were taken 10 days post agro-infiltration. White asterisks indicate plants undergoing systemic HR.

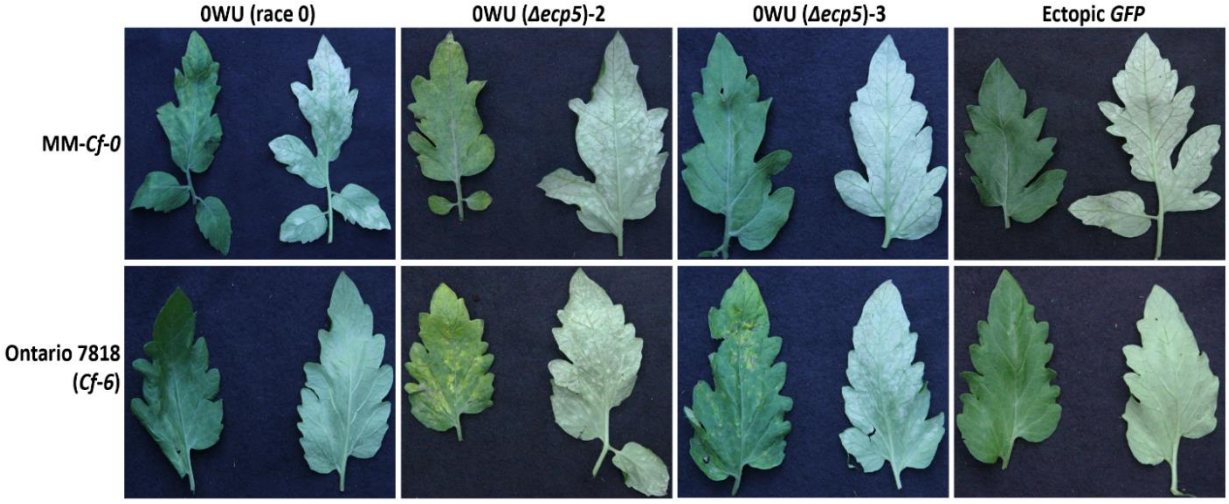
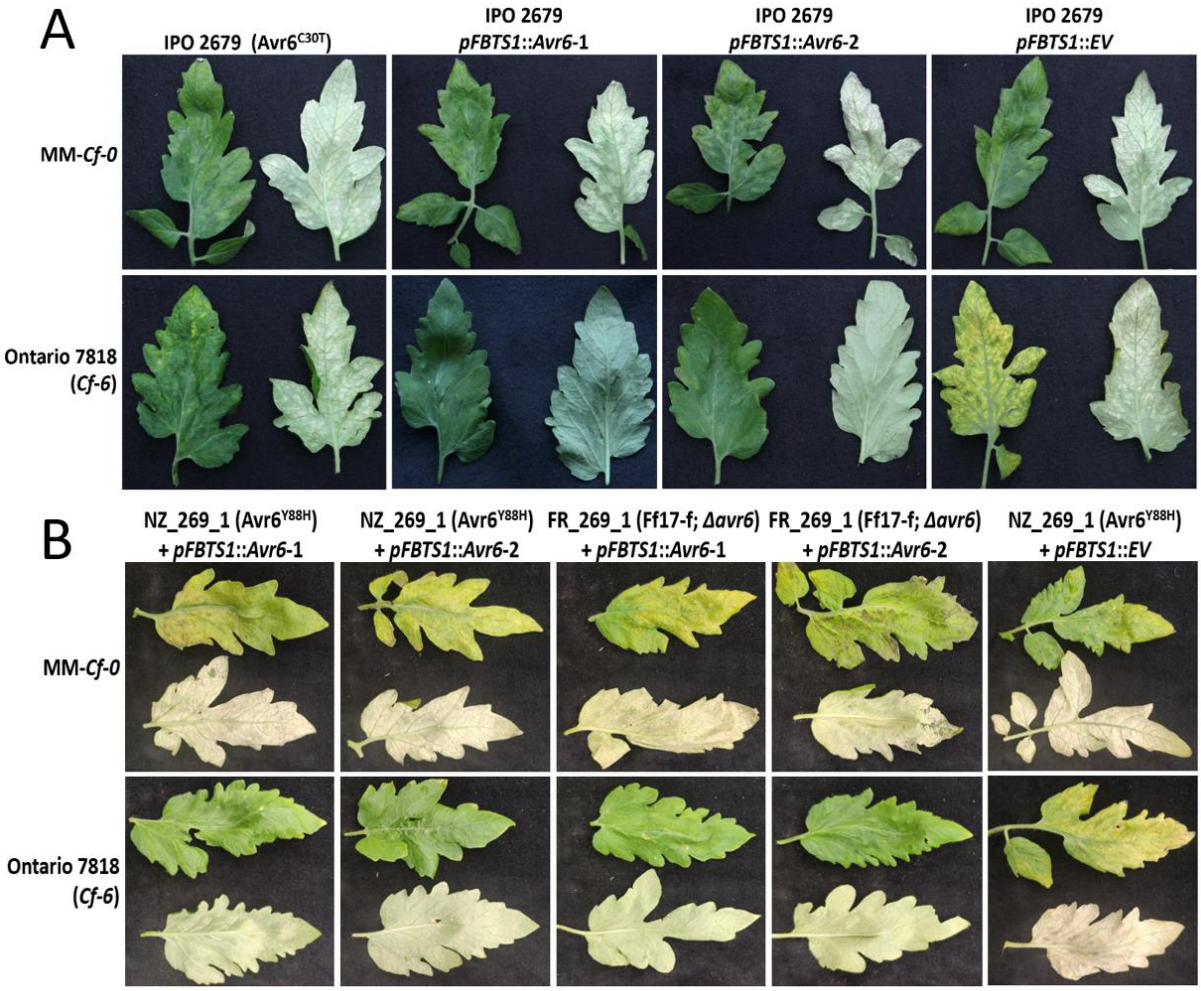


Figure 3: Deletion of *Ecp5* in the *F. fulva* race 0 strain 0WU confers virulence on Ontario 7818 (*Cf-6*) tomato plants. The 0WU wild-type strain, two independent *F. fulva Ecp5* knockout strains (*Δecp5*-2 and *Δecp5*-3) and an ectopic *green fluorescent protein* (*GFP*) transformant control were inoculated on 4-week-old Ontario 7818 (*Cf-6*) and MM-*Cf-0* (no *Cf* genes) tomato plants. The upper and lower surfaces of leaflets were photographed at 14 days post-inoculation.

Polymorphisms in *Avr6* allows *F. fulva* to overcome *Cf-6*-mediated resistance

Next, we validated that polymorphisms in Avr6 observed in race 2.6.9 strains allow circumvention of *Cf-6*-mediated resistance. *F. fulva* strain IPO 2679 carries a p.Cys30Tyr mutation in Avr6, which we hypothesized would allow it to break *Cf-6*-mediated resistance. To test this, we inoculated transgenic IPO 2679 strains complemented with the wild-type *Avr6* gene from strain 0WU, generated by de la Rosa & Schol et al. (2024), onto MM-*Cf-0* and Ontario 7818 (*Cf-6*) plants. We observed that the wild-type IPO 2679 strain is virulent on both lines, whereas the two complemented transformants have lost the ability to infect Ontario 7818 (*Cf-6*), but not MM-*Cf-0* plants (**Figure 4A**). Similarly, when strains FR_269_1 (del*Avr6*) and NZ_269_1 (p.Tyr88His) were complemented with wild-type *Avr6*, virulence was abolished on *Cf-6* plants but not on MM-*Cf-0* (**Figure 4B**). We conclude that these three mutations in Avr6 each allow the fungus to break *Cf-6*-mediated resistance. This contrasts with previous results, in which no allelic variation had been described in *Avr6* across a large core collection of *F. fulva* strains (Iida et al., 2015; Stergiopoulos, de Kock, et al., 2007). To determine whether there is evidence for further selection pressure on *Avr6* in response to *Cf-6* deployment, we sequenced *Avr6* in 155 additional *F. fulva* strains that were predominantly collected over the past two decades and mostly originate from Europe and Japan. A wild-type *Avr6* gene was present in almost all strains (**Table S1**), suggesting that these are unable to cause disease in *Cf-6* plants. Only in one recently collected German strain (P3), we identified a predicted splice site mutation at the end of the second intron of *Avr6* which resulted in (partial) restoration of virulence on *Cf-6* plants (**Figure S4**). Although the observed

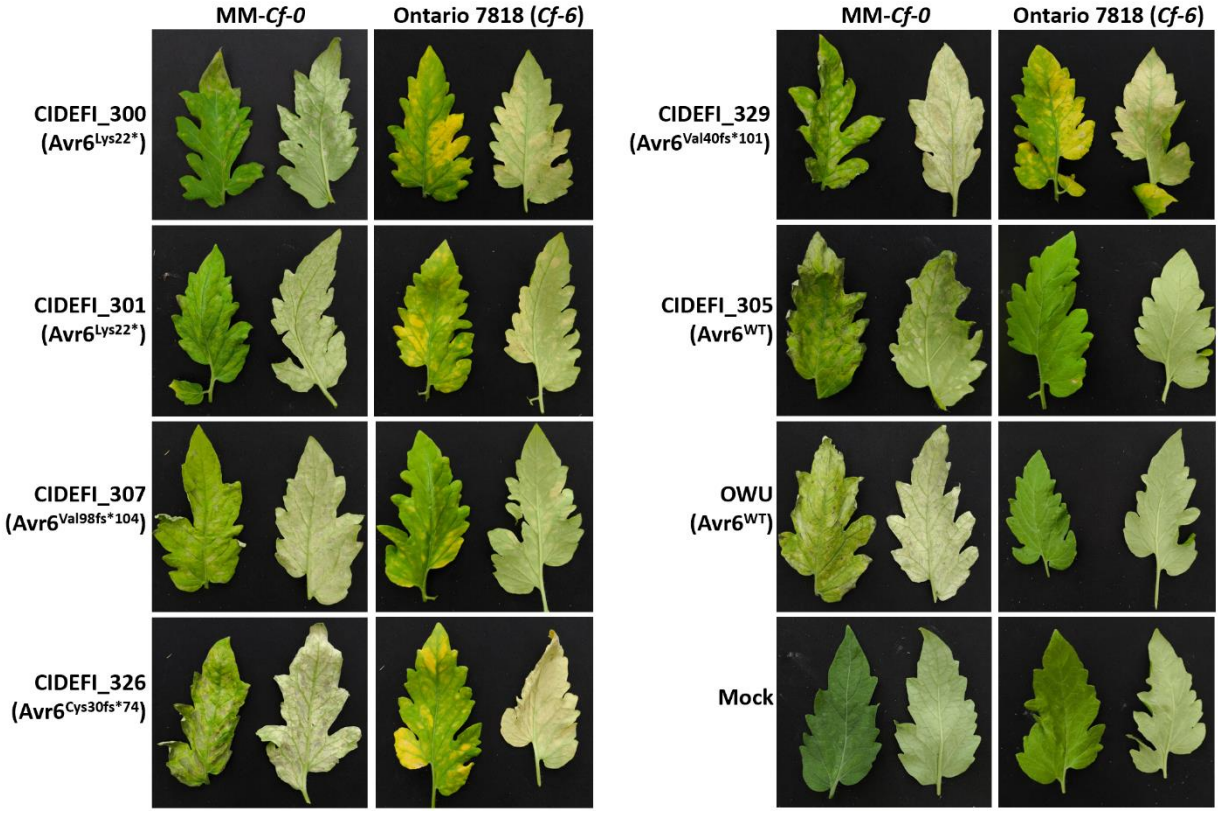
402 symptoms were not as pronounced as on MM-*Cf-0*, they were more severe compared to the symptoms
403 caused by strain P30, which has a wild-type *Avr6* gene, indicating that this mutation impairs the
404 recognition of Avr6 by Cf-6. This partial restoration of virulence is anticipated to be due to a reduction
405 in the amount of correctly spliced *Avr6* transcript and, thus, Avr6 protein available for recognition by
406 Cf-6. In all 154 other strains, we found a wild-type *Avr6* gene, suggesting that *Cf-6*-breaking strains
407 recently emerged.



408

409 **Figure 4: Complementation of *Cf-6*-breaking *F. fulva* strains with wild-type *Avr6* from strain 0WU**
410 **results in loss of virulence on Ontario 7818 (*Cf-6*) tomato plants.** The IPO 2679 (Avr6, p.Cys30Tyr) wild-
411 type strain, two independently generated IPO 2679 *Avr6*-complemented *F. fulva* (*Avr6*-1 and *Avr6*-2)
412 transformants, and an empty vector (EV) control transformant were inoculated on 4-week-old Ontario
413 7818 (*Cf-6*) and MM-*Cf-0* (no *Cf* genes) tomato plants (Panel A). Two independently generated
414 NZ_269_1 and FR_269_1 *Avr6*-complemented *F. fulva* (*Avr6*-1 and *Avr6*-2) transformants, and an EV
415 control, were inoculated on 4-week-old Ontario 7818 (*Cf-6*) and MM-*Cf-0* (no *Cf* genes) tomato plants
416 (Panel B). Leaflets were photographed at 14 days post-inoculation.

417 Recently, however, non-synonymous and frame-shift mutations were reported in *Avr6* of several *F. fulva*
418 strains collected in Argentina (Lucentini et al., 2021; Medina et al., 2015). To investigate whether these
419 mutations also result in virulence on *Cf-6* tomato, we inoculated strains expressing Avr6 carrying the
420 mutations p.Lys22*, p.Cys30fs*74, p.Val40fs*101 and p.Val98fs*104 (**Table S1**) onto MM-*Cf-0* and
421 Ontario 7818 plants. This resulted in visible disease symptoms on both genotypes for strains harboring
422 these mutant alleles, but not for the control strains 0WU or CIDEFI 305, which both contain wild-type
423 *Avr6* and are therefore unable to overcome *Cf-6* (**Figure 5**). As all mutations are predicted to result in
424 either frameshifts or cysteine mutations, it is not surprising that recognition by Cf-6 is abolished.
425 Unfortunately, we could not test strain CIDEFI 318 (p.Cys48Tyr) due to its poor growth and sporulation
426 *in vitro*, but it is likely that the p.Cys48Tyr mutation also abolishes recognition. In all, apart from
427 deletion of the *Avr6* ORF and the splice site mutation in P31, we identified eight different non-
428 synonymous and frame-shift mutations in *Avr6* across strains (**Figure 6**), of which seven were confirmed
429 to result in breakdown of *Cf-6* by *F. fulva*.

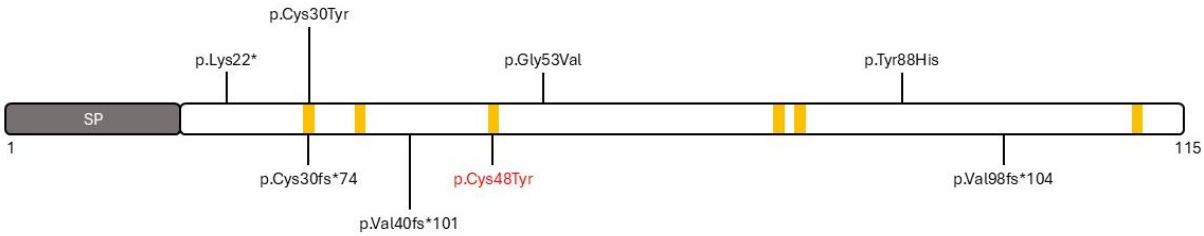


430

431 **Figure 5**: **Non-synonymous and frame-shift mutations in *Avr6* of Argentinian *F. fulva* strains confer**
432 **virulence on *Cf-6* tomato plants**. Four-week-old MM-*Cf-0* (no *Cf* genes) and Ontario 7818 (*Cf-6*) tomato
433 plants were inoculated with several of the Argentinian *F. fulva* strains reported by Medina et al. (2015)
434 and Lucentini et al. (2021) to harbor mutations in *Avr6* (race 2.6 (initially reported as race 2): CIDEFI
435 300, 301, 307 and 329; race 6 (initially reported as race 0): CIDEFI 326). As controls, strain 0WU (race

436 0) and CIDEFI 305 (race 2), which can infect MM-*Cf-0* but not *Cf-6* plants, were also inoculated.
437 Symptoms were photographed at 14 days post-inoculation.

438



439

440 **Figure 6: Schematic representation of the Avr6 protein showing the location of mutations that allow**
441 ***F. fulva* to overcome *Cf-6*-mediated resistance**. The signal peptide (SP) is indicated in grey and yellow
442 bars indicate the positions of cysteine residues. The strain carrying the predicted p.Cys48Tyr mutation
443 (in red font) could not be tested.

444 **Many plant-pathogenic Dothideomycete and Sordariomycete fungi are predicted to produce an Avr6-**
445 **like protein**

446 To investigate whether other fungi produce Avr6-like proteins, BLAST searches were performed against
447 fungal genomes (tBLASTn) and their associated protein catalogues (BLASTp) in publicly available
448 sequence databases using *F. fulva* Avr6 as a query. This resulted in the identification of several Avr6-
449 like proteins, all from plant-pathogenic Dothideomycetes and Sordariomycetes (**Figure S5**). One such
450 protein was identified in *Pseudocercospora fuligena* (**Figure S5**), a Dothideomycete responsible for
451 black leaf mold disease of tomato (Phengsintham et al., 1938; Zaccaron and Stergiopoulos, 2020). All
452 identified Avr6-like proteins were predicted to possess an N-terminal signal peptide for extracellular
453 targeting (**Notes S1**). Furthermore, based on a sequence alignment, the six cysteine residues present
454 in Avr6 were conserved in all Avr6-like proteins (**Figure S5**). Aside from these conserved cysteine
455 residues, several other amino acid residues were highly conserved, including Tyr88 and Gly53, which
456 were mutated in *F. fulva* strains NZ_269_1 (p.Tyr88His) and FR_269_4 (p.Gly53Val), indicating that they
457 may play a crucial role in the overall structure of Avr6 and/or its role in virulence (**Figure S5**).

458 **The predicted tertiary structure of Avr6 shows similarity to Leptosphaeria AviRulence and**
459 **Suppressing (LARS) effectors**

460 To gain insight into possible functions and constraints on Avr6, we predicted its tertiary structure
461 (without the signal peptide) using AlphaFold2 and compared it to experimentally determined fungal
462 structures in the RCSB Protein Data Bank using Foldseek. The predicted structure consists of six β-
463 strands and one α-helix, stabilized by three putative disulfide bonds (Cys13-Cys18, Cys31-Cys59 and

Cys61-Cys94), consistent with a compact, cysteine-rich fold (**Figure 7**). One predicted disulfide bond (Cys61–Cys94) was not explicitly called by AlphaFold2 but is strongly suggested by the close spatial proximity of the two residues. Mapping the positions of the Gly53 and Tyr88 substitutions found in *Cf-6*-breaking strains indicates that Gly53 lies in a surface-exposed loop linking the sole α-helix to β-strand 2, whereas Tyr88 is predicted to be buried, suggesting that these substitutions are likely to affect protein stability and/or interaction surfaces in distinct ways.

Foldseek analysis identified the Leptosphaeria AviRulence and Suppressing (LARS) effectors AvrLm4-7 (PDB ID: 4FPR) and AvrLm5-9 (PDB ID: 7B76) from *Leptosphaeria maculans* (Blondeau et al., 2015; Lazar et al., 2022), as the closest structural matches to Avr6, with moderate similarity (TM-scores ~0.55) (**Figure 7**). These proteins share an overall β-sheet–rich architecture but differ in elongation and loop topology, and exhibit limited primary sequence similarity. Thus, Avr6 appears to adopt a LARS-like fold, although it forms a distinct structural variant within this effector family. The biological functions and host targets of AvrLm4-7 and AvrLm5-9 remain unknown, and further biochemical work will be needed to determine whether Avr6 shares mechanistic features with these effectors or uses this fold for entirely different virulence activities.

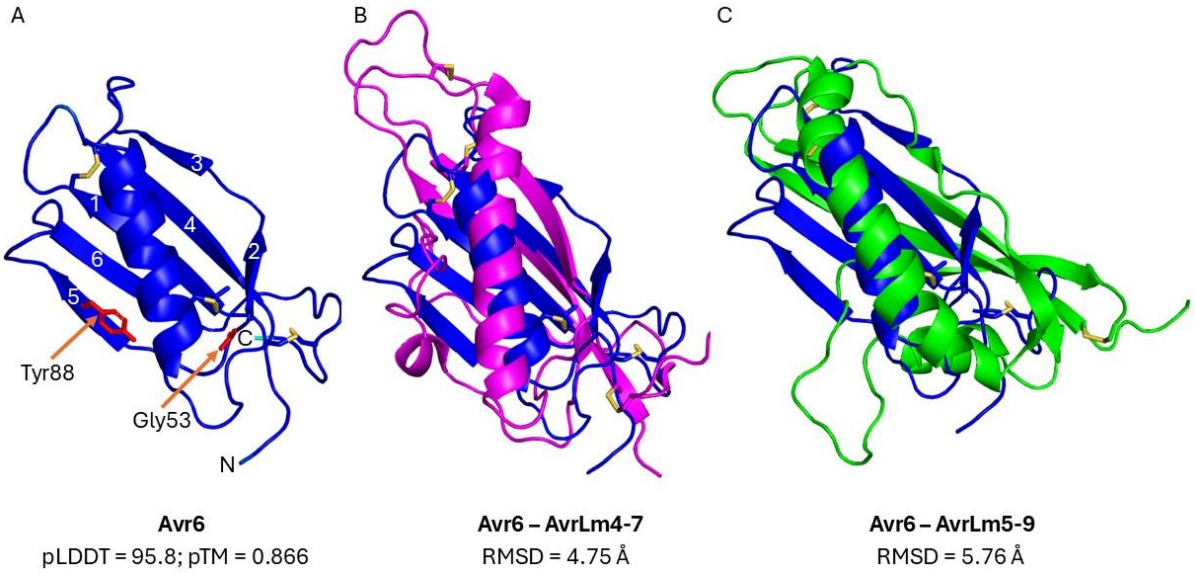


Figure 7: Avr6 is predicted to adopt a LARS-like fold. A. Predicted tertiary structure of Avr6. The tertiary structure is colored according to the predicted Local Distance Difference Test (pLDDT) score (confidence: cyan = high; dark blue = very high). Gly53 and Tyr88 are shown as sticks and are colored red. β-strands are numbered sequentially. Predicted disulfide bonds are colored yellow. N- and C-termini are shown. **B.** Alignment of Avr6 (blue) with AvrLm4-7 (magenta; RCSB Protein Data Bank (PDB) ID: 4FPR) from *L. maculans.* **C.** Alignment of Avr6 (blue) with AvrLm5-9 (green; PDB ID: 7B76) from *L. maculans*. pTM: predicted Template Modelling score. RMSD: Root Mean Square Deviation.

487 **Bulked segregant analysis maps *Cf-6* in Ontario 7818 to a 2 Mb interval on the short arm of**
488 **chromosome 12**

489 Currently, three distinct loci originating from different *S. pimpinellifolium* accessions that mediate a
490 gene-for-gene relationship with *Avr6* have been reported, i.e. *Cf-Ecp5.1* on the short arm of
491 chromosome 1 (Haanstra et al., 2000; Iakovidis et al., 2020), *Cf-Ecp5.7* on the long arm of chromosome
492 7, and *Cf-Ecp5.12* on the short arm of chromosome 12 (Iakovidis et al., 2020). To validate the location
493 of *Cf-6* on the short arm of chromosome 12 in Ontario 7818 as reported by Kanwar et al. (1980), we
494 applied a Bulked Segregant Analysis (BSA)-sequencing approach in an F2 population derived from a
495 cross between Ontario 7818 (*Cf-6*) and MM-*Cf-0*. For this, 133 F2 seedlings were subjected to systemic
496 PVX-based expression of *Avr6* and subsequently phenotyped based on presence or absence of a
497 systemic HR (**Figure S6**). gDNA from 88 (out of 92) plants that underwent a systemic HR, indicating
498 Avr6-responsiveness, was bulked into a responsive (R) pool, and from 40 (of 41) non-responders into a
499 non-responsive (NR) pool. The expected 3R:1NR segregation pattern, under the hypothesis of *Cf-6*
500 being a single dominant gene, was confirmed with a χ^2 test (χ^2=2.41, *p*-value=0.12) (**Table S6**). After
501 short-read sequencing of the pools, we applied a Comparative Subsequence Sets analysis (CoSSa)
502 bioinformatics pipeline (Prodhomme et al., 2019), to identify R-bulk-specific *k*-mers, which were
503 subsequently mapped to the *S. lycopersicum* Heinz SL4.0 reference genome (Hosmani et al., 2019). We
504 identified a small 2 Mb interval spanning from approximately 2.2 Mb to 4.2 Mb on the short arm of
505 chromosome 12 that was significantly enriched for R-bulk-specific *k*-mers, indicating the presence of
506 *Cf-6* (**Figure 8**). Potential resistance gene-encoding candidates for *Cf-6* within this region include eight
507 RLP-encoding genes, divided over two distinct clusters, and two nucleotide-binding leucine-rich repeat
508 (NB-LRR)-encoding genes. All these genes show significant allelic variation in Ontario 7818 compared
509 to the Heinz 1706 SL4.0 reference and MM-*Cf-0*, indicating that they could be involved in recognition
510 of Avr6 in Ontario 7818 (**Figure S7**). The region coincides with previous findings mapping *Cf-6* to the
511 short arm of chromosome 12 (Kanwar et al., 1980), and the later finding for the two *Cf-Ecp5.12* alleles
512 (Iakovidis et al., 2020).

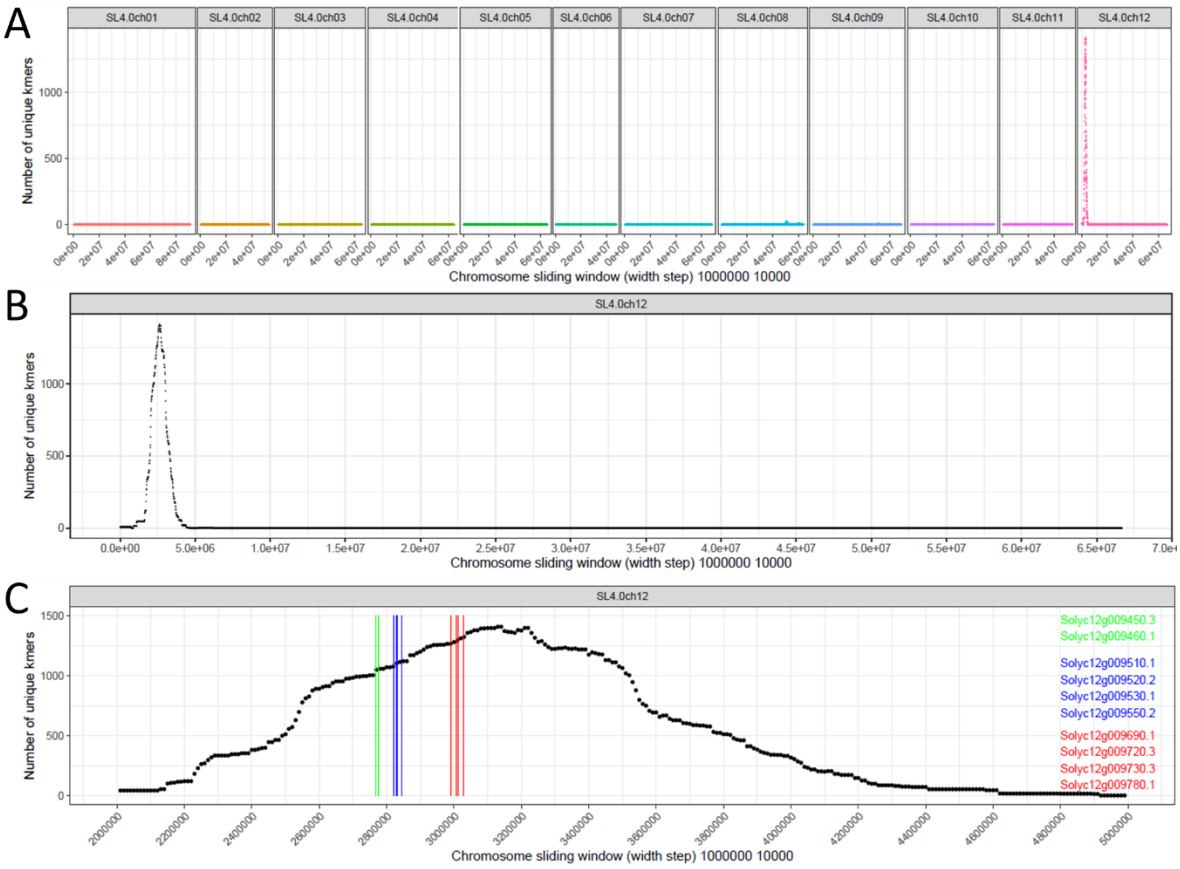


Figure 8: BSA-mapping of *Cf-6* (based on responsiveness to Avr6) in an Ontario 7818 x Moneymaker F2 population using Comparative Subsequence Sets analysis (CoSSa). Plots show R-pool-specific *k*-mers (resulting from intersection with Ontario 7818 specific *k-mers* and subtraction of NR-pool and MM-*Cf-0* specific *k*-mers), with respect to their position on the *S. lycopersicum* Heinz 1706 SL4.0 reference genome (Panel A). We found a strong correlation with a region spanning 2.2 to 4.2 Mb on the short arm of chromosome 12 (Panel B). One cluster containing two candidate NB-LRR-encoding genes (green lines) and two clusters both containing four RLP-encoding genes (blue and red lines, respectively) within this mapping interval are indicated (Panel C).

To determine which of these genes could be *Cf-6*, we predicted the domain structure of all ten encoded candidate Cf-6 proteins based on their predicted protein sequences from the Heinz 1706 ITAG4.0 reference annotation (**Figure S8, Tables S7 and S8**). Both NB-LRR proteins are predicted to encode for coiled-coil(CC)-NB-LRRs, whilst of the eight RLPs, only Solyc12g009510 and Solyc12g009520 are predicted to harbor an N-terminal SP for secretion, followed by an LRR domain and a transmembrane domain (TM), features that are deemed essential for RLP functionality. Interestingly, Solyc12g009510 is predicted to harbor a cytoplasmic LRR domain with a second C-terminal TM. The other RLPs do not show a complete predicted domain organization based on the reference, but because the structures of these proteins in Ontario 7818 remain unknown, they all remain candidates for Cf-6.

531 Finally, to verify the correlation between Avr6 recognition and resistance to *F. fulva* in the F2
532 population, we inoculated 52 new individuals from the same F2 population with PVX::*Avr6*, and
533 inoculated cuttings of these individuals with the *F. fulva* race 0 strain 0WU (**Figure S9**). For every
534 individual, we found a strict co-segregation of response to Avr6 with resistance to *F. fulva*, and of non-
535 responsiveness with susceptibility. Again, we observed a 3R:1NR (40 R, 12 NR) segregation of the
536 response to Avr6 and of disease resistance (χ^2=0.103, *p*-value=0.75) (**Table S6**). This indicates that *Cf-6*
537 is the only resistance gene providing resistance to *F. fulva* in the Ontario 7818 F2 population and further
538 confirms that this gene confers resistance to *F. fulva* upon recognition of Avr6.

539

540 **Discussion**

541 **Parallel loss and mutation of *Avr6* highlight repeatable paths to virulence**

542 Avr6 encodes a 115-amino acid protein of unknown function that was originally cloned as Ecp5 (Laugé
543 et al., 2000). Removal of its 17-amino acid N-terminal signal peptide for secretion yields a 98-amino
544 acid mature apoplastic protein containing six cysteine residues. Avr6 expression is strongly induced *in*
545 *planta* relative to expression in culture (Mesarich et al., 2014). Consistent with earlier reports (Iida et
546 al., 2015; Stergiopoulos, de Kock, et al., 2007), we observed virtually no allelic variation in *Avr6* across
547 a panel of recently collected Japanese strains and in all but four French race 2.6.9 strains and one
548 additional European strain (P31). This pattern suggests that *Avr6* has largely evolved under purifying
549 selection, with positive selection only recently imposed upon its deployment as an Avr determinant
550 through *Cf-6*. As *Cf-6* becomes more widely deployed, it is reasonable to expect that additional
551 virulence alleles of *Avr6* will emerge. The p.Cys30Tyr mutation in Avr6 of strain IPO 2679 is puzzling
552 because this strain was collected in New Zealand in the 1980s (de la Rosa & Schol et al. 2024), when
553 *Cf-6* is not known to have been commercially deployed. One possibility is that this mutation arose in a
554 different genetic background or host context and only later encountered *Cf-6*. Interestingly, a previous
555 study reported that an Avr6 p.Cys30Ala substitution (p.Cys13Ala in the mature protein) did not
556 compromise the HR in the *Cf-Ecp5* donor line G1.1161 (Luderer et al., 2002). This difference suggests
557 either that the Cf-Ecp5 receptor encoded by the G1.1161 chromosome 1 allele and Cf-6 encoded by
558 the Ontario 7818 chromosome 12 allele differ in specificity, or that replacing Cys30 with a bulky, polar
559 tyrosine has a more pronounced structural effect than substitution with a small, hydrophobic alanine.
560 Distinguishing between these scenarios will require direct comparison of *Cf-Ecp5* alleles and their
561 recognition specificities.

562 The diversity of polymorphisms in *Avr6* reported in Argentinian strains (Lucentini et al., 2021; Medina
563 et al., 2015) raises questions about the origin of selection on this effector, because the sampled tomato

564 cultivars are unlikely to harbor *Cf-6*/*Cf-Ecp5* alleles, given their susceptibility to strains expressing wild-
565 type *Avr6*. Nevertheless, both *Ecp4* and *Avr6* were inferred to be under positive selection in the
566 Argentinian *F. fulva* population, despite modest overall nucleotide diversity (Lucentini et al., 2021). One
567 possible explanation is that selection on *Avr6* originated in wild tomato populations, where Cf-Ecp5-
568 like receptors are common and widespread (Iakovidis et al., 2020), with subsequent spill-over of
569 adapted *F. fulva* strains into cultivated tomato. A similar scenario could apply to IPO 2679, which may
570 have acquired the p.Cys30Tyr mutation in a South American population before moving to New Zealand.
571 However, given the current sampling bias towards cultivated tomato, we cannot rule out alternative
572 explanations such as drift, founder effects, or selection by other, as yet unidentified host factors. A
573 targeted sampling campaign of *F. fulva* and wild hosts in centres of tomato diversity, followed by
574 genome- and population-level analyses (Bartoli & Roux, 2017; Lebeda & Burdon, 2023), will be required
575 to clarify the evolutionary origin of *Avr6* variation.

576 **Stacked *Cf* genes can potentially be overcome by recombination between strains carrying different**
577 **mutant *Avr* genes**

578 Our data indicate that closely related *F. fulva* lineages can independently acquire virulence against
579 different *Cf* genes via distinct mutations in multiple *Avr* genes. In particular, the clades comprising five
580 race 2.6.9 and six race 2.9 strains appear to have overcome both *Cf-6* and *Cf-9B* through different
581 mutations in *Avr6* and *Avr9B*: four race 2.6.9 strains carry a deletion of the *Avr6* open reading frame
582 combined with Avr9B p.del129–133,p.L135V, the race 2.9 strains have a wild-type *Avr6* and Avr9B
583 p.Cys107*, and one race 2.6.9 strain has Avr6 p.Gly53Val together with Avr9B p.Cys107* (**Figure 1**).
584 These observations show that single *Cf* gene-mediated resistances can be eroded by multiple,
585 independent effector mutations within relatively few fungal generations.

586 We also observe identical Avr mutations in strains with opposite mating types, including Avr2
587 p.Tyr41fs42, Avr2 p.Lys23fs34, Avr2 p.Asp54–Gly78del, Avr4 p.Cys109* and Avr4E p.Phe82Leu /
588 p.Met93Thr. Similar patterns have been reported in a Turkish *F. fulva* population, where Avr alleles and
589 both mating types occur at roughly equal frequencies (Gül et al., 2022; Gül et al., 2024). The most
590 parsimonious explanation for these observations is that recombination occurs within *F. fulva*
591 populations, consistent with earlier suggestions of cryptic sexual reproduction (Stergiopoulos, de Kock,
592 et al., 2007; Stergiopoulos, Groenewald, et al., 2007; de la Rosa & Schol et al., 2024). Although we
593 cannot yet formally distinguish sexual from parasexual recombination, either mechanism would
594 facilitate the accumulation of multiple effector mutations in single genotypes and thereby increase the
595 likelihood that *Cf* gene stacks are overcome over time. Nonetheless, the French strains we sequenced
596 appear largely clonal: most cluster into a single, tight phylogenetic branch and show no evidence of

597 recombination over the timeframe sampled. This suggests that, while recombination likely occurs in
598 the broader species, it may be infrequent or episodic, and not necessarily detectable in all regional
599 populations.

600 **Avr6 has sequence and structural similarity to putative effectors of other plant-pathogenic fungi**

601 Sequence queries suggest that many plant-pathogenic Dothideomycete and Sordariomycete fungi
602 produce one or more Avr6-like proteins, of which one, *Pf*Avr6, was identified in *P. fuligena*, a pathogen
603 closely related to *F. fulva* that causes black leaf mold of tomato (Phengsintham et al., 1938). Given that
604 the mature version of *Pf*Avr6 has a high level of sequence identity to the mature version of Avr6
605 (~71.4%), including Gly53 and Tyr88 (**Figure S5**), and that there is evidence of resistance to *P. fuligena*
606 in cultivated and wild tomato (Hartman & Wang, 1993; Wang et al., 1995), it would be interesting to
607 determine if it is recognized by *Cf-6* tomato plants. If so, *Cf-6* could also provide protection against *P.*
608 *fuligena*. However, the emergence of *Cf-6*-breaking *F. fulva* strains suggests that any such protection
609 will likely be short-lived as *P. fuligena* may adapt in a similar way. Searches based on the predicted
610 tertiary structure of Avr6 revealed similarity to LARS effectors, which have so far been identified in at
611 least 13 plant-pathogenic fungi (Lazar et al., 2022). Examples include four Avr effectors from *L.*
612 *maculans*, as well as *F. fulva* Ecp11-1 (Lazar et al., 2022; Mesarich et al., 2018). In the *L. maculans*–
613 *Brassica* pathosystem, recognition of the LARS effectors AvrLm3 and AvrLm5-9 by their corresponding
614 R proteins can be masked by another LARS effector, AvrLm4-7 (Ghanbarnia et al., 2018; Plissonneau et
615 al., 2016). However, it is not yet known whether Avr6 (or Ecp11-1) has the capacity to mask the
616 recognition of other *F. fulva* effectors in susceptible tomato plants. The predicted tertiary structure of
617 Avr6 displays several notable differences to the LARS effectors described above, reflected in a lack of
618 sequence similarity. LARS-like effectors lacking such similarity or possessing subtle differences in their
619 predicted tertiary folds have been identified in other plant-pathogenic fungi including in *Venturia*
620 *inaequalis*, which causes apple scab disease (Rocafort et al., 2022).

621 ***Cf-6* maps to the chromosome 12 allele of *Cf-Ecp5* at the *ANDROMEDA* locus**

622 Initially, an HR was observed upon elicitation with the then-called Ecp5 protein (Laugé et al., 2000) in
623 the introgression line G1.1161, an F_3BC_1 resulting from a cross between *S. pimpinellifolium* CGN15529
624 and MM-*Cf-0* (Haanstra et al., 2000). The *Cf-Ecp5* gene in this accession was mapped to the *AURORA*
625 (*AU*) locus on the short arm of chromosome 1 (Haanstra et al., 2000), but a later study found that
626 rather, it was tightly linked with *Cf-9* at the *MW* locus (Iakovidis et al., 2020). This study also reported
627 two other independent *Cf-Ecp5* loci, i.e. *Cf-Ecp5.7* at the *CENTAURI* (*CE*) locus on the long arm of
628 chromosome 7 in *S. pimpinellifolium* LA0722, and *Cf-Ecp5.12* at the *ANDROMEDA* (*AN*) locus located
629 within a region on the short arm of chromosome 12 spanning from approximately 2.1 to 3.0 Mb in *S.*

630 *lycopersicum* Ontario 7522 and *S. pimpinellifolium* LA2852 (Iakovidis et al., 2020). Our BSA–CoSSa
631 mapping of *Cf-6* in Ontario 7818 to a ~2 Mb interval on the short arm of chromosome 12 that overlaps
632 the *Cf-Ecp5.12* mapping interval strongly suggests that *Cf-6* and *Cf-Ecp5.12* represent alleles of the
633 same underlying gene, although allelic diversification between Ontario 7818 and the other *Cf-Ecp5.12*
634 donors cannot yet be excluded.

635 The study of Iakovidis *et al*. (2020) employed a marker at 3.014 Mb, which showed 4.5% recombination
636 with the *Cf-Ecp5.12* locus. Assuming that the *Cf-6* allele from Ontario 7818 and the *Cf-Ecp5* allele from
637 Ontario 7522 are the same and encode an RLP, it is more likely to reside within the "blue" RLP-encoding
638 gene cluster at 2.85 Mb, rather than at the "red" cluster at around 3.0 Mb (**Figure 8C**), which is roughly
639 at the same location as the mentioned marker of Iakovidis et al. (2020). Although originating from the
640 same breeding program, Ontario 7818 and 7522 do appear to have different donors, namely *S.*
641 *pimpinellifolium* PI 211839 and PI 124161, respectively. PI 124161 appears to originate from Guatemala
642 (**Table S2**), whilst the origin of PI 211839 is unknown. Since all three mapped *Cf-6/Cf-Ecp5* loci identified
643 to date originate from *S. pimpinellifolium*, it would be valuable to determine the genomic locations of
644 corresponding alleles in other tomato species such as *S. peruvianum* or *S. chilense*, as several
645 accessions from these species also respond to Avr6 (Iakovidis et al., 2020). Mapping these alleles may
646 reveal additional, distinct *Cf-6*/Cf-Ecp5 loci and thereby provide further insight into the evolution,
647 diversification, and geographic distribution of *Cf* genes across wild tomato species.

648 Finally, our results help resolve earlier conflicting reports on the chromosomal position of *Cf-6*: neither
649 chromosome 6 (Grushetskaya et al., 2007) nor chromosome 11 (Wang et al., 2007) appear to
650 contribute to Avr6 recognition. Instead, our data align with the more recent findings of Iakovidis et al.
651 (2020), which map *Cf-Ecp5.12*—now strongly supported to be allelic with *Cf-6*—to the short arm of
652 chromosome 12.

653 The identified candidate RLP-encoding genes within our mapping region in Ontario7818 are prime
654 candidates for *Cf-6*, particularly *Solyc12g009510* and *Solyc12g009520*, given their predicted complete
655 protein structures in Heinz 1706, although it is also possible that *Cf-6* of Ontario 7818 is not annotated
656 in the Heinz 1706 genome. This could be resolved by generating a *de novo* genome assembly of Ontario
657 7818, or by resistance gene enrichment sequencing (RENseq) (Lin et al., 2020; Witek et al., 2016),
658 revealing any structural differences that are often found across different accessions/species in RLP-
659 encoding genes (de Kock et al., 2005; Dixon et al., 1998; Dixon et al., 1996; Man et al., 2020). For
660 example, the recognition of Avr9 in wild *S. chilense* accessions is highly region-dependent and the *Cf-9*
661 locus is highly polymorphic amongst these accessions (Kahlon et al., 2020).

662 **Future strategies for developing novel, more durable resistance to tomato leaf mold**

663 Future breeding efforts should focus on achieving more durable resistance against *F. fulva*, as
664 breakdown of the recently deployed *Cf-6* gene appears to have occurred more rapidly than the
665 breakdown of the multi-gene *Cf-9* locus (de la Rosa & Schol et al., 2024), underscoring that single *Cf*
666 gene strategies are unlikely to be durable. Wild tomato germplasm that triggers specific HR responses
667 to one or more of the nine newly identified Ecp effectors (ten including Ecp17-1) (Mesarich et al., 2018)
668 likely contains an equivalent number of untapped *Cf-Ecp* genes that could be incorporated into
669 breeding programs. However, before deployment, the durability of such genes should be carefully
670 assessed. One promising approach is to generate *Ecp* knockout strains to determine their contribution
671 to *F. fulva* virulence, as recently demonstrated for *Ecp20-2* (Karimi-Jashni et al., 2022). The long-
672 standing assumption that Ecp effectors are more conserved—and therefore more essential for
673 virulence—than Avr effectors (Ökmen & de Wit, 2012; Stergiopoulos, de Kock, et al., 2007) is
674 challenged by our findings, which show that *Avr6*/*Ecp5* can be readily lost or mutated without
675 substantial fitness cost.

676 At present, pyramiding multiple *Cf* genes appears to be the most durable strategy, as sequential loss of
677 several effector genes is expected to occur far more slowly than loss of individual effectors, even when
678 sporadic recombination is considered. Nevertheless, truly durable resistance will require an innovative
679 approach, such as *Cf* genes matching effectors that are essential for pathogenicity, or the discovery of
680 susceptibility factors that can be targeted to remove essential host functions exploited by the pathogen
681 (Koseoglou et al., 2022; Sun KaiLe et al., 2014).

682 Progress toward durable resistance can be accelerated by comprehensive population-level studies of *F.*
683 *fulva* across its full geographic range, including wild host populations, to identify which effectors and
684 *Cf* genes are most constrained, which are most variable, and how rapidly new alleles arise. Such
685 ecological and evolutionary data should be integrated with pathogen–host interaction modelling (Susi
686 et al., 2020; Märkle et al., 2024; McMullan et al., 2025), enabling predictions of which *Cf* genes are
687 under the strongest purifying selection and, consequently offer the greatest long-term durability.

688

689 **Acknowledgements**

690 We would like to thank Bert Essenstam and colleagues of the Unifarm greenhouse facilities at
691 Wageningen University for excellent plant care, Dr Pedro Ballati of the Universidad Nacional de La Plata,
692 Buenos Aires, Argentina for kindly providing the Argentinian *F. fulva* strains, Dr Phillipe Nicot of INRAE
693 Centre de Recherche PACA, Montfavet, France for providing a selection of *F. fulva* strains from the

Southern region of France and the growers of the Cerafel association, St. Martin-des-Champs, France, for providing *F. fulva* strains from the Brittany region. This research was largely funded by the EU Horizon 2020 HARNESSTOM project (Grant agreement no.: 101000716).

Competing interests

None declared.

Author contributions

CRS, LF, YB, MHAJ, IS, pJGMdW and CHM designed the research; CRS, LF, AH, RJ, SdlR, BÖ, AMH, SLvZ, LDHV, CM, KM, AZZ, DE and CHM performed the research; CRS, LF, AH, AMH, SLvZ, MT, YI, AZZ, DE, A-MAW and CHM carried out the data analysis, collection or interpretation; CRS, LF and CHM wrote the manuscript. All authors reviewed the manuscript and approved it for publication.

Data availability

All *F. fulva* sequencing data generated in this study is deposited in GenBank (PRJNA1271984 and PRJNA961892), with specific BioSample and Sequence Read Archive (SRA) accession numbers listed in **Table S3**.

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Supporting information (brief legends)

Figure S1: Phylogeny and sequence comparison of effector genes across a panel of 33 *F. fulva* genomes.

Figure S2: Novel strains of *F. fulva* can infect Ontario 7818 tomato plants harboring the *Cf-6* gene.

994 **Figure S3:** *Ecp5* is part of an 18 kb deletion in four Race 6 strains of *F. fulva*.

995 **Figure S4:** An intro-exon border mutation in *Avr6* allows *F. fulva* to partially overcome *Cf-6*-mediated
996 resistance.

997 **Figure S5:** Avr6-like proteins are predicted to be produced by many plant-pathogenic Dothideomycete
998 and Sordariomycete fungi.

999 **Figure S6:** The Ontario 7818 x MM-*Cf-0* F2 BSA mapping population segregates for an HR to Avr6.

1000 **Figure S7:** Receptor-encoding candidates within the mapping interval of *Cf-6* exhibit significant
1001 numbers of R-pool-specific SNPs compared to the Heinz 1706 SL4.0 reference genome and the NR-
1002 pool.

1003 **Figure S8:** Domain predictions of receptor proteins within the mapping interval of *Cf-6*, based on the
1004 Heinz 1706 reference annotation (ITAG 4.0).

1005 **Figure S9:** Recognition of Avr6 correlates with tomato leaf mold disease resistance in Ontario 7818 x
1006 MM-*Cf-0* F2 individuals.

1007 **Notes S1:** Predicted Avr6-like sequences from fungi.

1008 **Table S1:** *F. fulva* strains used in this study for whole genome sequencing (strains with an alternate ID;
1009 as used for clarity in the manuscript) and for *Avr6* allelic variation analysis (strains without alternate
1010 ID).

1011 **Table S2:** Detailed information regarding the plant accessions used and discussed in this study as
1012 provided by the Centrum voor Genetische Bronnen Nederland (CGN).

1013 **Table S3:** Quality statistics for *F. fulva* genome assemblies generated in this study.

1014 **Table S4:** Quality statistics for publicly available *F. fulva* genome assemblies.

1015 **Table S5:** Fastp statistics for the R- and NR-pools used for BSA mapping of the *Cf-6* locus in the Ontario
1016 7818 x MM-*Cf-0* F2 tomato mapping population.

1017 **Table S6:** Statistical analysis of the observed segregation ratios based on PVX::*Avr6* response in the
1018 Ontario 7818 x MM-*Cf-0* F2 tomato populations by means of $\chi 2$ tests.

1019 **Table S7:** Interpro domain prediction results for Cf-6 candidate receptor proteins, based on their Heinz
1020 1706 ITAG4.0 annotation.

1021 **Table S8:** Summary of Interpro domain prediction results for Cf-6 candidate receptor proteins, based
1022 on their Heinz 1706 ITAG4.0 annotation.