



# *Fulvia fulva* [syn. *Cladosporium fulvum*, *Passalora fulva*] races in Argentina are evolving through genetic changes and carry polymorphic *avr* and *ecp* gene sequences

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**Abstract** The aim of this work was to study further the population of *Fulvia fulva* [syn. *Cladosporium fulvum*, *Passalora fulva*] in Argentina particularly in terms of diversity at the genetic level by means of ISSR. Also, we studied polymorphisms within *avr* and *ecp* sequences, what incidentally might lead to the development of new races. Argentinian tomato cultivars only are affected by two races of *F. fulva* and the data we provide here indicates that the populations of these two races are under an evolutionary process. Representatives of them had several

polymorphisms within the coding sequences of effectors, but they are not constrained to *avr* genes, probably because, based on field data, resistant cultivars are not available. Most polymorphisms observed in *avr* and *ecp* genes were deletions or insertions (INDELs) or single nucleotide polymorphisms (SNPs). Therefore, probably the environment and management practices are the most important factors driving evolution of these races in Argentina and this might explain why the pathogen population is not driven at the pathogenic or race level.

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## Introduction

*Fulvia fulva* (Cooke) Cif. [syn. *Cladosporium fulvum* Cooke, the name traditionally quoted in the literature for this fungus and because of this used throughout this paper], and *Passalora fulva* (Cooke) U. Braun & Crous] (Mycosphaerellaceae, Ascomycota) is a hemibiotrophic fungal pathogen that causes leaf mould of tomato (*Solanum lycopersicum* L.; Thomma et al. 2005). This is a disease that occurs mainly in greenhouse-grown tomatoes, where high relative humidity and temperatures around 20 °C prevail (Thomma et al. 2005). Conidia on the adaxial leaf surface germinate and runner hyphae penetrate through stomata (De Wit 1977; Lazarovits and Higgins 1976). Within a week, diffuse light green to yellowish spots develop on the abaxial leaf surface that gradually increase in size. Within two weeks the fungus starts to sporulate on the abaxial leaf surface and the lesions gradually become necrotic (Blancard and Iglesias 1988; Thomma et al. 2005).

The *C. fulvum*-tomato interaction complies with the gene-for-gene relationship, in which each pathogen avirulence (*avr*) gene product is recognized by a receptor protein encoded by a corresponding dominant host Cf resistance gene (Stergiopoulos and De Wit 2009). AVR proteins serve dual functions; in the absence of a matching Cf receptor protein in the plant, they contribute to virulence and serve as effector proteins facilitating apoplastic leaf colonization (compatible interaction). However, in tomato plants carrying Cf receptors, matching effector proteins are recognized and trigger a Cf-mediated resistance response (incompatible interaction). This is usually associated with a hypersensitive response (HR) (Heath 2000) and by the accumulation of callose, phytoalexins and pathogenesis-related proteins (De Wit 1982; De Wit 1992), which are effective tools against hemibiotrophic pathogens like *C. fulvum* (De Wit et al. 2009a; De Wit et al. 2009b; Dixon et al. 1996; Wulff et al. 2009; De Wit 2016). Many effector proteins of *C. fulvum* have been isolated to date (Van Kan et al. 1991; Van den Ackerveken et al. 1993; Joosten et al. 1994; Laugé et al. 2000; Luderer et al. 2002a; Westerink et al. 2004a; Bolton et al. 2008;

Stergiopoulos et al. 2012; Ökmen et al. 2013; Mesarich et al. 2014; Mesarich et al. 2018).

Plant-pathogenic fungi can be characterized by their lifestyle, which is the result of complex interactions with their hosts with whom they coevolved. Plant immune receptors impose a high selection pressure on pathogen effectors that mostly evolved in such a way that they avoid or escape recognition though retaining their function, which has been found in many pathosystems including the *C. fulvum*-tomato interaction (Allen et al. 2004; Dodds et al. 2006; Ma and Guttman 2008; Soanes and Talbot 2008; Stavrinos et al. 2008; Van Den Hooven et al. 1999, 2001). Also, horizontal gene transfer might increase pathogens aggressiveness, expanding in this way their host range (Stergiopoulos and De Wit 2009). Since the 1930s, different Cf resistance genes against *C. fulvum* have been introduced in tomato cultivars (Lindhout et al. 1989; Rivas and Thomas 2005), which raised the selection pressure on the fungal population, leading to various types of adaptations in effector proteins. Regarding this, gene loss or pseudogenization might contribute to the adaptation of fungal pathogens to a hemibiotrophic lifestyle like that revealed by *C. fulvum*. An example of this might be the limited number of genome encoded proteases that are expressed during *C. fulvum* infection (Jashni et al. 2019).

In *C. fulvum*, five *avr* genes (*avr2*, *avr4*, *avr4E*, *avr5* and *avr9*) have been cloned (Joosten et al. 1994; Luderer et al. 2002b; Van den Ackerveken et al. 1992; Van Kan et al. 1991; Westerink et al. 2004a, b; Mesarich et al. 2014), all encode proteins that trigger HR in tomato plants carrying the cognate Cf-2, Cf-4, *Hcr9-4E*, Cf-5 and Cf-9 genes, respectively (Dixon et al. 1996; Jones et al. 1994; Takken et al. 1999; Thomas et al. 1997; Dixon et al. 1998). *Cladosporium fulvum* Avr proteins are small, secreted, cysteine-rich, and the nomenclature of the races of the strains of *C. fulvum* is according to their virulence, which depends on the presence/absence of Avr proteins (Gabriëls 2006). In addition to these five *avr* genes, seventy genes coding for extracellular proteins (*ecp*) from *C. fulvum* have now been cloned recently (Mesarich et al. 2018). A total of fourteen Ecps have been shown to trigger HR in wild species of tomato (*Solanum* spp.). These were Ecp1, Ecp2–1, Ecp4, Ecp5, Ecp6, Ecp8, Ecp9–1, Ecp10–1, Ecp11–1, Ecp12, Ecp13, Ecp14–1, Ecp15 and Ecp16 that carry the cognate Cf-*ecp* genes (Laugé et al. 1998; Laugé et al. 2000; Van der Hoorn et al. 2001; Kruijt et al. 2005; Thomma et al. 2011; Mesarich et al. 2018;

Iakovidis et al. 2020; Kahlon et al. 2020). Recently, Iakovidis et al. (2020) described the genes encoding recognition of the *C. fulvum* Ecp5 that are encoded by several loci within the genome of tomato.

Various types of mutations in *avr* genes of *C. fulvum* have been reported, including deletions and changes that can either lead to their loss or provide stability/instability to effector proteins that might be no longer recognized by their cognate Cf receptors (Westerink et al. 2004b; Van Kan et al. 1991, Van Den Burg et al. 2006). In this respect, Stergiopoulos et al. (2007) found that nucleotide variation in *ecp* genes occurred at a lower frequency than in *avr* ones and also that they are mostly silent substitutions or modifications in non-protein-coding sequences (Stergiopoulos et al. 2007), which might be explained by the fact that Cf-*ecp* genes have not been introduced in commercial tomato varieties regularly (Laugé et al. 1998).

Hybrid cultivars of tomato grown in Argentina are susceptible to *C. fulvum* and this fungus causes significant economic losses in greenhouse-grown tomatoes in the provinces Corrientes and Buenos Aires (Rollan et al. 2013). Medina et al. (2015) isolated, characterized and analysed several isolates of this fungus collected from diseased tomatoes in these provinces. We monitored the disease in recent years and collected additional isolates, all belonged either to race 0 or 2.

The aim of the present work was to increase our knowledge regarding the population of *C. fulvum* in Argentina particularly in terms of its diversity. We analysed diversity of *C. fulvum* isolates by means of ISSR and then we studied polymorphisms within *avr* and *ecp* sequences that code for virulence factors, which might lead to the appearance of new races.

## Materials and methods

### Isolates of *Cladosporium fulvum*

Eight new isolates of *C. fulvum* were obtained from tomato cultivars with typical leaf mould disease symptoms. They were collected in different locations of the two main tomato production areas of Argentina (Table 1). Monosporic cultures of isolates were maintained on potato dextrose agar (PDA) at 4 °C (Rollan et al. 2013), and deposited in the culture collection of the Centro de Investigaciones de Fitopatología (CIDEFI), Universidad Nacional de La Plata (UNLP). In addition,

12 previously described isolates were also included in this study (Medina et al. 2015).

### Genomic DNA extraction

Total genomic DNA was extracted from monosporic cultures using the Wizard® Genomic DNA Purification kit. The quality and quantity of genomic DNA was evaluated by electrophoresis at 90 V for 30 min in a 0.7% (w/v) agarose gel that was stained with ethidium bromide (10% v/v). Gels were visualized using UV light and images were captured with the GeneSnap software. Genomic DNA was quantified by comparing DNA bands with those of a molecular marker lambda genome digested with *HindIII* (Invitrogen) through the GeneTools in an image analyser (SynGene, Cambridge, UK), according to the methodology described by Franco et al. (2017).

### Genetic diversity of *C. fulvum* isolates

Diversity among the isolates was done by analysis of Inter Simple Sequence Repeat (ISSR) fingerprints. As outgroup *S. lycopersici* CIDEFI 216 was included in the analysis (Franco et al. 2017). Seven ISSR primers were used (AA<sub>5</sub>, AN, GA<sub>5</sub>, IA<sub>5</sub>, BA<sub>3</sub>, FA<sub>5</sub>, and LA<sub>5</sub>, Additional Table 1) to perform PCR amplifications as described by Franco et al. (2017). PCRs were run in a PTC-0150 MiniCycler (MJ. Research) thermocycler programmed as follows: an initial denaturation step at 94 °C for 7 min, followed by 33 cycles of 94 °C for 1 min, 48 °C (primers AA<sub>5</sub>, AN, GA<sub>5</sub>, IA<sub>5</sub> and BA<sub>3</sub>) or 53 °C (primers FA<sub>5</sub> and LA<sub>5</sub>) for 75 s and 72 °C for 4 min, and then a final extension of 72 °C for 7 min. ISSR-PCR products were resolved in 1.5% agarose gels stained with ethidium bromide. Gels were run at 70 V, and then were exposed to UV illumination and images were captured with GeneSnap software (SynGene). The ISSR-PCR banding patterns obtained from stable amplified bands were analysed according to statistical methods used by Franco et al. (2017).

### PCR of internal transcribed spacer (ITS)

The ITS region was amplified by ITS-4 and ITS-5 primers (Additional Table 2, White et al. 1990), using fungal genomic DNA as template. Reaction mixtures contained 1× amplification buffer (Inbio Highway), 1.5 mM MgCl<sub>2</sub> (Inbio Highway), 40 ng of each primer,

**Table 1** Isolates of *C. fulvum* used in this study and their sampling geographic source as well as tomato cultivar of origin

Isolate CIDEFI	Race	Tomato cultivar	Year of sampling	Site of sampling	Reference
300	2	Elpida	2011	Los Hornos, La Plata	Medina et al. 2015
301	2	Elpida	2011	Abasto, La Plata	Medina et al. 2015
303	0	Elpida	2012	Los Hornos, La Plata	Medina et al. 2015
305	2	Colibri	2012	Arana, La Plata	Medina et al. 2015
307	2	Cherry	2012	Arana, La Plata	Medina et al. 2015
309	0	Elpida	2012	Arana, La Plata	Medina et al. 2015
310	0	Compak	2012	Arana, La Plata	Medina et al. 2015
311	2	Colibri	2012	Arana, La Plata	Medina et al. 2015
312	2	Cherry-Colly	2012	Arana, La Plata	Medina et al. 2015
315	0	Elpida	2012	Olmos, La Plata	Medina et al. 2015
318	0	Elpida	2012	Etcheverry, La Plata	Medina et al. 2015
320	2	Keitor	2013	Corrientes	Medina et al. 2015
322	2	Yigido	2014	Abasto, La Plata	<i>This study</i>
323	2	Verde Cebra	2014	Abasto, La Plata	<i>This study</i>
325	2	Elpida	2015	Gorina, La Plata	<i>This study</i>
326	0	Pantano	2015	Abasto, La Plata	<i>This study</i>
327	2	Banano	2015	Abasto, La Plata	<i>This study</i>
329	2	Banano	2012	Abasto, La Plata	<i>This study</i>
330	0	Elpida	2012	Los Hornos, La Plata	<i>This study</i>
332	0	Elpida	2015	Abasto, La Plata	<i>This study</i>

200 mM each deoxynucleoside triphosphate (Inbio Highway), 25 ng of DNA template, and 1 U of Taq DNA polymerase (Inbio Highway) in a 15- $\mu$ l volume. Reactions were performed in a PTC-1152 Mini Cycler (MJ Research) programmed as follows: an initial step at 94 °C for 4 min; followed by 33 cycles of a denaturing step at 94 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min; with a final extension at 72 °C for 5 min.

PCR products were resolved by electrophoresis in 1% (w/v) agarose gels stained with 10% ethidium bromide (v/v). A 100 to 1000 bp DNA ladder marker (Inbio Highway) was used to estimate the size of the amplicons. Gels were observed and documented in a GeneGenius analyser (Syngene) and the bands were processed by means of GeneSnaps, GeneTools, and GeneDirectory software (Syngene). Amplicons were purified as described by Sambrook et al. (1989), precipitated, and sequenced at MACROGEN Inc. (Seoul, South Korea). ITS sequences were deposited at the National Centre for Biotechnology Information (NCBI) GenBank (Additional Table 3).

#### PCR amplification of *avr* genes and race identification

The primers used in the amplification reactions were those described by Stergiopoulos et al. (2007), which were modified since the universal M13 sequence was removed (Additional Table 2). PCRs were performed in a 15- $\mu$ l volume with a thermocycler (Model Multigene gradient; Labnet International, In) programmed as follows: an initial denaturing step at 94 °C for 5 min; then, 40 cycles of 30 s at 94 °C; an annealing step of 1 min at 63, 65, 53.5 and 62.5 °C for *avr2*, *avr4*, *avr4E* and *avr9* amplification, respectively; and 1 min of extension at 72 °C according to the procedure described by Medina et al. (2015). Reactions contained 40 ng of genomic template DNA isolated from monospore cultures (Table 1), 1.5 mM MgCl<sub>2</sub> (Inbio Highway), 0.40 mM of each primer pair (corresponding to *avr2*, *avr4*, *avr4E* and *avr9*, respectively; Additional Table 2), 0.2 mM dNTPs (Inbio Highway), and 1 U of T-Plus DNA polymerase (Inbio Highway) in 1 $\times$  reaction buffer (Inbio Highway). Amplified PCR products were

**Table 2** Allelic variation in the avirulence (*Avr*) and extracellular protein (*Ecp*) effector genes of *Cladosporium fulvum*. In black font are listed the variations found in the isolates of *C. fulvum* presented in this study

Gene	Polymorphism at DNA level	Isolates	Gene region	Predicted mutation in protein
<i>Avr 2</i>	15 <i>del. G</i>	318	Exon 1	–
	22 <i>del. C</i>	309	Exon 1	–
	<b>26 A &gt; C</b>	<b>330</b>	<b>Exon 1</b>	–
	55 T > G	309	Exon 1	Trp > Gly Signal peptide
	56 G > T	315	Exon 1	Trp > Leu Signal peptide
	191 A > G	309	Intron 1	–
	387 <i>del. G</i>	309	Exon 2 after stop	–
	424 <i>del. T</i>	309	Exon 2 after stop	–
	441 <i>del. A</i>	309	Non coding region	–
	460 <i>del. A</i>	309	Non coding region	–
	469 <i>del. A</i>	309	Non coding region	–
	<i>Avr 4</i>	1 C > A	310	Non coding region
23 <i>ins. C</i>		301	Non coding region	–
48 G > T		309	Non coding region	–
<b>63 del. C</b>		<b>325</b>	<b>Non coding region</b>	–
75 A > C		309	Non coding region	–
77 A > T		309	Non coding region	–
80 G > A		300, 301, 305, 307, 311	Non coding region	–
<b>80 G &gt; A</b>		<b>322, 323, 327, 329, 332</b>	<b>Non coding region</b>	–
82 A > C		309	Non coding region	–
83 C > T		309	Non coding region	–
89 A > C		309	Non coding region	–
90 A > G		309	Non coding region	–
103 C > T		309	Non coding region	–
114 A > C		309	Non coding region	–
220 C > A		309	Exon 1	Synonymous (Signal peptide)
<b>493 G &gt; A</b>		<b>325</b>	<b>Exon 1</b>	Synonymous
678 A > T		305	Non coding region	–
<i>Avr 4E</i>	364 C > T	All	Exon 1	Phe > Leu
	<b>364 C &gt; T</b>	<b>All</b>	<b>Exon 1</b>	Phe > Leu
	398 C > T	All	Exon 1	Met > Thr
	<b>398 C &gt; T</b>	<b>All</b>	<b>Exon 1</b>	Met > Thr
<i>Avr 9</i>	<b>18 G &gt; T</b>	<b>326</b>	<b>Non coding region</b>	–
	137 T > G	311	Exon 1	–
	<b>225 A &gt; T</b>	<b>325</b>	<b>Intron 1</b>	–
	363 <i>del. C</i>	312	Exon 2	–
	412 <i>del. G</i>	311	Exon 2 after stop	–
	426 <i>del. T</i>	311	Exon 2 after stop	–
	<b>501 G &gt; C</b>	<b>332</b>	<b>Exon 2 after stop</b>	–
	<b>505 ins. T</b>	<b>332</b>	<b>Exon 2 after stop</b>	–
	<b>516 T &gt; C</b>	<b>332</b>	<b>Exon 2 after stop</b>	–
	<b>518 ins. G</b>	<b>332</b>	<b>Exon 2 after stop</b>	–
<b>535 ins. A</b>	<b>332</b>	<b>Exon 2 after stop</b>	–	
<i>Ecp1</i>	93 A > T	307	Non coding region	–

**Table 2** (continued)

Gene	Polymorphism at DNA level	Isolates	Gene region	Predicted mutation in protein
	97 A > G	307	Non coding region	–
	120 <i>ins.</i> C	300, 305, 307, 318, 320	Non coding region	–
	<b>120 <i>ins.</i> C</b>	<b>323, 325, 326, 327, 329, 330, 332</b>	<b>Non coding region</b>	–
	217 <i>del.</i> G	307	Non coding region	–
	487 <i>ins.</i> G	All	Non coding region	–
	<b>487 <i>ins.</i> G</b>	<b>All</b>	<b>Non coding region</b>	–
	522 C > A	323	Non coding region	–
	<b>801 <i>ins.</i> T</b>	<b>322</b>	<b>Intron 1</b>	–
	826 C > T	307	Intron 1	–
	915 C > T	All (except 305)	Intron 2	–
	<b>915 C &gt; T</b>	<b>All (except 323)</b>	<b>Intron 2</b>	–
	943 <i>del.</i> C	All (except 305)	Intron 2	–
	<b>943 <i>del.</i> C</b>	<b>All (except 322)</b>	<b>Intron 2</b>	–
	944 G > C	329	Intron 2	–
	949 C > A	329	Intron 2	–
	964 G > A	329	Exon 3	–
	969 T > G	329	Exon 3	Asp > Asn
	970 A > T	329	Exon 3	–
	974 C > G	329	Exon 3	–
	979 <i>ins.</i> A	329	Exon 3	Frameshift
	<b>1003 <i>ins.</i> G</b>	<b>329</b>	<b>Exon 3 after stop</b>	–
	1025 <i>ins.</i> G	318	Exon 3 after stop	–
	<b>1076 <i>ins.</i> G; <i>ins.</i> TT</b>	<b>326, 329</b>	<b>Exon 3 after stop</b>	–
	1078 G > A	329	Exon 3 after stop	–
	1079 G > A	326, 329	Exon 3 after stop	–
	1085 G > A	329	Exon 3 after stop	–
	1086 C > G	329	Exon 3 after stop	–
	1090 G > A	329	Exon 3 after stop	–
	1092 G > C	329	Exon 3 after stop	–
	1095 <i>ins.</i> A	325, 326, 329	Exon 3 after stop	–
	1107 A > T	329	Exon 3 after stop	–
	1108 C > G	329	Exon 3 after stop	–
	1122 <i>ins.</i> A	326	Exon 3 after stop	–
	1123 G > T	329	Exon 3 after stop	–
	1126 G > C	329	Exon 3 after stop	–
	1129 G > T	329	Exon 3 after stop	–
	1134 G > A	326, 329	Exon 3 after stop	–
	1135 A > G	326	Exon 3 after stop	–
	1137 T > A; T > C	326, 329	Exon 3 after stop	–
	1138 G > T; G > A	326, 329	Exon 3 after stop	–
	1139 T > G; T > A	326, 329	Exon 3 after stop	–
	1140 G > T	326, 329	Exon 3 after stop	–
	1141 C > G	326, 329	Exon 3 after stop	–
	1142 C > T	329	Exon 3 after stop	–

**Table 2** (continued)

Gene	Polymorphism at DNA level	Isolates	Gene region	Predicted mutation in protein
	1144 T>G; T>C	326, 329	Exon 3 after stop	–
	1146 C>T	326	Exon 3 after stop	–
	1148–1153 ACGTCG>CTACGT	326	Exon 3 after stop	–
	1154–1157 <i>ins.</i> TCAA; <i>ins.</i> TCGA	326, 327	Exon 3 after stop	–
	1158 A>G	326, 327	Exon 3 after stop	–
	1161 G>T	329	Exon 3 after stop	–
	1162 G>C; G>T	326, 329	Exon 3 after stop	–
	1163 C>G; C>A; C>T	323, 326, 329	Exon 3 after stop	–
	1165 G>A	326	Exon 3 after stop	–
	1167 G>A	320	Exon 3 after stop	–
	1167 G>C	326	Exon 3 after stop	–
	1169 G>T	329	Exon 3 after stop	–
	1170 A>C	329	Exon 3 after stop	–
	1177 G>C	326	Exon 3 after stop	–
	1178 C>C	329	Exon 3 after stop	–
	1179 C>A	329	Exon 3 after stop	–
	1182 <i>ins.</i> T	326	Exon 3 after stop	–
	1188 G>A	326, 329	Exon 3 after stop	–
	1190 C>A	326	Exon 3 after stop	–
	1192 A>C	326	Exon 3 after stop	–
	1193 C>T	326, 329	Exon 3 after stop	–
	1195 A>C	326, 329	Exon 3 after stop	–
	1197–1198 <i>ins.</i> AA	326	Exon 3 after stop	–
	1201 A>T	329	Exon 3 after stop	–
	1202 A>C	307	Exon 3 after stop	–
	1206 <i>ins.</i> A	326	Exon 3 after stop	–
	1210 G>A	326, 329	Exon 3 after stop	–
	1212 C>A	326	Exon 3 after stop	–
	1213 <i>Ins.</i> C	320	Exon 3 after stop	–
	1213 <i>ins.</i> C; <i>ins.</i> G	326, 322, 332	Exon 3 after stop	–
	1214 G>T	322, 332	Exon 3 after stop	–
	1216 <i>del.</i> T	322	Exon 3 after stop	–
	1218 A>C	329	Exon 3 after stop	–
	1219 G>A	326	Exon 3 after stop	–
	1223 T>C	326	Exon 3 after stop	–
	1224 G>T	326, 329	Exon 3 after stop	–
	1225 <i>ins.</i> G	326	Exon 3 after stop	–
	1229 T>C	329	Exon 3 after stop	–
	1231 G>A	329	Exon 3 after stop	–
	1238 T>A	301	Exon 3 after stop	–
	1238 T>A	326, 329	Exon 3 after stop	–
	1239 C>A	301	Exon 3 after stop	–
	1239 C>A	322, 326, 332	Exon 3 after stop	–
	1242 C>T	329	Exon 3 after stop	–



**Table 2** (continued)

Gene	Polymorphism at DNA level	Isolates	Gene region	Predicted mutation in protein
	1249 T > C	326	Exon 3 after stop	
	1251 <i>ins.</i> C	326	Exon 3 after stop	
	1266 C > T	326	Non coding region	–
	1269 T > C	326	Non coding region	–
	1270 T > C	326	Non coding region	–
	1271–1273 <i>ins.</i> TTG	326	Non coding region	–
	1284 A > G	326	Non coding region	–
	1287–1288 <i>ins.</i> AC	326	Non coding region	–
	1293 C > G	326	Non coding region	–
	1294 G > C	326	Non coding region	–
	1296 A > C	326	Non coding region	–
	1302 <i>del.</i> T	326	Non coding region	
	1311 <i>ins.</i> A	326	Non coding region	–
	1312 C > T	326	Non coding region	–
	1319–1321 AGC > CCG	326	Non coding region	–
	1323 C > T	326	Non coding region	–
	1326 C > G	326	Non coding region	–
	1328 T > C	326	Non coding region	–
	1329 <i>del.</i> G	326	Non coding region	–
	1331 A > G	326	Non coding region	–
	1332 G > A	326	Non coding region	–
	1335 G > T	326	Non coding region	–
	1336 A > G	301	Non coding region	–
	1336 A > G	327	Non coding region	–
	1337 C > A	301	Non coding region	–
	1337 C > A; C > T	326, 327	Non coding region	–
	1338 C > T	326	Non coding region	–
<i>Ecp2</i>	22 <i>del.</i> T	318	Non coding region	–
	22 <i>del.</i> T	323	Non coding region	–
	22–25 <i>del.</i> TGCA	300	Non coding region	–
	28 <i>del.</i> C	329	Non coding region	–
	30–32 <i>del.</i> TAC	320	Non coding region	–
	35 T > A	320	Non coding region	–
	41 C > T	300	Non coding region	–
	42 A > C	320	Non coding region	–
	75 <i>del.</i> G	329	Non coding region	–
	75–76 <i>del.</i> GA	300	Non coding region	–
	79 G > C	320	Non coding region	–
	82 A > T	320	Non coding region	–
	89–90 GT > CC	305	Non coding region	–
	123–124 TT > AC	326	Non coding region	–
	193 <i>ins.</i> C	326	Non coding region	–
	205 C > A	326	Non coding region	–
	257 T > C	326	Exon 1	Synonymous (Signal peptide)



**Table 2** (continued)

Gene	Polymorphism at DNA level	Isolates	Gene region	Predicted mutation in protein
	<b>305 C &gt; A</b>	<b>326</b>	<b>Exon 1</b>	Synonymous
	<b>409 G &gt; T</b>	<b>326</b>	<b>Exon 1</b>	Arg > Ile
	<b>414 T &gt; G</b>	<b>326</b>	<b>Exon 1</b>	Ser > Ala
	663 <i>ins.</i> A	300	Exon 2	Frameshift
	680 <i>ins.</i> G	301	Exon 2	Frameshift
	783–791 <i>ins.</i> GCCCGCGGC	300	Non coding region	–
<i>Ecp4</i>	380 G > T	300, 301, 305, 307, 320	Exon 2	Cys > Trp
	<b>380 G &gt; T</b>	<b>All</b>	<b>Exon 2</b>	<b>Cys &gt; Trp</b>
	<b>395 ins. A</b>	<b>326</b>	<b>Exon 2</b>	Gly > Val
	<b>408 ins. A</b>	<b>326</b>	<b>Exon 2</b>	Frameshift
	<b>423 ins. T</b>	<b>326</b>	<b>Non coding region</b>	–
<i>Ecp5</i>	65 A > T	300, 301	Exon 1	Premature stop codon
	<b>516 del. G</b>	<b>322, 323, 327, 329</b>	<b>Exon 2</b>	<b>Frameshift</b>
	541 G > A	318	Exon 2	Cys > Tyr
	<b>541 G &gt; A</b>	<b>325, 330, 332</b>	<b>Exon 2</b>	<b>Cys &gt; Tyr</b>
	691 <i>ins.</i> G	307	Exon 2	Frameshift +premiure stop codon
	<b>863 del. A</b>	<b>323</b>	<b>Non coding region</b>	–
	867 <i>del.</i> A	305	Non coding region	–
	<b>867 del. A</b>	<b>323, 327</b>	<b>Non coding region</b>	–

processed as previously described. The molecular race identification was based on the presence/absence/sequence of *avr* genes (Medina et al. 2015).

*Avr* gene sequences were deposited at National Centre for Biotechnology Information (NCBI) GenBank (Additional Table 3).

**Table 3** Calculated indexes of molecular diversity based on the sequence variation in the *Avr* and *Ecp* effector genes of *C. fulvum*

Genes	N <sub>a</sub>	Nucleotide diversity parameters			
		Nucleotide diversity	π	θ <sub>S</sub>	(ω)
<i>Avr2</i>	8	0.005658 (0.003810)	2.7500	1.54270	/
<i>Avr4</i>	19	0.002459 (0.016880)	1.6670	4.00562	0
<i>Avr4E</i>	19	0 (0)	0.0000	0.00000	0
<i>Avr9</i>	19	0.002050 (0.001569)	1.1110	1.45368	1.5464
<i>Ecp1</i>	14	0.018247 (0.009580)	24.7250	29.4405	0.7045
<i>Ecp2</i>	14	0.006648 (0.003827)	5.3850	4.40233	0.3414
<i>Ecp4</i>	14	0.001171 (0.001131)	0.5710	0.31445	21,952
<i>Ecp5</i>	13	0.002178 (0.001486)	1.9230	0.64449	23,095

N<sub>a</sub>: Number of isolates used for the analysis. CIDEFI 326 was excluded from calculation of ECP5 due the large deletion

π: Nucleotide diversity based on the average number of nucleotide differences between two random sequences estimated for the entire length of the sequences analyzed for only non-synonymous substitutions, and synonymous ones

θ<sub>S</sub>: The statistic of the number of segregating sites, estimated for the entire length of the sequences analyzed, for non-synonymous and synonymous substitution

(ω) = Ka/Ks = ratio of non-synonymous (Ka) to synonymous (Ks) nucleotide substitution rates. is an indicator of selective pressures on genes

## PCR amplification of *ecp* genes

Primers used in the reactions were those described by Stergiopoulos et al. (2007), except the *ecp1* and *ecp4* forward primers, which were modified to increase the specificity of the reaction (Additional Table 2). PCRs were performed in a 15- $\mu$ l volume with a thermocycler (Model Multigene gradient; Labnet International, In) programmed as follows: an initial denaturing step at 94 °C for 5 min; then, 25 cycles of 30 s at 94 °C; 30 s of annealing at 55, 60, 64 and 64 °C for *ecp1*, *ecp2*, *ecp4* and *ecp5* amplification, respectively; and a 1 min extension stop at 72 °C according to Stergiopoulos and co-workers (Stergiopoulos et al. 2007). All reactions included a final extension at 72 °C for 7 min.

Reactions were performed with 40 ng of genomic template DNA from monosporic cultures (Table 1), containing 1.5 mM MgCl<sub>2</sub> (Inbio Highway), 0.40 mM of each primer pair (corresponding to *ecp1*, *ecp2*, *ecp4* and *ecp5*, respectively; Additional Table 2), 0.2 mM dNTPs (Inbio Highway), and 1 U of T-Plus DNA polymerase (Inbio Highway) in 1 $\times$  reaction buffer (Inbio Highway). PCR amplifications were processed as previously described. *Ecp* sequences obtained were deposited in the NCBI GenBank (Additional Table 3).

## Polymorphism within *avr* and *ecp* genes

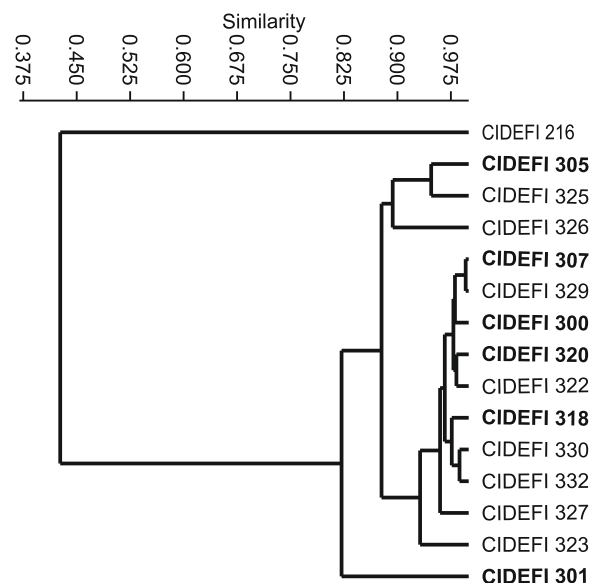
Polymorphisms within *avr* and *ecp* genes of the 8 new isolates of *C. fulvum* as well as those of 12 isolates stored at the CIDEFI collection were identified by comparing nucleotide sequences as well as alignments of the PCR amplicons sequenced (Table 1). DNA sequences were aligned with ClustalW (Thompson et al. 1994) whereas multiple alignments of predicted proteins were made using Muscle (Edgar 2004) in Geneious v9.1.2 (Kearse et al. 2012). Nucleotide diversity were calculated using ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010). The ratio of nonsynonymous (Ka) to synonymous (Ks) nucleotide substitution rates is an indicator of selective pressures on genes and was calculated through the Ka/Ks service provided by Universitetet I Bergen ([services.cbu.uib.no/tools/kaks](http://services.cbu.uib.no/tools/kaks)).

## Results

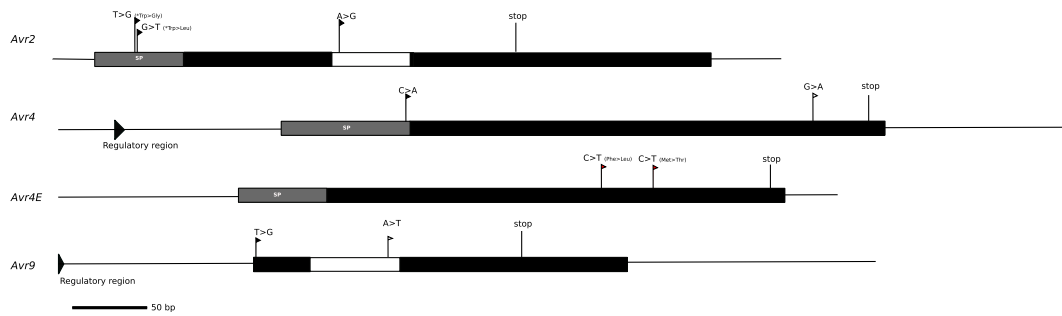
### Genetic diversity within isolates of *Cladosporium fulvum*

The eight isolates described in this study were identified as *Fulvia fulva* (Syn. *Cladosporium fulvum*; *Passalora fulva*) based on the homology of the amplified ITS sequences. This was further supported by the growth of these isolates on PDA, where they developed colonies as well as conidia with morphological characteristics typical of *C. fulvum* (data not shown), and by the fact that we successfully amplified *C. fulvum* *avr2*, *avr4*, *avr4E* and *avr9* genes, providing in this way evidence that they belonged either to race 0 or race 2 of *C. fulvum* (Table 1; Medina et al. 2015). While isolates CIDEFI 326, CIDEFI 330 and CIDEFI 332 were identified as representatives of race 0, CIDEFI 322, CIDEFI 323, CIDEFI 325, CIDEFI 327 and CIDEFI 329 were identified as representatives of race 2.

The seven ISSR primers selected amplified bands that ranged from 250 bp to 6500 bp and were used to assess genetic diversity. We found that the isolates were genetically diverse since 54 amplicons were polymorphic (30.86%). Based on the ISSR data we built a dendrogram using the UPGMA algorithm and Dice.



**Fig. 1** Dendrogram generated by UPGMA cluster analysis using the Dice similarity coefficient based on the ISSR fingerprint of *Cladosporium fulvum* isolates. *S. lycopersici* CIDEFI 216 was included in this analysis as an outgroup



**Fig. 2** Sequence variation at the nucleotide level in *Cladosporium fulvum* avirulence (*Avr*) effector genes. Signal peptides are shown in grey boxes, coding sequences as black boxes and introns as white boxes. The DNA modifications in the isolates described by

Medina et al. (2015) are indicated by black flags and by white flags for isolates described in this study. Of the non-synonymous DNA modifications, the changes in amino acid sequence are indicated

All the isolates were clustered in two groups at a similarity coefficient of 0.883 (Fig. 1). CIDEFI 305 and CIDEFI 325; CIDEFI 307, CIDEFI 329, CIDEFI 300, CIDEFI 320, CIDEFI 322, CIDEFI 318, CIDEFI 330, CIDEFI 332 and CIDEFI 327 isolates were clustered with higher homologies (>95%); however, no relationship was found between clusters and geographical origin, cultivar of pathogen isolation, or race of the isolates.

#### Polymorphism within *avr* and *ecp* genes

Polymorphisms within *avr* genes were analysed by aligning sequences, including those available in public databases (Fig. 2, Table 2, and *See supporting electronic material*). We analysed a 486 bp fragment of *avr2*, which was 100% homologous to the sequence of a European isolate (accession number: AJ421629). Only one variation was found within the *avr2* sequence of the isolates presented in this study. This was a single nucleotide polymorphism (SNP) due to a transversion (26A > C) in the 5' *exon-1* sequence before the start codon of strain CIDEFI 330.

Also we analysed a 678 bp DNA fragment of *avr4* gene and compared it with the sequence obtained by Joosten et al. (1997; accession number: Y08356). The CIDEFI strain sequences presented seven variations, which were found in non-coding sequences (over 98%); they included five SNPs of nucleotide transitions (80G > A) and one deletion (63*delC*). Furthermore, a nucleotide transition was detected in the *avr4* coding sequence of isolate CIDEFI 325, which resulted in a synonymous modification, 493G > A.

We also amplified, analysed and compared the sequence of a 520 bp fragment of *avr4E* with the available sequence of a European isolate (AY546101). Within our

isolates we found 16 polymorphisms, which were located within the coding region. Interestingly, all Argentinian isolates presented an SNP in one codon by a transition (364C > T; 398C > T), which resulted in a non-synonymous modification. One modification consisted of a substitution of Phe by Leu (F82L), while another consisted of a substitution of a Met by Thr (M93T).

The 545 bp partial sequence of the *avr9* gene, was compared with that of a European (accession number: X60284). Interestingly, the isolates described in this study were polymorphic within the non-coding sequence, which has not been reported before in isolates from Argentina. One SNP was found in a non-coding region of *avr9* of CIDEFI 326 (18G > T) and one within the *intron* sequence of CIDEFI 325 (225A > T), both due to transversions. Most mutations were found within the *exon-2* sequence of *avr9*. Two SNPs occurred as transition and transversion of a nucleotide: 497G > C and 516 T > C and three indels (503-504*insT*; 516-517*insG* and 534-535*insA*) were observed in the sequence of CIDEFI 332 isolate. These changes did not provoke changes in the protein sequence since they occurred after the stop codon (382\_384).

Based on nucleotide diversity analysis the *avr2* gene presented the highest number of changes with the analyzed sequence (Table 3), but since they did not lead to amino acid substitutions, we cannot infer if they occur in response to evolutionary forces. In contrast with this, within the *avr9* gene sequence the substitutions observed by Medina et al. (2015) most probably were due to the positive selective pressure since for the sequence of the *Avr9* effector  $\omega$  was greater than 1.

Polymorphisms within *ecp* genes were analysed by aligning our sequences and those available in public databases (Fig. 3, Table 2, and *See supporting*

*electronic material*). The 1338 bp partial sequence of *ecp1* was compared with the only sequence available at the NCBI (accession number: Z14023). The sequences of the Argentinian isolates presented one hundred seventy-five polymorphisms compared to the sequence of a European isolate, 111 SNPs and 64 indels. Interestingly, 166 variations occurred within non-coding sequences of CIDEFI isolates. All the isolates carried two nucleotide insertions (120–121*insC* and 487–488*insG*), except CIDEFI 322 and CIDEFI 301 that only carried the latter insertion. Strain CIDEFI 307, carried two SNPs (93A > T and 97A > G) and a deletion (217*delG*) in a non-coding region. CIDEFI 318 carried a SNP (522C > A). CIDEFI 307 and CIDEFI 322 carried one SNP and one insertion, respectively (826C > T and 801–802*insT*) in the *intron-1* sequence. All isolates, but CIDEFI 305 and CIDEFI 323, carried a SNP causing a transition (915C > T) and a deletion (943*delC*), respectively, in the *intron-2* sequence. Also, CIDEFI 329 showed two additional transversions (944G > C and 949C > A). Again, variations were found within the *exon-3* sequence. The sequence of isolate CIDEFI 329 contained one transition (964G > A) and three transversions (969 T > G, 970A > T and 974C > G). While the transition led to a synonymous modification, transversions resulted in non-synonymous modifications, where an Asp was replaced by Asn (D62N). Additionally, we found one insertion in CIDEFI 318 (1025–1026*insG*) and two (979–980*insA* and 1003–1004*insG*) in the sequence of CIDEFI 329 that caused frameshifts. While the isolates reported by Medina et al. (2015) presented, six SNPs and one indel after the stop codon, the new isolates described in this work contained twenty indels and eighty-one SNPs in the same region.

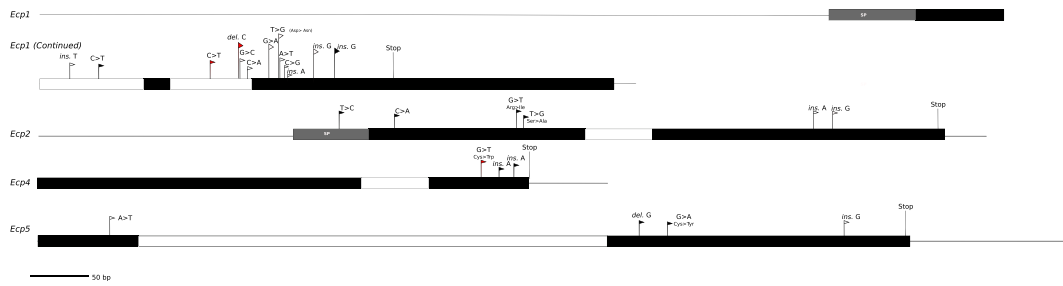
The 811 bp *ecp2* gene fragment of CIDEFI isolates was analysed and compared to other available sequences of *C. fulvum* like as European isolate accession number: Z14024 and twenty-three variations were found. Seventeen of these polymorphisms were located within non-coding regions; among them, six occurred in sequences of the new isolates described in this study. Deletions were found in non-coding sequences of both previously reported and new isolates of *C. fulvum* (22*delT*; 24*delG*; 22\_25*delTTGCA*; 28*delC*; 30\_32*delTAC*; 75*delG*; 75\_76*delGA*). Furthermore, an insertion was found within the same DNA sequence (783–791*insGCCGCGGC*) of CIDEFI 300. Two insertions were found within the coding region of CIDEFI 300 and CIDEFI 301, respectively, (663\_664*insA* and

680\_681*insG*) and the analysis predicted a frameshift (>130 and >137). CIDEFI 326 was the isolate that presented the highest level of variation within the coding sequence. It presented two SNP that led to synonymous mutations, one in the signal peptide sequence (257 T > C) and one within *exon-1* (305C > A). Additionally, *exon-1* had two SNPs by transversion of nucleotides that resulted in non-synonymous changes (409G > T and 414 T > G). Arg was substituted by Ile (R64I) and Ser by Ala (S66A).

Within the 488 bp sequence of the *ecp4* gene, 16 polymorphisms were detected within the coding region of all the isolates analysed that were compared to the only available sequence (EF104526). Except for the sequence of isolate CIDEFI 318, a transversion was detected, (380G > T), that resulted in a non-synonymous change, where Cys was replaced by Trp (C108W). Isolate CIDEFI 326 showed three single nucleotide insertions (395–396*insA*, 408–409*insA* and 423–424*insT*) that resulted in a non-synonymous change, where Gly was replaced by Val (G109V) and with a predicted frameshift (>113).

The 883 bp sequence of *ecp5* showed 17 polymorphisms within both non-coding (over 36%) and coding (over 64%) regions compared to the available sequence of a European isolate (accession number: EF104527). The *ecp5* sequences of CIDEFI 300 and CIDEFI 301 showed a codon change, where AAG (65A > T) was replaced by TAG leading to a premature stop codon (>21, as shown in Fig. 4), which might lead to a truncated protein. In the coding region, isolates CIDEFI 322, CIDEFI 323, CIDEFI 327 and CIDEFI 329 presented a deletion (516*delG*) with a frameshift, whereas the isolates CIDEFI 318, CIDEFI 325, CIDEFI 330 and CIDEFI 332 had a transition (541G > A) that led to a non-synonymous mutation, where Cys was replaced by Tyr (C48Y). Within the same region, CIDEFI 307 had an insertion (691*insG*), that causes a frameshift and premature stop. Besides, deletions were also detected after the stop codon, in isolates CIDEFI 305 (866*delA*), CIDEFI 332 (863*delA* and 866*delA*) and CIDEFI 327 (866*delA*). Finally, CIDEFI 326 carried a 358 bp sequence deletion (Fig. 5), that corresponded to the 3' terminal *intron* and 5' start of *exon-2* of the *ecp5* gene (213–572*del*).

Based on nucleotide diversity analysis, *ecp1* was the extracellular protein where changes occurred the most within the analyzed sequences and also presented the sites segregating the most (Table 3). Interestingly, by the



**Fig. 3** Sequence variation at the nucleotide level in *Cladosporium fulvum* extracellular protein (*Ecp*) effector genes. Signal peptides are shown as grey boxes, coding sequences as black boxes and introns as white boxes. The number of DNA modifications in the

isolates described by Medina et al. (2015) are indicated by black flags and by white flags for isolates described in this study. Of the non-synonymous DNA modifications, the changes in aminoacid sequence are indicated

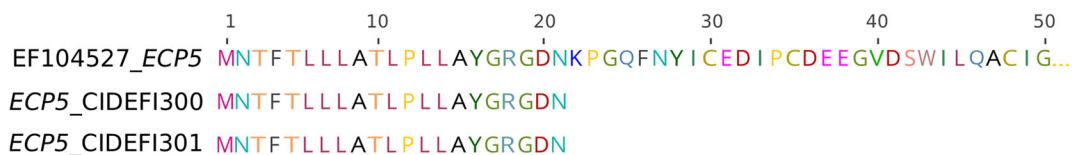
*Ka/Ks* ratio analysis, it appears that *ecp1* and *ecp2* are under a negative pressure so that the protein sequence is conserved whereas *ecp4* and *ecp5* are under positive selective pressure (Table 3).

**Discussion**

*Cladosporium fulvum* collected from production areas in Argentina belonged either to race 0 or 2 (Medina et al. 2015) and the new isolates described here also belonged to these two races. However, we found that genetic diversity was considerable and unrelated to the tomato cultivar and the area of collection. Identification of pathogens races is critical to develop a strategy to manage diseases through resistance genes. Identification of races of *C. fulvum* can be done either by inoculating isolates on a set of tomato *cv* Money Maker differentials carrying known *Cf* genes (*Cf*-2, *Cf*-4, *Cf*-5, *Cf*-9; Rollan et al. 2013) as well as by the amplification of *avr* genes in a PCR multiplex (Medina et al. 2015). Such amplicons can be sequenced and in this way we might identify mutations that eventually might overcome known *Cf*- genes. Until now the Argentinian population of *C. fulvum* belong to race 0 or 2 (Medina et al. 2015), the fact that most cultivars are susceptible to the disease suggest that in Argentina tomato hybrids lack the necessary resistance genes to prevent the disease.

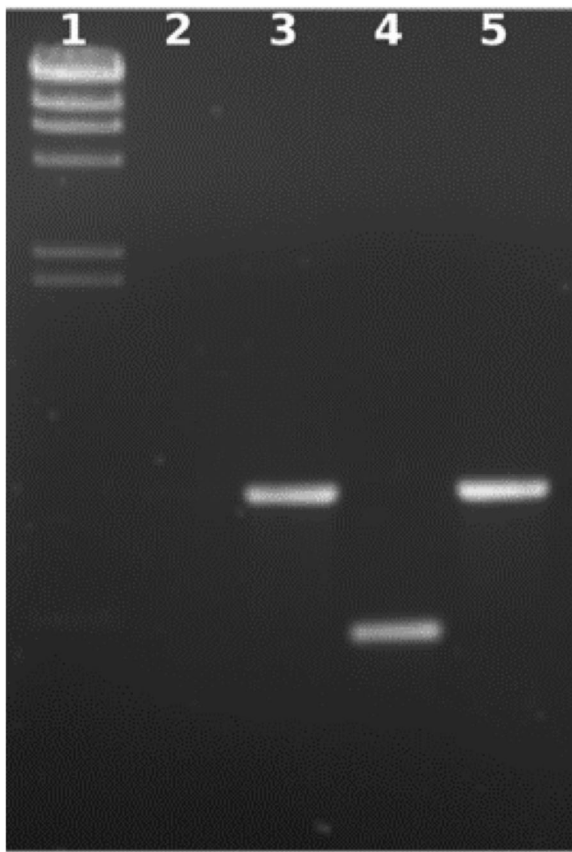
Despite the fact that many polymorphisms were found within the partial sequences of *avr* genes, the *avr4E* gene was the only one that presented a non-synonymous variation within the coding sequence, which was found in all the isolates from Argentina suggesting that the parental strains already carry the mutation and then additional genetic changes occurred in the strains of the CIDEFI collection. Two-point mutations led to two amino acid residue substitutions in AVR4E proteins, such changes have already been reported in isolates from other countries (Westerink et al. 2004b; Medina et al. 2015; Iida et al. 2015). Westerink et al. (2004b) found that the substitution of Phe by Leu within AVR4E protein was enough to avoid recognition by Cf-4E (Westerink et al. 2004b). Furthermore, Iida et al. (2015) found within Japanese isolates two substitutions within the AVR4E protein, the first one was similar to the non-synonymous change described in this work, whereas in the other one a Met was replaced by an aromatic amino acid, Tyr. In the isolates analysed here, Met was replaced by Thr. The fact that a non-synonymous substitution provoked the replacement within AVR4E protein sequence, where a hydrophobic amino acid (Met) was replaced by a hydrophilic one (Thr) should modify the peptide function, suggesting this that this is under positive selection (Stergiopoulos et al. 2014).

*Ecp* genes encode proteins that have been considered virulence factors, although their roles remain to be



**Fig. 4** Alignment of the predicted Ecp5 protein sequence from strains CIDEFI 300 and CIDEFI 301 with the reference Ecp5 protein sequence, EF104527 (115 aa)





**Fig. 5** Agarose gel (1% w/v) of the amplification product of the *ecp5* gene (882 bp). Line 1: molecular weight marker. Line 2: negative control. Line 3: Product amplified from CIDEFI 305 strain. Line 4: Product amplified from CIDEFI 326 strain. Line 5. Product amplified from CIDEFI 322 strain

elucidated (Stergiopoulos et al. 2010). However, these proteins play an essential role in infection and colonization, since their deletion results in less virulent *C. fulvum* mutants (De Wit et al. 2009a; De Wit et al. 2012; Iakovidis et al. 2020). Stergiopoulos et al. (2007) stated that the frequency of loss of these genes within fungal populations is low, which might explain their presence in Argentinian isolates as well as their key role in pathogenesis. The biological role of ECP6 has been described; it codes a protein with three lysin motif domains that alter chitin-triggered immunity (Bolton et al. 2008; Bolton and Thomma 2008; De Jonge et al. 2010; Sánchez-Vallet et al. 2013). Luderer et al. (2002b) predicted that ECP1 and ECP5 might be rich in Cys residues and many of them form intramolecular disulphide bonds in protein, which is required for stability and function in the protease-rich leaf apoplast of tomato. Regarding the function of the other ECP proteins, fungal

transformants where *Ecp1* or *Ecp2* were knocked out were impaired in aggressiveness in mature tomato plants (Laugé et al. 1997). Based on this and on the changes reported within the gene sequence of the predicted ECP1 protein, of CIDEFI 318 and CIDEFI 329 most probably these sequence changes impaired aggressiveness of the isolates. Furthermore, the change of Asp by Asn in the deduced sequence from CIDEFI 329; where an electrophilic amino acid was replaced by a hydrophilic one might alter the peptide's function, which deserves to be studied further.

Stergiopoulos et al. (2014) found many polymorphisms within *ecp2* genes from *Mycosphaerella fijiensis*. The authors found a change at position 66, where Ala was substituted by His or Gly. In our study, we found that Ser, a hydrophilic amino acid, was replaced by Ala, that is hydrophobic, which, at this position, might affect protein folding and probably its activity. Furthermore, the aggressiveness of CIDEFI 300 and CIDEFI 301, while interacting with tomato, should be affected due to the change in the C-terminal region of *Ecp2*. So, it appears that it is crucial to evaluate the role of such changes in virulence, which should be done by generating mutants.

Regarding the ECP4 protein, Mesarich et al. (2018) found that all six Cys residues are mostly conserved between *Ecp4* and its homolog, *Ecp7*. Interestingly, in Argentinian isolates, one Cys residue at position 108 was replaced by Trp, a change that might affect their aggressiveness as well as fitness. Additionally, ECP4 of CIDEFI 326 most probably has no activity due to the loss of a stop codon; consequently, fitness of the isolate to interact with tomato might have been negatively affected.

Regarding the ECP5 protein, it has six cysteine residues and is the least polymorphic *C. fulvum* effector (Stergiopoulos et al. 2007; De Wit et al. 2009b). Iakovidis et al. (2020) found that genes encoding ECPs that inducing an HR segregated as a monogenic trait, mapping to distinct loci in the tomato genome and identified at least three loci within chromosomes that might harbour distinct *Cf-ecp5* genes. Based on an analysis of nucleotide and amino acid sequences, we can infer that the predicted change of Cys by Tyr as well as the premature stop codons that produce truncated ECP5 products and the frameshifts that change N-terminal or C-terminal regions might harm fitness of *C. fulvum*, to infect and colonize the apoplastic space.

It is possible that the large variation (SNP, indels) that occurred in non-coding sequences of *ecp* genes provide fungal isolates with an adaptive advantage in

the environments assayed though this has to be studied in further detail.

## Conclusions

Argentinian tomato cultivars are affected by only two races of *C. fulvum* whose populations are under an evolutionary process. Representatives of these two races present several polymorphisms in effectors, which are not constrained to *avr* genes, probably because, based on field data, resistant cultivars are not available. Variations found in the gene sequences of *avr2*, *avr4* and *avr9* within CIDEFI isolates were neutral for pathogenesis on tomato. This is the first report showing polymorphisms within *ecp* genes and the changes observed in Argentinian isolates suggest that they might not be such relevant factors of pathogenesis. There seems to be different evolutionary forces acting upon *ecp* genes, since, while *ecp1* and *ecp2* seemed to be under a negative pressure for changes, suggesting that they might probably play a key biological role, *ecp4* and *ecp5* are under a positive selective pressure, reflected by the amino acid changes and nonfunctional predicted peptides generated by frameshift and premature stop codons. Still additional studies regarding virulence and aggressiveness of the isolates described should be done in order to evaluate the effect of polymorphisms within *ecp* genes. Therefore, probably the environment and management practices are the most important factors driving evolution of these races in Argentina and this explains why the pathogen population is evolving but not at the pathogenic or race level.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s10658-020-02181-9>.

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**Compliance with ethical standards**

**Declaration of competing interest** The authors declare that they have no competing interests.

This work does not include research involving human participants and/or animals.

## References

- Allen, R. L., Bittner-Eddy, P. D., Grenville-Briggs, L. J., Meitz, J. C., Rehmany, A. P., Rose, L. E., & Beynon, J. L. (2004). Host-parasite coevolutionary conflict between Arabidopsis and downy mildew. *Science*, *306*(5703), 1957–1960. <https://doi.org/10.1126/science.1104022>.
- Blancard, D., & Iglesias, A. P. (1988). Enfermedades del tomate: observar, identificar, luchar (Nº. 635.6429 B535E.). *Mundi-Prensa*.
- Bolton, M. D., & Thomma, B. P. H. J. (2008). The complexity of nitrogen metabolism and nitrogen-regulated gene expression in plant pathogenic fungi. *Physiological and Molecular Plant Pathology*, *72*(4–6), 104–110. <https://doi.org/10.1016/j.pmp.2008.07.001>.
- Bolton, M. D., Van Esse, H. P., Vossen, J. H., De Jonge, R., Stergiopoulos, I., Stulemeijer, I. J. E., et al. Bolton M.D., van Esse H.P., Vossen J.H., de Jonge R., Stergiopoulos I., Stulemeijer I.J.E., van den Berg J.C.M., Borrás-Hidalgo O., Dekker H.L., de Koster C.G., de Wit P.J.G.M., Joosten M.H.A.J., & Thomma B.P.H.J. (2008). The novel *Cladosporium Fulvum* lysin motif effector Ecp6 is a virulence factor with orthologues in other fungal species. *Molecular Microbiology*, *69*(1), 119–136. <https://doi.org/10.1111/j.1365-2958.2008.06270.x>.
- De Jonge R., van Esse, H.P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., van der Krol, S., Shibuya, N., Joosten M.H.A.J., & Thomma B.P.H.J. (2010). Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science*, *329*(5994), 953–955. <https://doi.org/10.1126/science.1190859>.
- De Wit, P. J. G. M. (1977). A light and scanning-electron microscopic study of infection of tomato plants by virulent and avirulent races of *Cladosporium Fulvum*. *Netherlands Journal of Plant Pathology*, *83*(3), 109–122. <https://doi.org/10.1007/BF01981556>.
- De Wit, P. J. G. M. (1982). Partial characterization and specificity of glycoprotein elicitors present in filtrates of cultures and cell walls of *Cladosporium fulvum*. *Active Defence Mechanisms in Plants*, 364–365.
- De Wit, P. J. G. M. (1992). Molecular characterization of gene-for-gene systems in plant-fungus interactions and the application of avirulence genes in control of plant pathogens. *Annual Review of Phytopathology*, *30*(1), 391–418. <https://doi.org/10.1146/annurev.py.30.090192.002135>.
- De Wit, P. J. G. M. (2016). *Cladosporium Fulvum* effectors: Weapons in the arms race with tomato. *Annual Review of Phytopathology*, *54*(1), 1–23. <https://doi.org/10.1146/annurev-phyto-011516-040249>.
- De Wit, P. J. G. M., Joosten, M. H. A. J., Thomma, B. H. P. J., & Stergiopoulos, I. (2009a). Gene for gene models and beyond: The *Cladosporium fulvum* – Tomato pathosystem. *Plant*



- Relationships*, 135–156. [https://doi.org/10.1007/978-3-540-87407-2\\_7](https://doi.org/10.1007/978-3-540-87407-2_7).
- De Wit, P. J. G. M., Mehrabi, R., Van Den Burg, H. A., & Stergiopoulos, I. (2009b). Fungal effector proteins: Past, present and future: Review. *Molecular Plant Pathology*, *10*(6), 735–747. <https://doi.org/10.1111/j.1364-3703.2009.00591.x>.
- De Wit, P. J. G. M., Van Der Burgt, A., Ökmen, B., Stergiopoulos, I., Abd-Elsalam, K. A., Aerts, A. L., Bahkali A.H., Beenen H.G., Chettri P., Cox M.P., Datema E., de Vries R.P., Dhillon B., Ganley A.R., Griffiths S.A., Guo Y., Hamelin R.C., enrissat B., Shahjahan Kabir M, Karimi Jashni M., Kema G., Klaubauf S., Lapidus A., Levasseur A., Lindquist E., Mehrabi R., Ohm R.A., Owen T.J., Salamov A., Schwelm A., Schijlen E., Sun H., van den Burg H.A., van Ham R.C.H.J., Zhang S., Goodwin S.B., Grigoriev I.V., Collemare J., & Bradshaw R.E. (2012). The genomes of the fungal plant pathogens *Cladosporium fulvum* and *Dothistroma septosporum* reveal adaptation to different hosts and lifestyles but also signatures of common ancestry. *PLoS Genetics*, *8*(11), 23209441. <https://doi.org/10.1371/journal.pgen.1003088>.
- Dixon, M. S., Jones, D. A., Keddie, J. S., Thomas, C. M., Harrison, K., & Jones, J. D. G. (1996). The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. *Cell*, *84*(3), 451–459. [https://doi.org/10.1016/S0092-8674\(00\)81290-8](https://doi.org/10.1016/S0092-8674(00)81290-8).
- Dixon, M. S., Hatzixanthis, K., Jones, D. A., Harrison, K., & Jones, J. D. G. (1998). The tomato *Cf-5* disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. *Plant Cell*, *10*, 1915–1925. <https://doi.org/10.1105/tpc.10.11.1915>.
- Dodds, P. N., Lawrence, G. J., Catanzariti, A. M., Teh, T., Wang, C. I. A., Ayliffe, M. A., Kobe, B., & Ellis, J. G. (2006). Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proceedings of the National Academy of Sciences*, *103*(23), 8888–8893. <https://doi.org/10.1073/pnas.0602577103>.
- Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. <https://doi.org/10.1093/nar/gkh340>.
- Excoffier, L., & Lischer, H. E. L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and windows. *Molecular Ecology Resources.*, *10*, 564–567. <https://doi.org/10.1111/j.1755-0998.2010.02847.x>.
- Franco, M. E. E., Troncozo, M. I., López, S. M. Y., Lucentini, C. G., Medina, R., Saparrat, M. C. N., Ronco B.L. & Balatti, P.A. (2017). A survey on tomato leaf grey spot in the two main production areas of Argentina led to the isolation of *Stemphylium lycopersici* representatives which were genetically diverse and differed in their virulence. *European Journal of Plant Pathology*, *149*(4), 983–1000. <https://doi.org/10.1007/s10658-017-1248-z>.
- Gabriëls, S. (2006). Functional analysis of tomato genes expressed during the *Cf-4/Avr4*- induced hypersensitive response. Thesis Wageningen University, The Netherlands. ISBN 90-8504-405-7, <https://pdfs.semanticscholar.org/b16a/f07b3e41d361bdee9d1a9c0807a2aa0d0628.pdf#page=63>.
- Heath, M. C. (2000). Hypersensitive response-related death. *Plant Molecular Biology*, no., *44*, 321–334.
- Iakovidis, M., Soumpourou, E., Anderson, E., Etherington, G., Yourstone, S., & Thomas, C. (2020). Genes encoding recognition of the *Cladosporium fulvum* effector protein Ecp5 are encoded at several loci in the tomato genome. *G3: Genes, Genomes, Genetics*, *10*(5), 1753–1763. <https://doi.org/10.1534/g3.120.401119>.
- Iida, Y., Van 'T Hof, P., Beenen, H., Mesarich, C. H., Kubota, M., Stergiopoulos, I., Rahim Mehrabi R., Notsu A., Fujiwara K., Bahkali A., Abd-Elsalam K., Collemare J., & de Wit P.J.G.M(2015). Novel mutations detected in avirulence genes overcoming tomato Cf resistance genes in isolates of a Japanese population of *Cladosporium fulvum*. *PLoS One*, *10*(4), 1–18. <https://doi.org/10.1371/journal.pone.0123271>.
- Jashni, M. K., Van Der Burgt, A., Battaglia, E., Mehrabi, R., Collemare, J., & De Wit, P. J. G. M. (2019). Transcriptome and proteome analyses of proteases in biotrophic fungal pathogen *Cladosporium fulvum*. *Journal of Plant Pathology*, *102*, 377–386. <https://doi.org/10.1007/s42161-019-00433-0>.
- Jones, D. A., Thomas, C. M., Hammond-Kosack, K. E., Balint-Kurti, P. J., & Jones, J. D. G. (1994). Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science*, *266*, 789–793. <https://doi.org/10.1126/science.7973631>.
- Joosten, M. H., Cozijnsen, T. J., & De Wit, P. J. (1994). Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. *Nature*, *367*(6461), 384–386. <https://doi.org/10.1038/367384a0>.
- Joosten, M. H., Vogelsang, R., Cozijnsen, T. J., Verberne, M. C., & De Wit, P. J. G. M. (1997). The biotrophic fungus *Cladosporium fulvum* circumvents *Cf-4*-mediated resistance by producing unstable AVR4 elicitors. *The Plant Cell*, *9*(3), 367–379. <https://doi.org/10.1105/tpc.9.3.367>.
- Kahlon, P. S., Seta, S. M., Zander, G., Scheikl, D., Hüchelhoven, R., Joosten, M. H., & Stam, R. (2020). Population studies of the wild tomato species *Solanum chilense* reveal geographically structured major gene-mediated pathogen resistance. *bioRxiv*, 020.05.29.122960. <https://doi.org/10.1101/2020.05.29.122960>.
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647–1649. <https://doi.org/10.1093/bioinformatics/bts199>.
- Kruijt, M., Kip, D. J., Joosten, M. H., Brandwagt, B. F., & de Wit, P. J. (2005). The *Cf-4* and *Cf-9* resistance genes against *Cladosporium fulvum* are conserved in wild tomato species. *Molecular Plant-Microbe Interactions*, *18*(9), 1011–1021. <https://doi.org/10.1094/MPMI-18-1011>.
- Laugé, R., Joosten, M. H. A. J., Van Den Ackerveken, G. F. J. M., Van Den Broek, H. W. J., & De Wit, P. J. G. M. (1997). The in planta-produced extracellular proteins ECP1 and ECP2 of *Cladosporium fulvum* are virulence factors. *Molecular Plant-Microbe Interactions : MPMI*, *10*(6), 725–734. <https://doi.org/10.1094/MPMI.1997.10.6.725>.
- Laugé, R., Joosten, M. H. A. J., Haanstra, J. P. W., Goodwin, P. H., Lindhout, P., & De Wit, P. J. G. M. (1998). Successful search for a resistance gene in tomato targeted against a

- virulence factor of a fungal pathogen. *Proceedings of the National Academy of Sciences*, 95(15), 9014–9018. <https://doi.org/10.1073/pnas.95.15.9014>.
- Laugé, R., Goodwin, P. H., De Wit, P. J. G. M., & Joosten, M. H. A. J. (2000). Specific HR-associated recognition of secreted proteins from *Cladosporium Fulvum* occurs in both host and non-host plants. *Plant Journal*, 23(6), 735–745. <https://doi.org/10.1046/j.1365-313X.2000.00843.x>.
- Lazarovits, G., & Higgins, V. J. (1976). Histological comparison of *Cladosporium fulvum* race 1 on immune, resistant, and susceptible tomato varieties. *Canadian Journal of Botany*, 54(3), 224–234. <https://doi.org/10.1139/b76-022>.
- Lindhout, P., Korta, W., Cislík, M., Vos, I., & Gerlagh, T. (1989). Further identification of races of *Cladosporium fulvum* (*Fulvia fulva*) on tomato originating from the Netherlands France and Poland. *Netherlands Journal of Plant Pathology*, 95(3), 143–148. <https://doi.org/10.1007/BF01999969>.
- Luderer, R. F. L., Takken, W., De Wit, P. J. G. M., & Joosten, M. H. A. J. (2002a). *Cladosporium Fulvum* overcomes Cf-2-mediated resistance by producing truncated AVR2 elicitor proteins. *Molecular Microbiology*, 45(3), 875–884. <https://doi.org/10.1046/j.1365-2958.2002.03060.x>.
- Luderer, R., De Kock, M. J. D., Dees, R. H. L., De Wit, P. J. G. M., & Joosten, M. H. A. J. (2002b). Functional analysis of cysteine residues of Ecp elicitor proteins of the fungal tomato pathogen *Cladosporium fulvum*. *Molecular Plant Pathology*, 3, 91–95. <https://doi.org/10.1046/j.1464-6722.2001.00095.x>.
- Ma, W., & Guttman, D. S. (2008). Evolution of prokaryotic and eukaryotic virulence effectors. *Current Opinion in Plant Biology*, 11(4), 412–419. <https://doi.org/10.1016/j.pbi.2008.05.001>.
- Medina, R., López, S. M. Y., Franco, M. E. E., Rollan, C., Ronco, B. L., Saparrat, M. C. N., de Wit, P. J. G. M., & Balatti, P. A. (2015). A survey on occurrence of *Cladosporium fulvum* identifies race 0 and race 2 in tomato-growing areas of Argentina. *Plant Disease*, 99(12), 1732–1737. <https://doi.org/10.1094/PDIS-12-14-1270-RE>.
- Mesarich, C. H., Griffiths, S. A., Van Der Burgt, A., Ökmen, B., Beenen, H. G., Etalo, D. W., Joosten M.H.A.J. & de Wit P.J.G. M. (2014). Transcriptome sequencing uncovers the Avr5 Avirulence gene of the tomato leaf mold pathogen *Cladosporium Fulvum*. *MolPlant Microbe Interact.*, 27(8), 846–857. <https://doi.org/10.1094/MPMI-02-14-0050-R>.
- Mesarich, C. H., Ökmen, B., Rovenich, H., Griffiths, S. A., Wang, C., Karimi Jashni, M., Mihajlovski, A., Collemare, J., Hunziker, L., Deng, C. H., van der Burgt, A., Beenen, H. G., Templeton, M. D., Bradshaw, R. E., & de Wit, P. J. G. M. (2018). Specific hypersensitive response-associated recognition of new apoplastic effectors from *Cladosporium fulvum* in wild tomato. *Molecular Plant-Microbe Interactions*, 31(1), 145–162. <https://doi.org/10.1094/MPMI-05-17-0114-FI>.
- Ökmen, B., Etalo, D. W., Joosten, M. H. A. J., Bouwmeester, H. J., De Vos, R. C. H., Collemare, J. & de Wit P.J.G.M. (2013). Detoxification of  $\alpha$ -tomatine by *Cladosporium fulvum* is required for full virulence on tomato. *New Phytologist*, 198(4), 1203–1214. <https://doi.org/10.1111/nph.12208>.
- Rivas, S., & Thomas, C. M. (2005). Molecular interactions between tomato and the leaf mold pathogen *Cladosporium Fulvum*. *Annual Review of Phytopathology*, 43(1), 395–436. <https://doi.org/10.1146/annurev.phyto.43.040204.140224>.
- Rollan, C., Protto, V., Medina, R., Lopez, S. M. Y., Bahima, J. V., Ronco, L., Saparrat M.C.N., & Balatti P.A. (2013). Identification of races 0 and 2 of *Cladosporium fulvum* (Syn. *Passalora fulva*) on tomato in the cinturón hortícola de La Plata, Argentina. *Plant Disease*, 97(7). <https://doi.org/10.1094/PDIS-10-12-0987-PDN>.
- Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (no. Ed. 2). New York: Cold Spring Harbor Laboratory Press.
- Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D. J., Thomma, B. P., & Mesters, J. R. (2013). Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *elife*, 2, e00790. <https://doi.org/10.7554/eLife.00790>.
- Soanes, D. M., & Talbot, N. J. (2008). Moving targets: Rapid evolution of oomycete effectors. *Trends in Microbiology*, 16(11), 507–510. <https://doi.org/10.1016/j.tim.2008.08.002>.
- Stavriniades, J., McCann, H. C., & Guttman, D. S. (2008). Host-pathogen interplay and the evolution of bacterial effectors. *Cellular Microbiology*, 10(2), 285–292. <https://doi.org/10.1111/j.1462-5822.2007.01078.x>.
- Stergiopoulos, I., & De Wit, P. J. G. M. (2009). Fungal effector proteins. *Annual Review of Phytopathology*, 47, 233–263. <https://doi.org/10.1146/annurev.phyto.112408.132637>.
- Stergiopoulos, I., De Kock, M. J. D., Lindhout, P., & De Wit, P. J. G. M. (2007). Allelic variation in the effector genes of the tomato pathogen *Cladosporium fulvum* reveals different modes of adaptive evolution. *Molecular Plant-Microbe Interactions : MPMI*, 20(10), 1271–1283. <https://doi.org/10.1094/MPMI-20-10-1271>.
- Stergiopoulos, I., van den Burg, H. A., Okmen, B., Beenen, H. G., Van Liere, S., Kema, G. H. J., & De Wit, P. J. G. M. (2010). Tomato Cf resistance proteins mediate recognition of cognate homologous effectors from fungi pathogenic on dicots and monocots. *Proceedings of the National Academy of Sciences*, 107(16), 7610–7615. <https://doi.org/10.1073/pnas.1002910107>.
- Stergiopoulos, I., Kourmpetis, Y. A., Slot, J. C., Bakker, F. T., De Wit, P. J., & Rokas, A. (2012). *In silico* characterization and molecular evolutionary analysis of a novel superfamily of fungal effector proteins. *Molecular Biology and Evolution*, 29(11), 3371–3384. <https://doi.org/10.1093/molbev/mss143>.
- Stergiopoulos, I., Cordovez, V., Ökmen, B., Beenen, H. G., Kema, G. H. J., & De Wit, P. J. G. M. (2014). Positive selection and intragenic recombination contribute to high allelic diversity in effector genes of *Mycosphaerella fijiensis*, causal agent of the black leaf streak disease of banana. *Molecular Plant Pathology*, 15(5), 447–460. <https://doi.org/10.1111/mpp.12104>.
- Takken, F. L. W., Thomas, C. M., Joosten, M. H. A. J., Golstein, C., Westerink, N., Hille, J., Nijkamp, H. J. J., de Wit, P. J. G. M., & Jones, J. D. G. (1999). A second gene at the tomato Cf-4 locus confers resistance to *Cladosporium fulvum* through recognition of a novel avirulence determinant. *The Plant Journal*, 20, 279–288. <https://doi.org/10.1046/j.1365-313X.1999.00601.x>.
- Thomas, C. M., Jones, D. A., Parniske, M., Harrison, K., Balint-Kurti, P. J., Hatzixanthis, K., & Jones, J. D. (1997). Characterization of the tomato Cf-4 gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognition specificity in Cf-4 and Cf-9. *Plant Cell*, 9, 2209–2224. <https://doi.org/10.1105/tpc.9.12.2209>.

- Thomma, B. P. H. J., Van Esse, H. P., Crous, P. W., & De Wit, P. J. G. M. (2005). *Cladosporium fulvum* (Syn. *Passalora fulva*), a highly specialized plant pathogen as a model for functional studies on plant pathogenic Mycosphaerellaceae. *Molecular Plant Pathology*, 6(4), 379–393. <https://doi.org/10.1007/BF00301063>.
- Thomma, B. P., Nürnberger, T., & Joosten, M. H. (2011). Of PAMPs and effectors: The blurred PTI-ETI dichotomy. *The Plant Cell*, 23(1), 4–15. <https://doi.org/10.1105/tpc.110.082602>.
- Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22(22), 4673–4680. <https://doi.org/10.1093/nar/22.22.4673>.
- Van den Ackerveken, G. F. J. M., Van Kan, J. A. L., & De Wit, P. J. G. M. (1992). Molecular analysis of the avirulence gene *Avr9* of the fungal tomato pathogen *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. *The Plant Journal*, 2, 359–366. <https://doi.org/10.1046/j.1365-3113.X.1992.t01-34-00999.x>.
- Van den Ackerveken, G. F. J. M., Vossen, P., & De Wit, P. J. G. M. (1993). The AVR9 race-specific elicitor of *Cladosporium fulvum* is processed by endogenous and plant proteases. *Plant Physiology*, 103(1), 91–96. <https://doi.org/10.1104/pp.103.1.91>.
- Van Den Burg, H. A., Harrison, S. J., Joosten, M. H. A. J., Vervoort, J., & De Wit, P. J. G. M. (2006). *Cladosporium fulvum* Avr4 protects fungal cell walls against hydrolysis by plant chitinases accumulating during infection. *Molecular Plant-Microbe Interactions*, 19(12), 1420–1430. <https://doi.org/10.1094/MPMI-19-1420>.
- Van Den Hooven, H. W., Appelman, A. W. J., Zey, T., De Wit, P. J. G. M., & Vervoort, J. (1999). Folding and conformational analysis of AVR9 peptide elicitors of the fungal tomato pathogen *Cladosporium fulvum*. *European Journal of Biochemistry*, 264(1), 9–18. <https://doi.org/10.1046/j.1432-1327.1999.00503.x>.
- Van Den Hooven, H. W., Van Den Burg, H. A., Vossen, P., Boeren, S., De Wit, P. J. G. M., & Vervoort, J. (2001). Disulfide bond structure of the AVR9 elicitor of the fungal tomato pathogen *Cladosporium fulvum*: Evidence for a cystine knot. *Biochemistry*, 40(12), 3458–3466. <https://doi.org/10.1021/bi0023089>.
- Van der Hoorn, R. A., Roth, R., & De Wit, P. J. (2001). Identification of distinct specificity determinants in resistance protein *Cf-4* allows construction of a *Cf-9* mutant that confers recognition of avirulence protein AVR4. *The Plant Cell*, 13(2), 273–285. <https://doi.org/10.1105/tpc.13.2.273>.
- Van Kan, J. A., Van Den Ackerveken, G. F. J. M., & De Wit, P. J. G. M. (1991). Cloning and characterization of cDNA of avirulence gene *Avr9* of the fungal pathogen *Cladosporium Fulvum*, causal agent of tomato leaf mold. *Molecular Plant-Microbe Interactions*, 8, e53937. <https://doi.org/10.1371/journal.pone.0053937>.
- Westerink, N., Joosten, M. H. A. J., & De Wit, P. J. G. M. (2004a). Fungal (a)virulence factors at the crossroads of disease susceptibility and resistance. In Z. K. Punja (Ed.), *Fungal disease resistance in plants: Biochemistry, molecular biology, and genetic engineering* (pp. 93–137). Binghamton: Food Products Press.
- Westerink, N., Brandwagt, B. F., De Wit, P. J. G. M., & Joosten, M. H. A. J. (2004b). *Cladosporium Fulvum* circumvents the second functional resistance gene homologue at the *Cf-4* locus (*Hcr9-4E*) by secretion of a stable *avr4E* isoform. *Molecular Microbiology*, 54(2), 533–545. <https://doi.org/10.1111/j.1365-2958.2004.04288.x>.
- White, T. J., Bruns, S., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal rna genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications*, citeulike-article-id:671166.
- Wulff, B. B. H., Chakrabarti, A., & Jones, D. A. (2009). Recognition specificity and evolution in the tomato–*Cladosporium fulvum* pathosystem. *Molecular Plant-Microbe Interactions*, 22(10), 1191–1202. <https://doi.org/10.1094/MPMI-22-10-1191>.