Mutation or deletion of the *Avr6* avirulence effector gene of *Fulvia fulva* leads to breakdown of the *Cf-6* leaf mold resistance locus in tomato

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Summary

Genetic resistance is an important mitigation strategy in commercial tomato cultivation against leaf mold disease caused by the fungal pathogen *Fulvia fulva*. In recent years, the widely deployed *Cf-9* resistance locus has been overcome, which has prompted breeders to deploy the novel resistance locus *Cf-6*. However, *Cf-6*-mediated resistance has already been overcome in several locations worldwide. To date, the identity of *Cf-6*, as well as the gene encoding the matching Avr6 effector from *F. fulva* have remained unknown. Here, we report the identification of *Avr6* using a comparative genomics strategy.

Whole genome sequencing of *Cf-6*-breaking *F. fulva* strains revealed that the gene coding for the known secreted effector Ecp5 is either deleted or mutated in these strains. *Ecp5* was subsequently confirmed to be *Avr6* through potato virus X-mediated expression in *Cf-6* tomato plants, as well as by gene knockout and complementation experiments. Using the Avr6-triggered hypersensitive response as a marker for disease resistance, we mapped the *Cf-6* locus to the short arm of chromosome 12 in tomato. In addition to identifying *Avr6* and setting the foundation for cloning of *Cf-6*, this work highlights the urgent need for more research into durable gene-for-gene resistance in tomato against *F. fulva*.

Keywords

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

Introduction

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

Upon widespread deployment of *Cf* resistance genes in tomato cultivars, strong selection pressure is exerted on *F. fulva* to circumvent Avr recognition to regain virulence. This is achieved through mutations in, or complete loss of the corresponding *Avr* gene (de la Rosa & Schol et al., 2024; Iida et al., 2015; Joosten et al., 1994; Mesarich et al., 2023; Stergiopoulos, De Kock, et al., 2007). To date, all known *F. fulva Avr* genes encode small, secreted proteins (SSPs) that are highly expressed during infection (Mesarich et al., 2023). Avr effectors are rich in cysteine residues and, often, through the mutation of

63 a cysteine residue and loss of the associated disulfide bond, recognition of an Avr effector by its
64 corresponding Cf receptor is lost (Joosten et al., 1997; Joosten et al., 1994). Most Avr effectors of *F.*
65 *fulva* show little homology to other proteins and lack known functional domains (Mesarich et al., 2018).
66 The only exceptions are Avr4 (chitin-binding domain) and Avr2 (protease inhibitor), which protect
67 against PR chitinases and proteases, respectively (Rooney et al., 2005; Van Esse et al., 2007). Recently,
68 a combined transcriptomics and proteomics approach was used to show that *F. fulva* secretes at least
69 75 SSPs, named extracellular proteins (Ecps), during infection, of which many are expected to be yet-
70 identified Avr effectors (Mesarich et al., 2018). In support of this, 10 Ecps were found to elicit an HR
71 upon expression in various wild tomato accessions (Mesarich et al., 2018).

72 We recently demonstrated that *F. fulva* overcame the widely deployed *Cf-9* resistance locus by
73 sequential loss of *Avr9* and loss or mutation of *Avr9B* (de la Rosa & Schol et al., 2024). Both of these
74 *Avr* genes encode SSPs and are recognized by the products of two RLP-encoding genes at the *Milky Way*
75 (MW) locus on the short arm of chromosome 1, *Cf-9C* and *Cf-9B*, respectively (Jones et al., 1994;
76 Parniske et al., 1997). This prompted breeders to deploy a novel resistance gene, *Cf-6*, which was
77 tentatively mapped to the short arm of chromosome 12 using the *S. lycopersicum* breeding line Ontario
78 7818 (CGN15839) and was reported to originate from *S. pimpinellifolium* PI211839 (Kanwar et al.,
79 1980). Recently, the International Seed Federation (ISF) updated the *F. fulva* and tomato differential
80 sets, used for determining virulence and resistance spectra, to include five new strains of *F. fulva* (races
81 9, 2.9, 4.9, 4.5.9 and 2.6.9) and Ontario 7818 as the differential line for *Cf-6* (Sangster, 2022). The
82 inclusion of a race 2.6.9 strain (breaking *Cf-2*, *Cf-6* and *Cf-9*-mediated resistance), indicates that the
83 resistance provided by *Cf-6* is race-specific. The first report of *Cf-6*-breaking *F. fulva* strains in Europe
84 came from Germany, where several race 2.6.9 strains were collected throughout the country in 2018
85 and 2019 (Meyer & Gärber, 2021). For the current study, we were provided with an *F. fulva* collection
86 containing several race 2.6.9 strains, collected between 2017 and 2021 in the Brittany region in France.
87 The emergence of these strains indicates that the recently deployed *Cf-6* locus has already been
88 overcome, which is vital information for breeders to adjust their breeding strategies accordingly.

89 To determine the genetic and molecular basis of *Cf-6* resistance breakdown, we set out to identify Avr6,
90 the Avr effector recognized by tomato plants carrying the *Cf-6* resistance locus, using comparative
91 genomics. Comparative transcriptomics and genomics have previously been successfully applied to
92 identify the *F. fulva Avr* genes *Avr5* and *Avr9B* but have been limited to comparison of a single
93 resistance-breaking strain with a single non-resistance breaking strain (de la Rosa & Schol et al., 2024;
94 Mesarich et al., 2014). To identify *Avr6*, we performed whole-genome sequencing and assembly of 15
95 *F. fulva* race 2, 2.9 and 2.6.9 strains, allowing us to compare polymorphisms in known SSP-encoding

96 genes, most of which are previously characterized *Ecp* genes (Laugé et al., 2000; Mesarich et al., 2018;
97 Van den Ackerveken et al., 1993).

98 Next, we validated the *Avr6-Cf-6* interaction through transient expression of *Avr6* in *Cf-6* tomato plants
99 and gene knockout and complementation assays in *F. fulva.* Then, we used Avr6 as a marker for
100 mapping the *Cf-6* gene in Ontario 7818 by means of bulked segregant analysis (BSA)-sequencing,
101 followed by a Comparative Subsequence Set Analysis (CoSSa) bioinformatics pipeline (Prodhomme et
102 al., 2019). Finally, we correlated resistance to *F. fulva* in the mapping population with the ability to
103 recognize Avr6. This work serves to alert breeders to the breakdown of the *Cf-6* gene, provide tools
104 that can be applied to quickly identify *Cf-6*-breaking *F. fulva* strains in the field, and to direct ongoing
105 resistance breeding efforts.

106 **Results**

107 **Deletion or mutation of *Extracellular protein 5* (*Ecp5*) is associated with *Cf-6*-breaking *F. fulva* strains**

108 As a starting point for identifying *Avr6*, we obtained 14 French *F. fulva* strains that were sampled from
109 diseased tomato plants in the French region of Brittany between 2017 and 2021, of which five were
110 reported to break *Cf-6,* and a strain from New Zealand that was reported to break *Cf-6* in 2012. We
111 confirmed that six of these strains indeed break *Cf-6*-mediated resistance with a disease assay on MM-
112 *Cf-0* and Ontario 7818 (*Cf-6*) tomato plants (**Figure S1**). Subsequently, all 15 *F. fulva* strains were
113 subjected to Illumina sequencing and we generated whole-genome assemblies (**Table S1**), and
114 compiled these with publicly available *F. fulva* assemblies (**Table S2**) into a dataset of 32 genomes.
115 Then, we inferred a phylogeny based on a concatenated alignment of 3484 conserved, single-copy
116 orthologs and found that the strains fall into four distinct clades, with most (11) clustering into a single,
117 closely related clade (**Figure 1**). Then, we inferred mating types for all strains in the dataset and found
118 that 11 had *Mat1-1* (36.4%) and 21 had *Mat1-2* (63.6%) at their mating type (MAT) locus. Further
119 inspection of the distribution of genotypes for known *Avr* genes *Avr2*, *Avr4*, *Avr4E*, *Avr5*, *Avr9* and *Avr9B*
120 revealed that predicted mutations in Avr2 (p. del54-78, Tyr41fs*34, Lys23fs*42), Avr4 (Cys109*) and
121 Avr4E (Phe82Leu and Met93Thr) occur in both mating types, which suggests occurrence of
122 recombination and thus sexual reproduction within *F. fulva* populations.

123 Next, we queried these genomes to identify candidate genes for *Avr6*: i.e. putative effector genes that
124 have non-synonymous mutations or are (partially) absent in all race 2.6.9 strains, but not in others.
125 Hypothesizing that *Avr6* should be an SSP, we used BLAST to compare the sequences of a set of known
126 and predicted Ecp genes from the *F. fulva* race 5 reference strain (Mesarich et al., 2018; Zaccaron et al.,
127 2022) to our dataset, and searched for those that were absent or exclusively had one or more mutations
128 in the race 2.6.9 strains (**Figure S2**). As previously reported/predicted, the *Ecp* genes, although

129 displaying some minor sequence variation, are present in most or all strains (Mesarich et al., 2018;
130 Stergiopoulos, De Kock, et al., 2007), with *Ecp24-2* and *Ecp5* being notable exceptions. Strikingly, we
131 found that *Ecp5* (Laugé et al., 2000) was deleted in four of the six *Cf-6*-breaking strains (**Figure 1**, **Figure**
132 **S2**). In these strains, we observed a deletion of approximately 18 kb on chromosome 2, resulting in the
133 loss of *Ecp5* (*CLAFUR5_03423*) and three adjacent genes, *CLAFUR5_20157*, *CLAFUR5_03424*, and
134 *CLAFUR5_03425* (**Figure S3**). As previously shown, *Avr6* is surrounded by transposable elements (TEs)
135 of various types (de Wit et al., 2012), which are likely responsible for its deletion. The latter three are
136 predicted to code for uncharacterized, non-secreted proteins and are therefore unlikely candidates for
137 *Avr6*. In the other two *Cf-6*-breaking strains, non-synonymous mutations in *Ecp5*, resulting in amino
138 acid substitutions Tyr88His and Gly53Val, were observed. Strain IPO 2679, which was used for the
139 identification of *Avr9B* (de la Rosa & Schol et al., 2024), was the only other strain showing sequence
140 variation for *Ecp5*, resulting in a Cys30Tyr substitution. In all other strains, a wild-type *Ecp5* allele was
141 present. For *Ecp24-2*, we did not observe a clear correlation with the ability to overcome *Cf-6* (**Figure**
142 **S2**), and it was omitted as a candidate for *Avr6*. Together, these results suggest that *Ecp5* is *Avr6*.

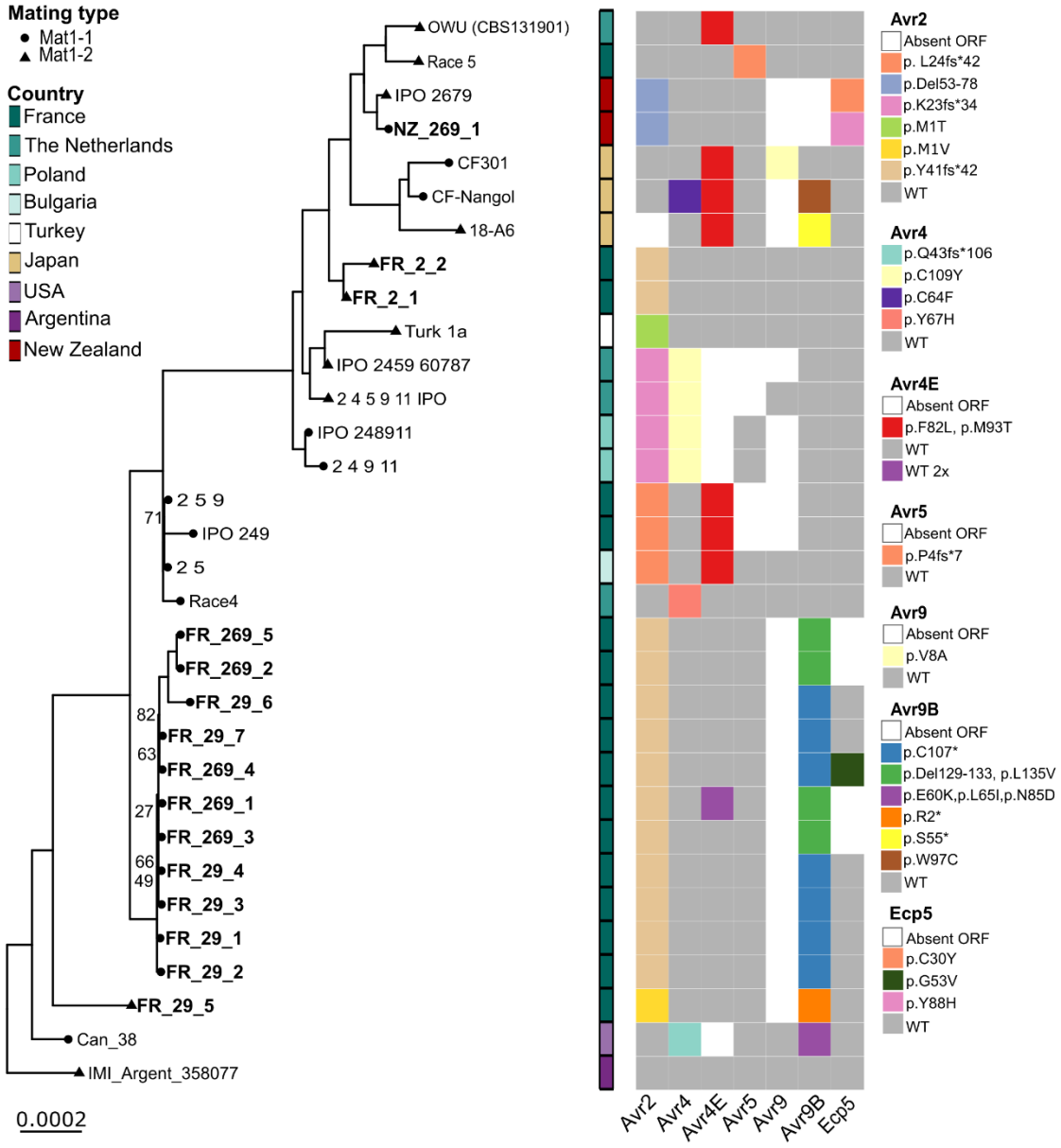

Figure 1: Phylogeny and predicted amino acid sequence comparisons of known Avrs, and Ecp5, across a panel of 32 *F. fulva* genomes.

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

155

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

157 To confirm that Ecp5 is Avr6, we systemically expressed it in *Cf-6* tomato (Ontario 7818) and *S.*
158 *lycopersicum* G1.1161, the line carrying the *Cf-Ecp5* locus which was mapped to the short arm of
159 chromosome 1 (Haanstra et al., 2000). Here, potato virus X (PVX)-based expression was employed,
160 whereby plants are transiently transformed with a recombinant version of PVX containing *Ecp5*
161 (PVX::*Ecp5*) using *Agrobacterium tumefaciens* (Haanstra et al., 2000; Laugé et al., 2000). As controls,
162 *Ecp5* was also expressed in MM-*Cf-0* plants that are universally susceptible to *F. fulva* and in MM-*Cf-9*
163 plants carrying the *Cf-9* locus. Concomitant with the systemic movement of the recombinant virus in
164 the plant, Ecp5 is produced systemically. Should Ecp5 be recognized by Cf-6, a systemic, visible HR,
165 resulting in chlorosis, necrosis, stunting, or death of the plant is expected to occur (Haanstra et al.,
166 2000; Laugé et al., 2000).

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

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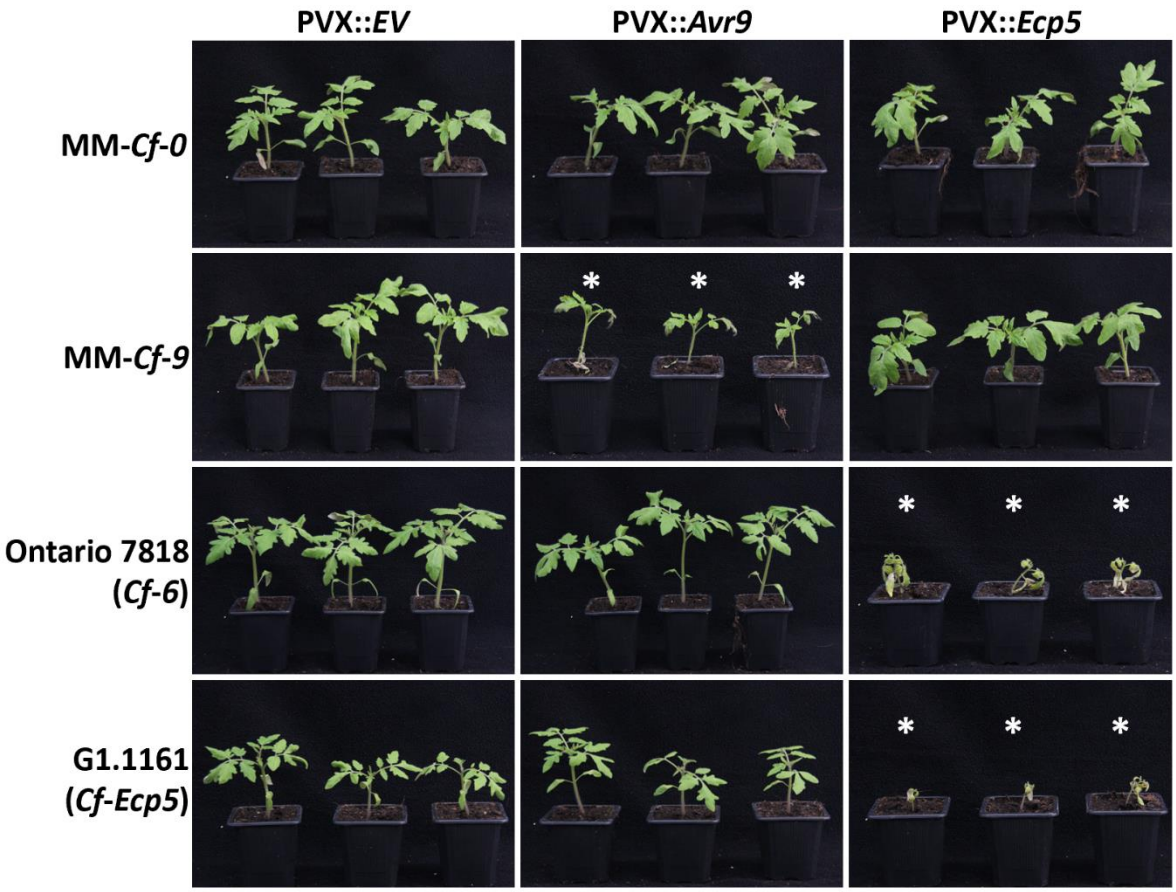


Figure 2: Ecp5 elicits a systemic HR in young tomato plants carrying *Cf-6* and *Cf-Ecp5*. Ecp5 was systemically expressed in seedlings of MM-*Cf-0* (no *Cf* genes), MM-*Cf-9* (*Cf-9B* and *Cf-9C*), Ontario 7818 (*Cf-6*), and G1.1161 (*Cf-Ecp5*), using the PVX-based expression system, with three replicates 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.

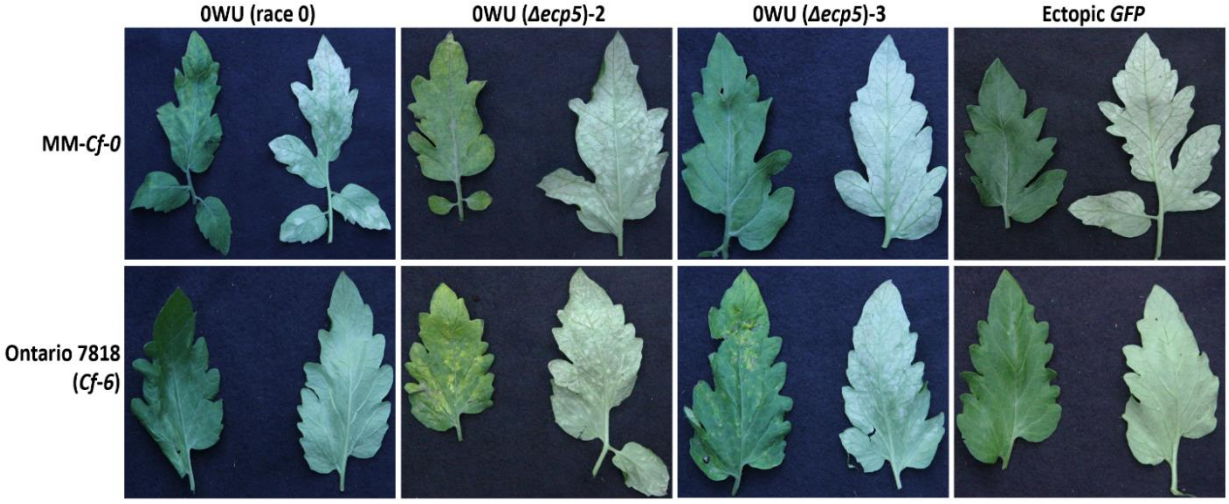
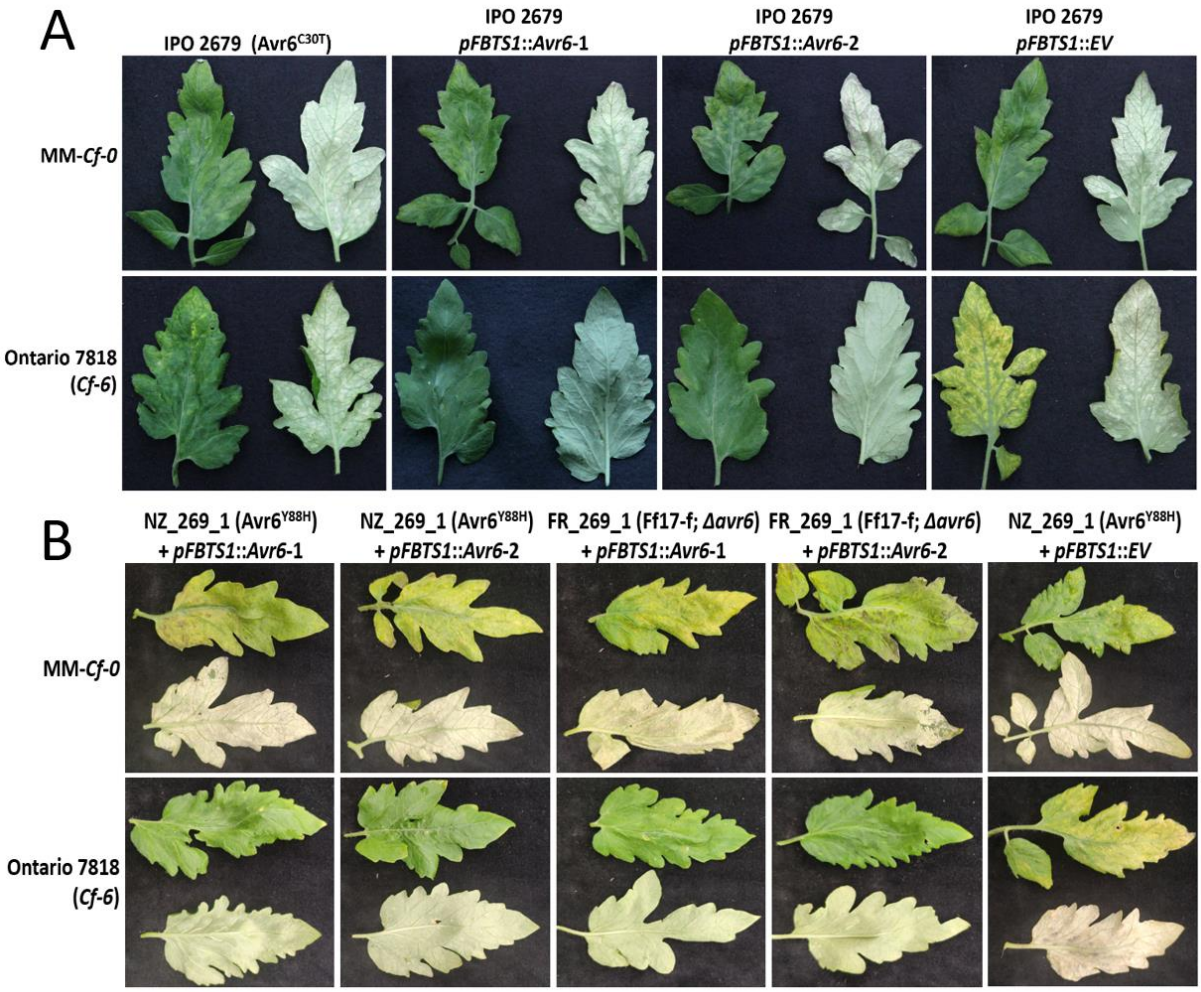


Figure 3: Deletion of *Ecp5* in the *F. fulva* race 0 strain 0WU confers virulence on Ontario 7818 (*Cf-6*) tomato plants. The 0WU wild-type strain, two independent *F. fulva Ecp5* knockout strains (*Δecp5*-2 and *Δecp5*-3) and an ectopic *green fluorescent protein* (*GFP*) transformant control were 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.

Allelic variation in *Avr6* allows specific *F. fulva* strains to overcome *Cf-6*

Next, we validated that mutations in Avr6 observed in race 2.6.9 strains allow circumvention of *Cf-6*-mediated resistance. *F. fulva* strain IPO 2679 carries a 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 (absent *Avr6* ORF) and NZ_269_1 ($Avr6^{Tyr88His}$) 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 S3**), 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

212 symptoms were not as pronounced as on MM-*Cf-0*, they were more severe compared to the symptoms
213 caused by strain P30, which has a wild-type *Avr6* gene, indicating that this mutation impairs the
214 recognition of Avr6 by Cf-6. In all 154 other strains, we found a wild-type *Avr6* gene, suggesting that *Cf-*
215 *6*-breaking strains recently emerged.

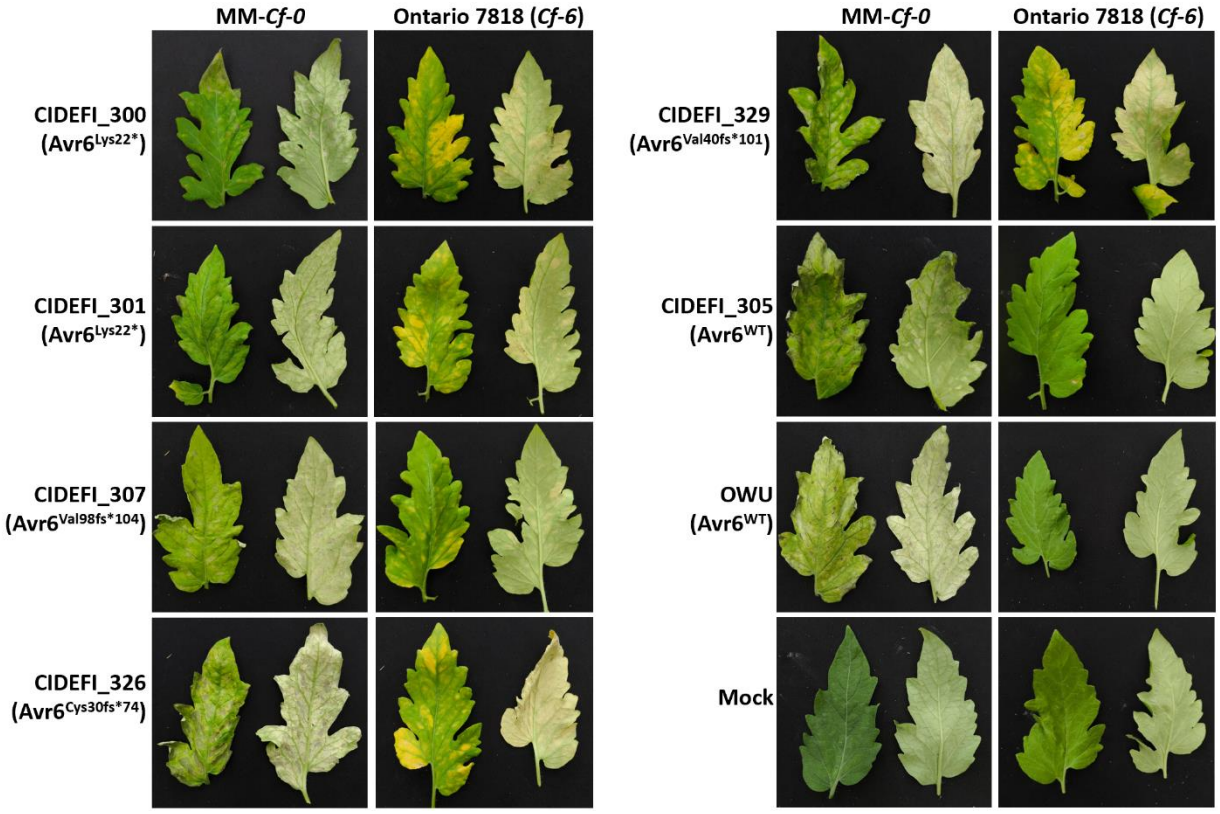


216

217 **Figure 4: Complementation of *Cf-6*-breaking *F. fulva* strains with wild-type *Avr6* from strain 0WU**
218 **results in loss of virulence on Ontario 7818 (*Cf-6*) tomato plants.** The IPO 2679 (Avr6^{Cys30Tyr}) wild-type
219 strain, two independently generated IPO 2679 *Avr6*-complemented *F. fulva* (*Avr6*-1 and *Avr6*-2)
220 transformants, and an empty vector (EV) control transformant were inoculated on 4-week-old Ontario
221 7818 (*Cf-6*) and MM-*Cf-0* (no *Cf* genes) tomato plants (Panel A). Two independently generated
222 NZ_269_1 and FR_269_1 *Avr6*-complemented *F. fulva* (*Avr6*-1 and *Avr6*-2) transformants, and an EV
223 control, were inoculated on 4-week-old Ontario 7818 (*Cf-6*) and MM-*Cf-0* (no *Cf* genes) tomato plants
224 (Panel B). Leaflets were photographed at 14 days post-inoculation.

225 Recently, however, non-synonymous and frame-shift mutations were reported in *Avr6* of several *F. fulva*
226 strains collected in Argentina (Lucentini et al., 2021; Medina et al., 2015). To investigate whether these

227 mutations also result in virulence on *Cf-6* tomato, we inoculated strains expressing Avr6^{Lys22*},
228 Avr6^{Cys30fs*74}, Avr6^{Val40fs*101} and Avr6^{Val98fs*104} (**Table S3**) onto MM-*Cf-0* and Ontario 7818 plants. This
229 resulted in visible disease symptoms on both genotypes for strains harboring these mutant alleles, but
230 not for the control strains 0WU or CIDEFI 305, which both contain wild-type *Avr6* and are therefore
231 unable to overcome *Cf-6* (**Figure 5**). As all mutations are predicted to result in either frameshifts or
232 cysteine mutations, it is not surprising that recognition by Cf-6 is abolished. Unfortunately, we could
233 not test strain CIDEFI 318 (Avr6^{Cys48Tyr}) due to its poor growth and sporulation *in vitro*, but it is likely
234 that the Cys48Tyr mutation also abolishes recognition. In all, apart from deletion of the *Avr6* ORF and
235 the splice site mutation in P31, we identified eight different non-synonymous and frame-shift
236 mutations in *Avr6* across strains (**Figure 6**), of which seven were confirmed to result in breakdown of
237 *Cf-6* by *F. fulva*.



238

239 **Figure 5**: **Non-synonymous and frame-shift mutations in *Avr6* of Argentinian *F. fulva* strains confer**
240 **virulence on *Cf-6* tomato plants**. Four-week-old MM-*Cf-0* (no *Cf* genes) and Ontario 7818 (*Cf-6*) tomato
241 plants were inoculated with several of the Argentinian *F. fulva* strains reported by Medina et al. (2015)
242 and Lucentini et al. (2021) to harbor mutations in *Avr6* (race 2.6 (initially reported as race 2): CIDEFI
243 300, 301, 307 and 329; race 6 (initially reported as race 0): CIDEFI 326). As controls, strain 0WU (race
244 0) and CIDEFI 305 (race 2), which can infect MM-*Cf-0* but not *Cf-6* plants, were also inoculated.
245 Symptoms were photographed at 14 days post-inoculation.

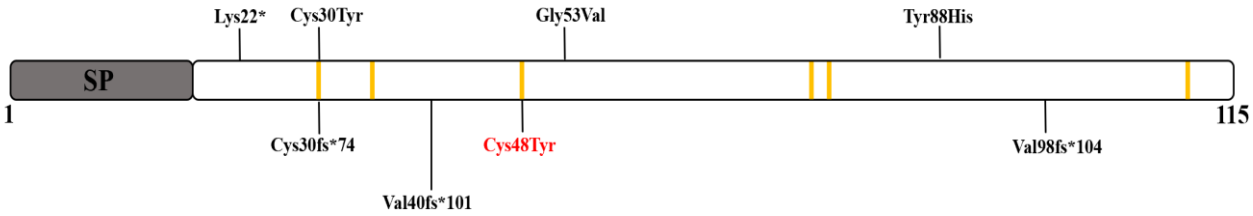


Figure 6: Schematic representation of the Avr6 protein showing the location of mutations that allow *F. fulva* to overcome *Cf-6*-mediated resistance. The signal peptide (SP) is indicated in grey and yellow bars indicate the positions of cysteine residues. The strain carrying the predicted Cys48Tyr mutation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

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

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

The predicted tertiary structure of Avr6 shows similarity to LEPTOSPHAERIA AVIRULENCE AND SUPPRESSING (LARS) effectors

To gain insight into the potential function of Avr6, its tertiary structure, without its SP for extracellular targeting, was predicted using AlphaFold2 and compared to characterized tertiary structures of fungal proteins in the RCSB Protein Data Bank using Foldseek. Based on this, Avr6 is predicted to adopt a tertiary fold made up of six β-strands and one α-helix, stabilized by three disulfide bonds (**Figure 7**). Following the numbering of the mature Avr6 sequence, the disulfide connectivity pattern is predicted to be Cys13-Cys18, Cys31-Cys59 and Cys61-Cys94. Of note, AlphaFold2 did not predict the Cys61-Cys94 bond, but instead this association is anticipated based on the close proximity of these two residues in the predicted Avr6 tertiary structure (**Figure 7**).

Next, the mutated, conserved amino acid positions identified in Avr6 of *F. fulva* strains NZ_269_1 (Tyr88His) and FR_269_4 (Gly53Val) that are virulent on *Cf-6* tomato, were mapped onto the predicted tertiary structure to examine whether they are likely to be surface-exposed and, thereby potentially accessible to the Cf-6 receptor (should recognition be direct) (**Figure 7**). Interestingly, Tyr88 is predicted to occupy an internal position within the Avr6 tertiary structure and therefore is not expected to be surface-exposed. Gly53, on the other hand, is predicted to form part of the loop linking the sole α-helix to β-strand 2 and is therefore predicted to be at least partially surface-exposed (**Figure 7**).

To investigate whether the predicted fold of Avr6 is found in other proteins, particularly those with a characterized biological function, we used FoldSeek to compare its predicted tertiary structure to experimentally determined tertiary structures of fungal proteins in the RCSB Protein Data Bank. Based on this approach, we identified the top hits to Avr6 as AvrLm4-7 (PDB ID: 4FPR; Probability score: 0.77, TM-Score: 0.549) and AvrLm5-9 (PDB ID: 7B76; Probability score: 0.75, TM-score: 0.0547), which are both Avr effectors from the LEPTOSPHAERIA AVIRULENCE AND SUPPRESSING (LARS) family of the *Brassica* blackleg pathogen *Leptosphaeria maculans* (Blondeau et al., 2015; Lazar et al., 2022). While these proteins adopt a similar fold to Avr6, the structures of AvrLm4-7 and AvrLm5-9 are more elongated and Avr6 possesses additional β-strands (**Figure 7**). Unfortunately, the specific host targets of AvrLm4-7 and AvrLm5-9 are not yet known.

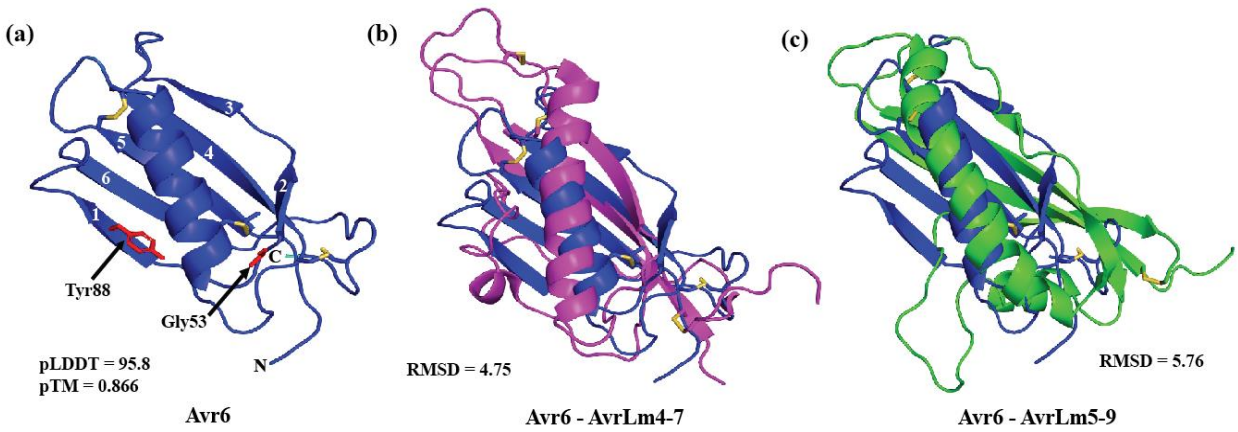


Figure 7: Avr6 is predicted to adopt a LARS-like fold. (a) Predicted tertiary structure of Avr6. The tertiary structure is colored according to the predicted Local Distance Difference Test (pLDDT) score (confidence: cyan = high; dark blue = very high). Gly53 and Tyr88 are shown as sticks and are colored 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.

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

302 Currently, three distinct loci originating from different *S. pimpinellifolium* accessions which mediate a
303 gene-for-gene relationship with *Avr6* have been reported: *Cf-Ecp5.1* on the top arm of chromosome 1
304 (Haanstra et al., 2000; Iakovidis et al., 2020), *Cf-Ecp5.7* on the bottom arm of chromosome 7, and *Cf-*
305 *Ecp5.12* on the top arm of chromosome 12 (Iakovidis et al., 2020). To validate the location of *Cf-6* on
306 the short arm of chromosome 12 in Ontario 7818 as reported by Kanwar et al. (1980), we applied a
307 Bulked Segregant Analysis (BSA)-sequencing approach in an F2 population derived from a cross
308 between Ontario 7818 (*Cf-6*) and MM-*Cf-0*. For this, 133 F2 seedlings were subjected to systemic PVX-
309 based expression of *Avr6* and subsequently phenotyped based on presence or absence of a systemic
310 HR (**Figure S6**, **Table S4**). Genomic DNA (gDNA) from 88 (out of 92) plants that underwent a systemic
311 HR, indicating Avr6-responsiveness, was bulked into a responsive (R) pool, and from 40 (of 41) non-
312 responders into a non-responsive (NR) pool (**Tables S4 and S5**). The expected 3R:1NR segregation
313 pattern, under the hypothesis of *Cf-6* being a single dominant gene, was confirmed with a χ^2 test
314 (χ^2=2.41, *p*-value=0.12) (**Table S6**). After short-read sequencing of the pools, we applied a Comparative
315 Subsequence Sets analysis (CoSSa) bioinformatics pipeline (Prodhomme et al., 2019), to identify R-bulk-
316 specific *k*-mers, which were subsequently mapped to the *S. lycopersicum* Heinz SL4.0 reference
317 genome (Hosmani et al., 2019). We identified a small 2 Mb interval spanning from approximately 2.2
318 Mb to 4.2 Mb on the short arm of chromosome 12 that was significantly enriched for R-bulk-specific *k*-
319 mers, indicating that presence of *Cf-6* (**Figure 8**). Potential resistance gene-encoding candidates for *Cf-*
320 *6* within this region include eight RLP-encoding genes, divided over two distinct clusters, and two
321 nucleotide-binding leucine-rich repeat (NB-LRR)-encoding genes. All of these genes show significant
322 allelic variation in Ontario 7818 compared with the Heinz 1706 SL4.0 reference and MM-*Cf-0*, indicating
323 that they could be involved in recognition of Avr6 in Ontario 7818 (**Figure S7**). The region coincides
324 with previous findings mapping *Cf-6* to the short arm of chromosome 12 (Kanwar et al., 1980), and the
325 later finding for the two *Cf-Ecp5.12* alleles (Iakovidis et al., 2020).

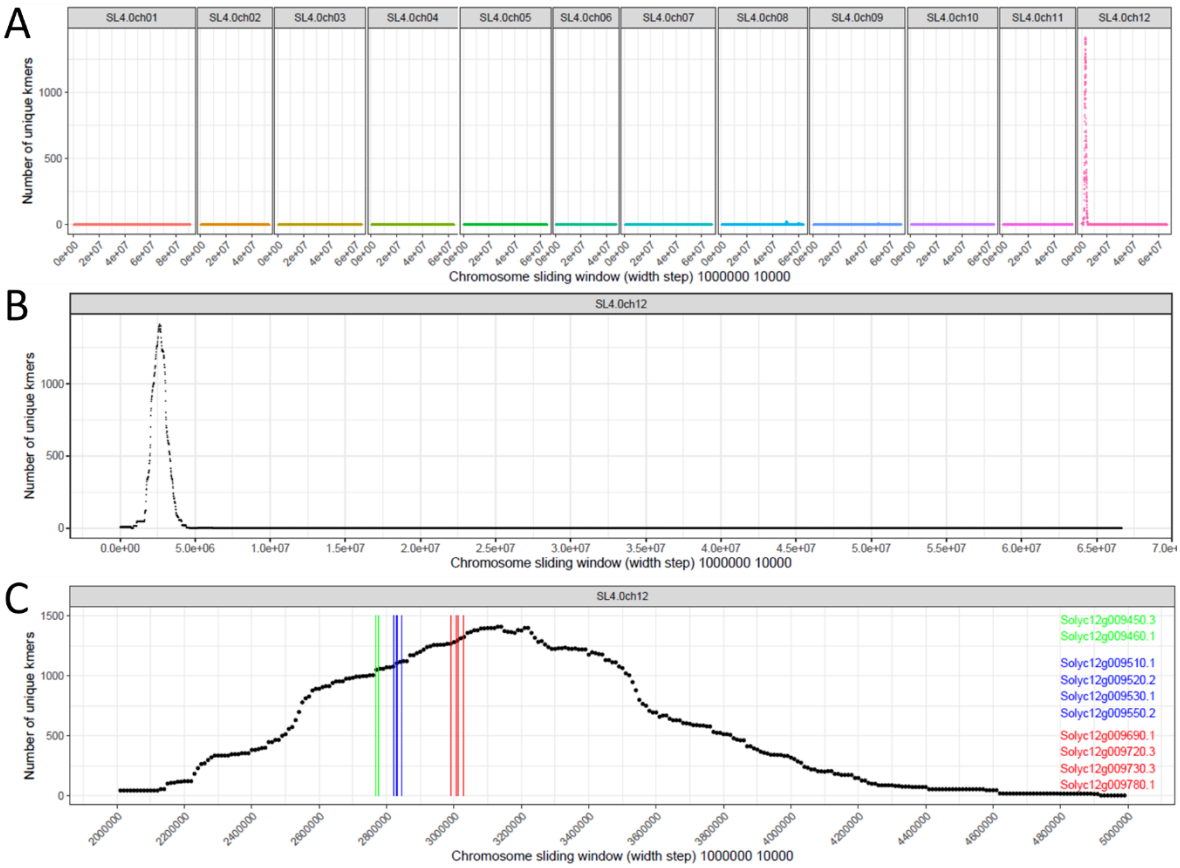


Figure 8: BSA-mapping of *Cf-6* (based on responsiveness to Avr6) in an Ontario 7818 x Moneymaker F2 population using Comparative Subsequence Sets analysis (CoSSa). Plots show R-pool-specific *k*-mers (resulting from intersection with Ontario 7818 specific *k-mers* and subtraction of NR-pool and MM-*Cf-0* 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 a 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 all of these proteins in Ontario 7818 remain unknown, they all remain candidates for Cf-6.

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

352 **Discussion**

353 ***F. fulva* overcomes *Cf-6* by loss or mutation of *Avr6***

354 *Avr6* codes for a 115-amino acid protein of (so-far) unknown function and was originally cloned as *Ecp5*
355 (Laugé et al., 2000). Upon removal of its 17-amino acid signal peptide, which is required for secretion,
356 Avr6 becomes a mature 98-amino acid SSP with six cysteine residues (Laugé et al., 2000). Expression
357 of *Avr6* is highly induced *in planta*, but not *in vitro* (Mesarich et al., 2014). Consistent with earlier
358 findings (Iida et al., 2015; Stergiopoulos, De Kock, et al., 2007), we observed a complete lack of allelic
359 variation in *Avr6* across a set of recently collected Japanese strains, as well as in all but the four French
360 race 2.6.9 strains and all but one (P31) of the other recently collected European strains we investigated.
361 This suggests that negative selection pressure on *Avr6* was only recently introduced, probably through
362 deployment of *Cf-6*, and it can be expected that more *Cf-6*-breaking strains will emerge when *Cf-6*
363 becomes more widespread in tomato cultivars. The Cys30Tyr mutation in Avr6 of strain IPO 2679 is
364 puzzling, as it was collected in New Zealand in the 1980s when, to our knowledge, *Cf-6* was not yet
365 commercially deployed (Iakovidis et al., 2020). Interestingly, a previous study reported an
366 uncompromised HR after expression of an Avr6^{Cys30Ala} mutant (Avr6^{Cys13Ala} in the mature protein) in *S.*
367 *lycopersicum Cf-Ecp5* donor line G1.1161 (Luderer et al., 2002), suggesting either a different
368 recognition specificity of the putative Cf-Ecp5 receptor encoded by the G1.1161 chromosome 1 allele
369 compared to Cf-6 encoded by the Ontario 7818 chromosome 12 allele, or that Avr6^{Cys30Tyr} but not
370 Avr6^{Cys30Ala} abolishes recognition. The variety of polymorphisms in *Avr6* in the previously reported
371 Argentinian strains raises questions regarding their origin, as the tomato varieties from which they were
372 sampled are unlikely to harbor *Cf-6/Cf-Ecp5* loci, since they are susceptible to strains with wild-type
373 *Avr6* (Lucentini et al., 2021; Medina et al., 2015). However, *Cf-6/Cf-Ecp5* is common and widespread
374 across wild tomato species (Iakovidis et al., 2020), so, given Argentina's close proximity to the tomato
375 center of origin, perhaps these mutations were acquired due to selection pressure exerted by wild
376 tomato. Subsequently, these strains could have spilled over into tomato cultivation areas. Potentially,

377 strain IPO 2679 also acquired Avr6^{Cys30Tyr} in South America before spilling over into New Zealand. Strains
378 originating from South America are so far underrepresented in current *F. fulva* collections, and
379 considering the variation that was found in the Argentinian strains of Lucentini et al. (2021) and Medina
380 et al. (2015), it would be interesting to determine the phylogeny and allelic variation of *Avr*/*Ecp* genes
381 in other South American strains by performing a sampling campaign for wild *F. fulva* strains (and
382 potentially their hosts) in the center of origin, followed by sequencing and population studies (Bartoli
383 & Roux, 2017; Lebeda & Burdon, 2023).

384 **Stacked *Cf* genes can potentially be overcome by recombination between different mutant *Avr* genes**

385 Surprisingly, the closely related clades consisting of five race 2.6.9 and six race 2.9 strains seem to have
386 overcome both *Cf-6* and *Cf-9B* by two distinct mutations in *Avr* and *Avr9B*: four race 2.6.9 strains show
387 a deletion of the *Avr6* ORF combined with Avr9B$^{Del129-133,\ L135V}$, whilst the race 2.9 strains have a wild-
388 type *Avr6* with Avr9B^{Cys107*}, and the last race 2.6.9 strain having Avr6^{Gly53Val} and Avr9B^{Cys107*} (**Figure 1**).
389 These observations show that single *Cf* gene-mediated resistance can rapidly be overcome
390 independently by closely related *F. fulva* lineages within a short time frame. It would be interesting to
391 determine which mutations in *Avr6* cause the recently identified German race 2.6.9 strains to overcome
392 *Cf-6* (Meyer & Gärber, 2021), and to investigate their phylogenetic relationship to the French clade, as
393 this would shed more light on the epidemiology of tomato leaf mold and the rate at which *Cf* genes are
394 overcome. Interestingly, all 14 sequenced French strains have Avr2^{Tyr41fs*42}, despite the only two race 2
395 strains having an opposing mating type (*Mat1-2*) of the other strains (*Mat1-1*). So far, Avr2^{Tyr41fs*42} had
396 only been described in an Italian race 2 strain with *Mat1-1*, collected in 1993 (Stergiopoulos, De Kock,
397 et al., 2007). Alarmingly, we see more of the same mutations in Avr proteins occurring in strains of
398 opposing mating types, specifically Avr2^{Tyr41fs*42}, Avr2^{Lys23fs*34}, Avr2$^{Asp54-Gly78del}$, Avr4^{Cys109*} and
399 Avr4E^{Phe82Leu,Met93Thr}, suggesting the occurrence of (sporadic) sexual recombination within the previously
400 considered to be asexual *F. fulva* population. This finding would impact future resistance breeding
401 strategies, as *F. fulva* would be more readily able to accumulate mutations in *Avr* genes, allowing it to
402 overcome stacked *Cf* genes. This is supported by previous findings, like the occurrence of Avr9B^{Ser55*} in
403 both mating types of some Japanese strains (de la Rosa & Schol et al., 2024), and that the two mating
404 types occur in a 1:1 ratio in a collection of Turkish strains (Gül et al., 2022). In fact, a study on allelic
405 variation in *Avr* genes in this Turkish population resulted in similar observations to ours (Gül et al.,
406 2024). The publication of a high-quality chromosome-level reference genome (Zaccaron et al., 2022)
407 followed by five additional near-complete assemblies provides a solid basis for genomic analyses
408 (Zaccaron & Stergiopoulos, 2024). Further development of an *F. fulva* pangenome, potentially including
409 strains originating from the wild, can elucidate whether this species is indeed sexually reproducing.

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

411 Sequence queries suggest that many plant-pathogenic Dothideomycete and Sordariomycete fungi
412 produce one or more Avr6-like proteins, of which one, *Pf*Avr6, was identified in *P. fuligena*, a pathogen
413 closely related to *F. fulva* that causes black leaf mold of tomato (Phengsintham et al., 1938). Given that
414 the mature version of *Pf*Avr6 has a high level of sequence identity to the mature version of Avr6
415 (~71.4%), including Gly53 and Tyr88 (**Figure S5**), and that there is evidence of resistance to *P. fuligena*
416 in cultivated and wild tomato (Hartman & Wang, 1993; Wang et al., 1995), it would be interesting to
417 determine if it is recognized by *Cf-6* tomato plants. If so, *Cf-6* could also provide protection against *P.*
418 *fuligena*. However, the emergence of *Cf-6*-breaking *F. fulva* strains suggests that any such protection
419 will likely be short-lived as *P. fuligena* may adapt in a similar way. Searches based on the predicted
420 tertiary structure of Avr6 revealed similarity to other LARS effectors, which have so far been identified
421 in at least 13 other plant-pathogenic fungi (Lazar et al., 2022). Examples include four Avr effectors from
422 *L. maculans*, as well as *F. fulva* Ecp11-1 (Lazar et al., 2022; Mesarich et al., 2018). In the *L. maculans*–
423 *Brassica* pathosystem, recognition of the LARS effectors AvrLm3 and AvrLm5-9 by their corresponding
424 R proteins can be masked by another LARS effector, AvrLm4-7 (Ghanbarnia et al., 2018; Plissonneau et
425 al., 2016). However, our experiments show no evidence to suggest that Avr6 masks the recognition of
426 other *F. fulva* effectors in susceptible tomato plants. The predicted tertiary structure of Avr6 displays
427 several notable differences to the LARS effectors described above, reflected in a lack of sequence
428 similarity. LARS-like effectors lacking such similarity or possessing subtle differences in their predicted
429 tertiary folds have been identified in other plant-pathogenic fungi including in *Venturia inaequalis*,
430 which causes apple scab disease (Rocafort et al., 2022).

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

432 Initially, an HR was observed upon elicitation with the then-called Ecp5 protein (Laugé et al., 2000) in
433 the introgression line G1.1161, an F_3BC_1 resulting from a cross between *S. pimpinellifolium* CGN15529
434 and MM-*Cf-0* (Haanstra et al., 2000). The *Cf-Ecp5* gene in this accession was mapped to the *AURORA*
435 (*AU*) locus on the short arm of chromosome 1 (Haanstra et al., 2000), but a later study found that
436 rather, it was tightly linked with *Cf-9* at the *MW* locus (Iakovidis et al., 2020). This study also reported
437 two other independent *Cf-Ecp5* loci: *Cf-Ecp5.7* at the *CENTAURI* (*CE*) locus on the bottom arm of
438 chromosome 7 in *S. pimpinellifolium* LA0722, and *Cf-Ecp5.12* at the *ANDROMEDA* (*AN*) locus located
439 within an 888 kb region on the short arm of chromosome 12 spanning from approximately 2.1 to 3.0
440 Mb in *S. lycopersicum* Ontario 7522 and *S. pimpinellifolium* LA2852 (Iakovidis et al., 2020). Of these,
441 the mapping interval for *Cf-Ecp5.12* coincides largely with our results for *Cf-6* in Ontario 7818. The study
442 of Iakovidis *et al*. (2020) employed a marker at 3.014 Mb which showed 4.5% recombination with the

443 *Cf-Ecp5.12* locus. Assuming that the *Cf-6* allele from Ontario 7818 and the *Cf-Ecp5* allele from Ontario
444 7522 are the same and that *Cf-6* is an RLP-encoding gene, it is more likely to reside within the "blue"
445 RLP-encoding gene cluster at 2.85 Mb, rather than at the "red" cluster at around 3.0 Mb (**Figure 9C**),
446 which is roughly at the same location as the mentioned marker of Iakovidis et al. (2020). Although
447 originating from the same breeding program, Ontario 7818 and 7522 do appear to have different
448 donors, namely *S. pimpinellifolium* PI 211839 and PI 124161, respectively. PI 124161 appears to
449 originate from Guatemala (**Table S10**), whilst the origin of PI 211839 is unknown. Since the three
450 mapped *Cf-6*/*Cf-Ecp5* alleles so far all originate from *S. pimpinellifolium*, it would be interesting to map
451 and clone additional alleles from different tomato species such as *S. peruvianum* or *S. chilense*, as some
452 accessions of these species also respond to Avr6 (Iakovidis et al., 2020). This could result in the
453 identification of more distinct *Cf-6/Cf-Ecp5* loci, shedding more light on the evolution and geographical
454 distribution of *Cf* genes in wild tomato. Finally, our study clarifies previous conflicting findings regarding
455 the location of *Cf-6*, as neither chromosome 6 (as reported by Grushetskaya et al. (2007)) nor 11 (as
456 reported by Wang et al. (2007)) seem to be involved in recognition of Avr6. (Iakovidis et al., 2020).

457 The identified candidate RLP-encoding genes within our mapping region in Ontario7818 are prime
458 candidates for *Cf-6*, particularly *Solyc12g009510* and *Solyc12g009520*, given their predicted complete
459 protein structures in Heinz 1706, although it is also possible that *Cf-6* of Ontario 7818 is not annotated
460 in the Heinz 1706 genome. This could be resolved by generating a *de novo* genome assembly of Ontario
461 7818, or by resistance gene enrichment sequencing (RENseq) (Lin et al., 2020; Witek et al., 2016),
462 revealing any structural differences that are often found across different accessions/species in RLP-
463 encoding genes (de Kock et al., 2005; Dixon et al., 1998; Dixon et al., 1996; Man et al., 2020). For
464 example, the recognition of Avr9 in wild *S. chilense* accessions is highly region-dependent and the *Cf-9*
465 locus is highly polymorphic amongst these accessions (Kahlon et al., 2020).

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

467 Future breeding efforts should be directed towards achieving more durable resistance against *F. fulva*,
468 as breakdown of the recently deployed *Cf-6* locus, to our knowledge, occurred much more rapidly than
469 breakdown of the *Cf-9* locus (de la Rosa & Schol et al., 2024), demonstrating that single *Cf* gene
470 strategies against *F. fulva* are likely insufficiently durable. The wild tomato germplasm that showed a
471 specific HR when exposed to one or multiple of in total nine novel Ecp effectors (ten if Ecp17-1 is
472 included) identified by Mesarich et al. (2018), likely harbors an equal number of unused *Cf-Ecp* genes
473 that could be deployed in breeding programs. However, the potential for durability of these genes
474 should be carefully considered before deployment and could potentially be predicted by generating
475 *Ecp* knockout strains to determine their contribution to *F. fulva* virulence, as was recently performed

476 for *Ecp20-2* (Karimi-Jashni et al., 2022). In general, the paradigm that Ecp effectors are more conserved
477 and thus would be more essential for virulence than Avr effectors (Ökmen & de Wit, 2012;
478 Stergiopoulos, De Kock, et al., 2007), seems to be broken by our findings described here.

479 In summary, *Cf* gene pyramiding currently seems to be the most durable resistance breeding strategy
480 against *F. fulva*, as sequential loss of multiple effector genes seems to occurs less rapidly than loss of
481 single effector genes (de la Rosa & Schol et al., 2024). To truly achieve long-lasting, broad-spectrum
482 resistance in tomato against *F. fulva*, and to serve as an example for other economically important
483 fungal crop diseases, it is now time to study the dynamics of the *F. fulva*-tomato pathosystem in the
484 wild. Such an ecology-informed approach, potentially combined with pathogen-host interaction
485 modeling (Märkle et al., 2024; McMullan et al., 2018), will lead to a better understanding of which
486 effectors are more important, and thus which corresponding (combination of) *Cf* genes could
487 potentially be more effective and durable, as tomato accessions from locations with naturally high
488 levels of *F. fulva* disease pressure have likely adapted to possess more advanced/effective resistance
489 mechanisms. This should be supported by molecular studies into the function of *F. fulva* effectors and
490 their virulence targets, which could also reveal mechanisms, or, potentially, susceptibility factors
491 (Koseoglou et al., 2022; Sun KaiLe et al., 2014), that can be exploited to achieve durable resistance.

492 **Materials and Methods**

493 **Fungal and plant material**

494 Information on the *F. fulva* strains used in this study can be found in **Table S3**. Novel French strains
495 (sample IDs starting with FR_) were collected by VEGENOV and their virulence spectrum was
496 determined by inoculation onto a tomato differential set carrying different *Cf* genes (including *Cf-6*),
497 according to the ISF (Sangster, 2022). Argentinean *F. fulva* strains were previously collected and
498 characterized by Medina et al. (2015) and Lucentini et al. (2021). Their virulence spectrum was
499 determined by the authors using a tomato differential set (excluding *Cf-6*) and, in this study we assessed
500 their virulence on Ontario 7818 (CGN15839; carrying *Cf-6*) and Moneymaker (MM) plants (CGN14330).
501 Transgenic *Avr6* knockout strains in the 0WU background were previously generated by Ökmen (2013).
502 Strain 0WU (race 0) (de Wit et al., 2012) was used as a control strain in the inoculation studies and
503 strain IPO 2679 (de la Rosa & Schol et al., 2024) was included as it carries Avr6^{Cys30Tyr}.

504 For BSA-sequencing, an F2 population of 133 individuals derived from a cross between Ontario 7818
505 (*Cf-6*) and MM-*Cf-0* was utilized. *S. lycopersicum* Ontario 7818 (*Cf-6*), breeding line G1.1161 (*Cf-Ecp5*)
506 (Haanstra et al., 2000) and MM-*Cf-9* (CGN15338) were utilized for PVX-mediated expression of Avr6,
507 Avr9 and Avr9B. Detailed information regarding CGN accessions as provided by the Center of Genetic
508 Resources Netherlands (CGN) in Wageningen, The Netherlands, can be found in **Table S10.**

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

510 Genomic DNA was isolated from *F. fulva* strains, MM-*Cf-0*, Ontario 7818 (*Cf-6*) and Ontario 7818 x MM-
511 *Cf*-0 F2 plants using a cetyltrimethylammonium bromide (CTAB) DNA protocol (Murray & Thompson,
512 1980) and purified using a Genomic DNA Clean & Concentrator (Zymo Research). For BSA-sequencing,
513 200 ng of gDNA per F2 plant was pooled into the according R- or NR-pool and then purified (**Tables S4**
514 **and S5**). gDNA quality and concentration were measured using the Nanodrop™ One (Thermo Scientific)
515 and Qubit 2.0 Fluorometer (Thermo-Scientific). All 150 bp paired-end (PE)-sequencing of *F. fulva* strains
516 was performed by Novogene (https://www.novogene.com) on an Illumina NovaSeq6000 sequencing
517 platform. All 150 bp PE-sequencing of (pooled) tomato gDNA was performed by BGI
518 (https://www.bgi.com) on a DNBseq sequencing platform. For each of the 15 *F. fulva* strains, reads
519 were trimmed with Trimmomatic v0.39 (Bolger et al., 2014) with parameters set to
520 SLIDINGWINDOW:4:20, MINLEN:100 using Truseq2 adapter sequences, and quality of trimmed reads
521 was checked with FASTQC (v0.12.1) (Andrews, 2019). Trimmed reads were assembled with Spades
522 v4.0.0 (Bankevich et al., 2012) and contigs < 1 kb were removed post assembly. Assembly quality was
523 assessed with Quast (v5.3.0) (Gurevich et al., 2013) and BUSCO (v5.7.1, genome-mode, with
524 dothideomycetes_odb10 as reference BUSCO set and metaEUK as gene predictor) (Manni et al., 2021)
525 (**Table S1**). All *F. fulva* sequencing data generated in this study is deposited in Genbank
526 (PRJNA1271984).

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

528 Publicly available genome sequences were downloaded from Genbank using the query '*Fulvia fulva*'
529 (**Table S2**). The genome of *Dothistroma septosporum* NZE10 (GCA_000340195.1) was downloaded
530 separately. We used BUSCO as described above to identify single-copy BUSCO genes in these
531 assemblies and those generated in this study. For each BUSCO query gene, we created a fasta file with
532 corresponding DNA sequences from each strain (33 *F. fulva* + 1 *D. septosporum*) if it was present as
533 single-copy in all assemblies using a custom Python script. Next, we used Muscle (v 5.3.linux64) (Edgar,
534 2022) with default settings to infer a multiple sequence alignment for each fasta and trimmed
535 alignments with TrimAl (v1.5.rev0) (Capella-Gutiérrez et al., 2009) with settings optimized for maximum
536 likelihood inference (-automated1). Trimmed alignments were then concatenated with a second
537 custom Python script into a single alignment with 6917 parsimony informative sites. We used IQtree
538 (Minh et al., 2020) with ModelFinder (Kalyaanamoorthy et al., 2017) and UFBoot (-m MFP -B 1000 -
539 bnni -alrt 1000) (Hoang et al., 2018) to identify an optimal substitution model (GTR+F+I+R10) and infer
540 a consensus maximum likelihood phylogeny from this concatenated alignment with 1000 bootstrap
541 replicates. For the figures, the outgroup (*Dothistroma septosporum* NZE10) and isolate CBS131901

542 (short-read assembly of 0WU) were removed. All custom scripts can be downloaded from
543 https://git.wur.nl/like.fokkens/clado_avr6.

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

545 To infer mating types of *F. fulva* strains, we used megablast (v 2.13.0+) (Morgulis et al., 2008) with
546 default settings to align *Mat1-1* and *Mat1-2* sequences (accessions DQ659350.2 and DQ659351.2
547 respectively). For identification of effector genes in the assemblies, we used a custom python script
548 implementing bedtools getfasta (v2.30.0) (Quinlan & Hall, 2010) and a list of predicted effector genes
549 from Zaccaron et al. 2022 to obtain a fasta file with sequences from the assembly of the Race 5
550 reference strain (GCA_020509005.2) including introns. We then used megaBLAST (v 2.13.0+) with –
551 default settings to search for homologs of each of these genes. To obtain a similarity score for each
552 strain/assembly and each effector gene, relative to the Race 5 reference, we divided the maximum
553 Smith-Waterman score of the effector gene against the assembly with its maximum score against the
554 assembly of Race 5. We used a custom Python script to parse the BLAST output table and calculate
555 these scores (**Figure S1**). For known *Avr* genes we generated MSAs to identify distinct genotypes. To
556 confirm absence of predicted effectors (**Figure S1**) and to determine the size of the deletion resulting
557 in loss of *Avr6*, we used Bowtie2 (v2.5.4, with -I 100 -X 1000) (Langmead & Salzberg, 2012) to align
558 reads to the assembly of the Race 5 strain, and checked the regions where effectors were predicted to
559 be absent in IGV (Robinson et al., 2011). For some strains certain effectors were not present in the
560 assembly, while we did find reads mapped to the gene in the Race 5 genome, which is indicated in
561 **Figure S1**. We used custom R scripts to plot the phylogeny with different *Avr* genotypes (**Figure 1**), and
562 with a heatmap with effector gene sequence similarity (**Figure S1**).
563

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

566 A Comparative Subsequence Sets Analysis (CoSSA) bioinformatics pipeline was applied to find the
567 resistance-specific haplotype (Prodhomme et al., 2019). Sequence reads were trimmed and sequencing
568 adaptors were removed using fastp (version 0.23.4) (Chen, 2023) (**Table S11**). *K*-mer Counter (KMC)
569 and KMC tools (version 3.2.4) were used to produce *k*-mer (*k*=31) tables from the trimmed reads, and
570 *k*-mer table subtraction, intersection and coverage-based filtering (Kokot et al., 2017). GenomeScope
571 2.0 (Ranallo-Benavidez et al., 2020) was used to visualize the *k*-mer coverage profiles. The NR-bulk
572 specific *k*-mer table was subtracted from the R-bulk specific table, and a *k*-mer table of the susceptible
573 parent (MM-*Cf-0*) was subtracted from the resulting R-bulk specific *k*-mers, and intersected by a *k*-mer
574 table of the resistant parent (Ontario 7818). *K*-mer sequences with coverage outside a range of 26 to

575 43 were removed to reduce error-to-noise ratio. Cutoff values are calculated as 25% margins of the
576 expected coverage of the *R* haplotype. Filtered *k*-mers were mapped to the SL4.00 tomato reference
577 genome (Hosmani et al., 2019) using BWA aln (version 0.7.18) (Li & Durbin, 2009) and plotted based
578 on genomic location and coverage. Used scripts reflect the workflow of (Prodhomme et al., 2019) and
579 are available on Github (https://github.com/PBR/CoSSA). Structural predictions of Cf-6 candidate
580 proteins were performed using predicted protein sequences from the ITAG4.0 annotation of the Heinz
581 1706 SL4.0 genome using InterPro (Paysan-Lafosse et al., 2023).

582 **Homolog searches and AlphaFold2 structural predictions**

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

593 ***F. fulva* disease assays**

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

602 **PVX-mediated expression assays**

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

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

608 **_F. fulva_ gene complementation**

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

612 **_Avr6_ allelic variation analysis**

613 *Avr6* was amplified from the genomic DNA of 90 *F. fulva* strains using primers Avr6 Fw2 (5'-
614 CTGACATAGCATTACACGAGCAG-3') and Avr6 Rev (5'-CGCGTCGCCTGATAGATTTG-3) by PCR, as previously
615 described for the *Avr9B* gene (de la Rosa & Schol et al., 2024). Amplicons were purified using ExoSAP-
616 IT™ PCR Product Cleanup Reagent (Thermo Scientific) and sequenced by Macrogen™ Europe
617 (https://www.macrogeneurope.com) by means of Sanger Sequencing using an ABI 3730xl DNA
618 analyzer (Applied Biosystems). Consensus sequences were generated and imported in Benchling
619 (Biology Software, https://benchling.com) for end-trimming and sequence alignment to the *Ecp5*
620 coding sequence (CLAFUR5_03423) from the *F. fulva* strain Race 5 reference genome (Zaccaron et al.,
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634

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