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1 **Population studies of the wild tomato species *Solanum chilense* reveal geographically**
2 **structured major gene-mediated pathogen resistance**
3
4 **Running title**: presence and loss of resistance in natural populations
5
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19 **Abstract**

20 Natural plant populations encounter strong pathogen pressure and defense-associated genes are
21 known to be under different selection pressure dependent on the pressure by the pathogens. Here
22 we use wild tomato *Solanum chilense* populations to investigate natural resistance against
23 *Cladosporium fulvum*, a well-known pathogenic fungus of domesticated tomatoes. We show that
24 populations of *S. chilense* differ in resistance against the pathogen. Next, we explored the
25 underlying molecular processes in a species wide-context. Then, focusing on recognition of the
26 two prominent avirulence factors secreted by *C. fulvum* (Avr4 and Avr9) in central and northern
27 populations of *S. chilense* we observed high complexity in the cognate *homologues of*
28 *Cladosporium resistance* (*Hcr9*) locus underlying the recognition of these effectors. Presence of
29 canonical genomic regions coding for *Cf-4* and *Cf-9*, two major dominant resistance genes in the
30 *Hcr9* locus recognizing Avr4 and Avr9, respectively, does not meet prediction from Avr
31 response phenotypes. We find both genes in varying fractions of the plant populations and we
32 show possible co-existence of two functionally active resistance genes, previously thought to be
33 allelic. Additionally, we observed the complete local absence of recognition of additional Avr
34 proteins of *C. fulvum*. In the southern populations we attribute this to changes in the coregulatory
35 network. As a result of loss of pathogen pressure or adaptation to extreme climatic conditions.
36 This may ultimately explain the observed pathogen susceptibility in the southern populations.
37 This work puts major gene mediated disease resistance in an ecological context.

38

39

40 Keywords: *Solanum chilense*, *Cladosporium fulvum, receptor-like proteins (RLPs), Cf-9,* tomato,
41 resistance.

42

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43 Funding: This work was funded through the German Science Foundation (DFG), SFB924

44

45 Introduction

46

47 How plants and their pathogens adapt to one another in their natural habitat is still poorly
48 understood. Some studies highlight the local adaptations in context to plant-pathogen
49 interactions: the wild flax-flax rust pathosystem is such an example, where more resistant wild
50 flax harbored more virulent strains of the rust (Thrall *et al.*, 2002; Thrall & Burdon, 2003).
51 Similar mechanisms of the co-occurrence of virulent strains of the powdery mildew
52 *Podosphaera plantaginis* and more resistant plants of *Plantago lanceolata* have also been
53 documented (Laine, 2005; Soubeyrand *et al*., 2009). Furthermore, complex multi-host and multi-
54 pathogen systems, with clear differences at a regional scale, have been observed in anther smut-
55 fungi-infecting *Dianthus* hosts in the southern European Alps (Petit *et al*., 2017). The molecular
56 mechanisms that is thought to drive these interactions is a gene-for-gene interaction (Flor, 1971),
57 where single gene encoded pathogen molecules, now referred to as effectors, are recognized by
58 specific receptors present in resistant host plants, also known as major resistance genes. The need
59 to recognize an invading pathogen and subsequently evade the recognition by the plant and
60 pathogen, respectively, leads to a constant co-evolutionary arms race, often described as the Red
61 Queen dynamics (Van Valen, 1973). These dynamics are thought to have led to the plethora of
62 different defense related genes in plants as we know it today. Wild crop relatives have been used
63 as a gene pool for isolating and introducing genetic resistance against many different fungal
64 pathogens. Genetic diversity of natural populations against microbes have been explored by
65 sequence analysis or by experimental biology often still as independent, though complementary,
66 approaches (Salvaudon *et al*., 2008), leading to a huge gap in our understanding of local

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67 adaptations and their role in shaping the current diversity of such resistance genes in an
68 ecological context.

69 Tomato leaf mold is caused by the non-obligate biotrophic fungus *Cladosporium fulvum* syn.
70 *Passalora fulva*. At the time of infection, *C. fulvum* enters through stomata into the leaf and
71 colonizes the apoplastic spaces (Stergiopoulos & de Wit, 2009). The fungus secretes various
72 small proteins, also referred to as effectors or avirulence factors (Avrs) when being recognized
73 by a resistant host, into the apoplast with the aim to manipulate the host for its successful
74 colonization of the host tissue. The infection leads to severe yellowing and wilting of the leaves,
75 which ultimately leads to a loss of photosynthetic capacity and thus a loss in yield or reduced
76 reproducible fitness. *C. fulvum* is a globally occurring pathogen with a clear genetic diversity
77 (Iida *et al*., 2015). It is thought to originate in the Andean region, where it likely has co-evolved
78 with one or several of wild tomato species that inhabit a mountain range from central Ecuador to
79 northern Peru (de Wit *et al*., 2012). During evolution, as a result of selection pressure imposed
80 by virulent strains of *C. fulvum*, several of the wild tomato species have evolved resistance genes
81 whose products mediate recognition of the Avrs secreted by *C. fulvum* (Joosten & de Wit, 1999).
82 This recognition facilitates host resistance following the gene-for-gene model. Resistance is
83 eventually achieved when Avr-activated defense leads to a hypersensitive response (HR), which
84 includes programmed cell death (PCD). This localized PCD is associated with various additional
85 defense responses such as a massive callose deposition, and prevents *C. fulvum* from obtaining
86 nutrients from the host, thereby limiting further pathogen ingress and multiplication (Lazarovits
87 & Higgins, 1976).

88 To date, 24 *C. fulvum* (*Cf*) resistance genes have been mapped (Kanwar *et al*., 1980). Due to the
89 complexity of the *Cf* genes and their loci, only a small number of *Cf* genes have been cloned and

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90 verified for their functionality. *Cf* genes are highly repetitive in their leucine-rich repeat encoding
91 parts and functional homologs are often accompanied by non-functional ones, with very little
92 sequence variation between them (Kruijt 2005). *Cf-9* from *S. pimpinellifolium* encodes a cell
93 surface receptor-like protein (RLP) and was the first *Cf* resistance gene to be cloned (Jones *et al.*,
94 1994). The *Cf-9* gene product recognizes the Avr9 protein of *C. fulvum*, which is a highly stable,
95 cysteine-knotted peptide of unknown function (Scholtens-Toma & de Wit 1988). The *Cf-9* gene
96 belongs to the *Hcr9* (homologs of *C. fulvum* resistance gene *Cf-9*) gene cluster, which is located
97 on chromosome 1. In addition to *Cf-9*, *Cf-4* is another well-studied *Cf* gene from the *Hcr9*
98 cluster. The *Cf-4* gene product recognizes Avr4 of *C. fulvum*, which is a chitin-binding protein
99 having eight cysteine residues (Joosten et al., 1994; van den Burg et al., 2004), and the *Cf-4* gene
100 originated from *S. habrochaites* (Thomas *et al*., 1997). Studies on MM-Cf9 which is an Avr9-
101 recognising introgression line of the domesticated tomato, *S. lycopersicum* cv Money Maker,
102 revealed presence of five *Cf-9* homologs, *Hcr9-9A* to *Hcr9-9E*, mapped at the short arm of
103 chromosome 1, with *Hcr9-9C* being the functional *Cf-9* gene (Parniske *et al*., 1997). Similarly,
104 *Hcr9-4A* to *Hcr9-4E* is present in the Avr4-recognising MM-Cf4 and *Hcr9-4D* corresponds to
105 *Cf-4* (Thomas *et al*., 1997). *Cf-4* and *Cf-9* lie at the same locus on chromosome 1 and are
106 assumed to be mutually exclusive or even allelic. Crossings between recombinant inbred lines
107 carrying *Cf-4* and *Cf-9* resulted in extreme genetic instability in the offspring (Parniske *et al.*,
108 1997, Thomas *et al.*, 1997).

109 Appreciating the important roles of these *Cf* genes and their assumed role in co-evolution
110 between wild tomato and native *C. fulvum*, it is surprising that only a few studies have sought to
111 investigate *Cf* gene diversity. An effectoromics approach was exploited by Mesarich *et al.*
112 (2017), to identify plants carrying novel *Cf* genes to be potentially used in plant breeding

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113 programs in the future. Studies have shown that in *S. pimpinellifolium* several putative homologs
114 are present, but their function remains unknown (Caicedo *et al*., 2004; Caicedo, 2008). Another
115 study identified four variants of *Cf-9* (originally isolated from *S. pimpinellifolium*), from its close
116 relative *S. habrochaites* and each one variant of *Cf-4* from *S. habrochaites*, *S. chilense*, *S.*
117 *chmielewskii*, *S. neorickii* and *S. arcanum* (Kruijt *et al*. 2005). In-spite of multiple single
118 nucleotide polymorphisms (SNPs) being present in the isolated *Cf* gene variants, all of them
119 showed the ability to induce an HR after recognition of Avr9 and Avr4, respectively, which led
120 the authors to conclude that Avr4 and Avr9 recognition is conserved among wild tomato.
121 However, a species-wide analysis of different accessions of *S. pimpinellifolium* revealed
122 intragenic recombination to have occurred between *Hcr9-9D* and *Hcr9-9C*/*Cf-9*. The resulting
123 allele, *9DC*, does not co-exist with the original *Cf-9* allele in the individual plants. Variant *9DC*
124 is the more common allele in the species and the product also recognizes Avr9 (Van der Hoorn *et*
125 *al*., 2001), indicating that one likely cannot speak of conservation of *Cf* alleles *sensu stricto.* In
126 addition, plants that recognize both Avr4 and Avr9 have not been identified. Detailed knowledge
127 on the relationship between *Cf-4* and *Cf-9* in other accessions or wild populations of tomato, and
128 on their actual roles in resistance is still lacking.
129 To perform more detailed studies on resistance provided by *Cf* genes in an ecological context, we
130 selected *S. chilense,* one of seventeen wild tomato species, as it covers a wide variety of habitats
131 on the western slopes of the Andes, ranging from Peru to Northern Chile (Nakazato *et al*., 2010).
132 The species range spreads from the edges of the Atacama Desert, as the southern edge of the
133 range, to relatively wet, high altitude regions (up to 3500 meter above sea level), as well as in
134 very specific coastal regions that experience regular sea fog (resulting in a relatively high
135 humidity) in the most northern part of Chile, as well as in south Peru (Cereceda & Schemenauer

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136 1991; Peralta *et al*., 2008; Chetelat *et al*., 2009). This highly varied distribution results in sub-
137 populations of this species that encounter different environmental challenges. Ultimately, these
138 different habitats lead to genetic differentiation within the species. The *S. chilense* population
139 can be clustered in four groups: north, central, southern highlands and southern lowlands. The
140 southern highlands and southern lowlands populations are derived from the central group
141 (Böndel*et al*., 2015). This divergence was confirmed by whole genome sequencing, and multiple
142 sequential markovian coalescent simulations revealed that migrations happened from the central
143 group southward 50,000 to 200,000 years ago (Stam *et al*., 2019b). The strong differentiation
144 between habitats leads to clearly observable adaptations. Southern populations respond faster to
145 drought (Fischer *et al*., 2013) whereas high altitude populations are more cold-tolerant (Nosenko
146 *et al*., 2016). In addition to adaptations to abiotic factors, these habitats are expected to be home
147 to different biotic stressors at different intensity levels, including various pathogen species,
148 which is anticipated to result in genetic variation in pathogen defense-associated genes. Indeed,
149 differences in resistance properties of the various *S. chilense* populations against three pathogens,
150 *Alternaria solani*, *Phytophthora infestans* and a *Fusarium* spp, are observed (Stam *et al*., 2017).
151 Moreover, large genetic variation has been observed within *S. chilense* populations in another
152 resistance-associated gene family; the nucleotide-binding leucine-rich repeat (NLR) resistance
153 genes. These genes show clear presence-absence-variation when compared to related tomato
154 species (Stam *et al*., 2019a), and an in-depth resequencing study, covering the whole species
155 range of *S. chilense,* shows evidence that the selection pressure imposed on the individual NLRs
156 differs for each of the populations (Stam *et al*., 2019b).

157 Little data exists showing biological interactions between *C. fulvum* and wild tomato species. In
158 this study we investigated the interaction of the fungal pathogen *C. fulvum* with the wild tomato

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159 species *S. chilense* throughout the geographical range of the species. We show that *S. chilense*
160 plants from different locations first of all show differences in their resistance to the fungus,
161 including complete loss of resistance in some populations. By investigating the well
162 characterized genes of the *Hcr9* locus, we furthermore place major gene mediated immunity in
163 an ecological context.
164
165 **Materials and methods:**
166
167 **Plants and fungal material**
168 Seeds of the fifteen accessions (populations) of *S. chilense* used in our studies were originally
169 obtained from the C. M. Rick Tomato Genetics Resource Center of the University of California,
170 Davis (TGRC UC-Davis) (http://tgrc.ucdavis.edu/). The selected accessions were LA1958,
171 LA1963, LA2747, LA2931, LA2932, LA2750, LA2959, LA3111, LA3784, LA3786, LA4107,
172 LA4117A, LA4118 and LA4330. Each Accession number represents a random collection of
173 seeds from a wild population and is propagated by TGRC in a way to maintain maximum genetic
174 diversity. Böndel et al (2015) have shown that this seed multiplication has negligible effect on
175 the genetic diversity of the accessions. Hence, each accession can be considered to truly
176 represent the diversity in the wild plant populations. Introgression lines *S. lycopersicum* cv
177 Moneymaker (MM) of *Cf-9, Cf-4* and *Cf-5* were generated by Tigchelaar *et al.* (1984) and *S.*
178 *pimpinellifolium* (LP12), in which the *9DC* was identified, and which was used as a control in all
179 our assays were provided by TGRC UC-Davis. Per *S. chilense* accession 8-17 plants were
180 grown. Plants were grown in a controlled glasshouse, with a minimum daytime temperature of
181 24°C and 16 hours of light and 8 hours of dark conditions. Adult plants were cut back at bi-

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182 weekly intervals to assure the presence of mature, fully developed, yet not senescent, leaves
183 during each repetition of the experiment.
184 A race 5 strain of *C. fulvum* was maintained on 1/2 potato dextrose agar (PDA) medium and
185 incubated at 16°C in the dark.
186

187 **Visualization of infection phenotype and quantification of *C. fulvum* biomass from tomato**
188 **leaves**

189 Spray inoculation of *C. fulvum* at a concentration 20,000 conidia/ml was performed on 3-week
190 old plant cuttings of population LA3111, LA4330, MM-Cf-9 and MM-Cf-5 plants; water-
191 inoculated plants served as a negative control in the experiment. Plants were maintained at 24°C
192 and 16 hours of light and 8 hours of dark, with 100% humidity for the first two days, after which
193 80% humidity was maintained throughout the experiment.
194 Photographs of leaflets of inoculated plants were collected at 14 days post inoculation (dpi) and
195 Microscopy was performed from 7dpi to 19dpi on bleached leaves following staining in acetic
196 acid (25%) 1:9 + ink (K□nigsblau, Pelikan, 4001) then washed in water and analysed under
197 brightfield microscope (Zeiss, Imager Z1m).
198 For quantification, leaflets of inoculated plants were collected at 14 days post inoculation (dpi)
199 and DNA was isolated using the protocol published by Yan *et al*. (2008). The infection loads or
200 approximate amounts of *C. fulvum* DNA present in inoculated leaves were quantified by qPCR
201 using the DNA-binding fluorophore Maxima SYBR Green Master Mix (2X) with ROX solution
202 (Thermo Scientific). All qPCR reactions were performed on an AriaMx Real-Time PCR system
203 (Agilent Technologies, Waldbronn, Germany). PCR were performed with the primer pairs
204 RS158 (5'-GTCTCCGGCTGAGCAGTT-3')/ ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and
205 RS001 (5'-GCCTACCATGAGCAGCTTTC-3')/ RS002 (5'-CAATGCGTGAGAAGACCTCA-

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206 3'), annealing to the ITS region and *elongation factor 1 alpha*, respectively, present in the *C.*
207 *fulvum* DNA. Templates used for this experiment were genomic DNA isolated from the leaves
208 inoculated with the pathogen and water. Amplifications using both primer pairs for each sample
209 were done on the same plate and in the run. The experiment was performed on three plants of
210 MM-Cf-9 and MM-Cf-5 each and five plants of LA3111 and LA4330 each. All the plants
211 samples well evaluated in three technical replicates and Cq differences higher than one among
212 technical replicates was not considered in the final evaluation. The Cq values and the linear
213 equations from the sensitivity graphs were used to calculate the quantities of pathogen and plant
214 DNA in each sample (Figure S1).
215
216 **Apoplastic washing fluid (AF), Avr9 and Avr4 infiltration assay**
217 AF containing the complete set of *C. fulvum* Avrs, except for Avr5, was obtained by isolating AF
218 from leaves of MM-Cf-5 plants colonized by race 5 of *C. fulvum*, at 10 to 14 days after
219 inoculation. Furthermore, a preparation of Avr9 concentrated from AF by acetone precipitation,
220 leaving the Avr9 peptide in the supernatant, and Avr4 produced in the yeast *Pichia pastoris*,
221 were employed.
222 Using a 1 ml syringe (Braun, Omnifix) without a needle, the AF, Avr9 and Avr4 preparations
223 were infiltrated from the lower side of fully expanded leaves of the different populations.
224 Experiments were performed in three independent biological replicates. Infiltrations were done
225 in fully expanded leaves of the same adult plant, with two technical replicates per plant.
226 Readings were performed between 2 to7 days post infiltration. For Avr9 infiltration, an HR was
227 typically observed within 1-4 days, whereas the AF-and Avr4-triggered HR were observed later.
228 Readings were not performed later than 7 days after infiltration.

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229

230 **Presence of *Cf-9*-, *9DC*-and *Cf-4*-specific regions in different *S. chilense* populations**

231 Screening of *S. chilense* populations for the presence of gene-specific regions of *Cf-9, 9DC* and
232 *Cf-4* was performed through PCR amplification using the gene-specific primers CS5-CS1 and
233 DS1-CS1 for *Cf-9* and *9DC*, respectively (Van der Hoorn *et al.*, 2001). Primer pair PSK047 (5'-
234 ACGACAGAAGAACTC-3')/ PSK050 (5'-GATGGAATTGGTCCTT-3'), was designed to
235 amplify the canonical *Cf-4* domain (Fig. S2). DNA was isolated using standard CTAB
236 extraction, using the same samples as used in a previous study (Stam *et al.*, 2016; Stam *et al.*,
237 2019b). PCR on gDNA was performed using Promega Green GoTaq® Polymerase and the
238 products were analyzed by 1% agarose gel electrophoresis. As a PCR control *elongation factor*
239 *1 alpha*, amplified with the primer pair 5'-GTCCCCATCTCTGGTTTTGA-3'/ 5'-
240 GGGTCATCTTTGGAGTTGGA-3', was included and MM-Cf-9 and MM-Cf-4, and LP12
241 served as positive and negative controls, respectively.

242

243 **Semi-quantitative evaluation of the expression of a *Cf-9* homolog in the southern**
244 **population LA4330**

245 We evaluated the transcript levels of the *Cf-9* homolog in LA4330 at eight hours after Avr9 or
246 water infiltration. RNA from the infiltrated leaves was extracted using the RNeasy plant mini kit
247 (Qiagen) and cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen).
248 Amplification of *Cf-9* the canonical region of the *Cf-9* homolog was performed with primer pair
249 binding on the start and end of the ORF, PSK009 (5'-ATGGATTGTGTAAAACTTGTATTCCT
250 -3') /PSK010 (5'-CTAATATCTTTTCTTGTGCTTTTTCA -3'), and the product was visualized
251 on 1% agarose gel.

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252

253 **Whole genome sequence analyses for *Cf* co-receptors in *S. chilense***

254 We extracted the sequences for four important components of the Cf protein signaling complex,
255 for which we chose SUPPRESSOR OF BIR1 (SORBIR1), SOMATIC EMBRYOGENESIS
256 RECEPTOR-KINASE 3A (SERK3a) and AVR9/CF-9-INDUCED KINASE 1 (ACIK1) (Wu,
257 2020), as well as for an additional potential co-receptor of the BAK1 signaling complex, BIR2
258 (Halter *et al*., 2014) from NCBI. To identify the genomic sequences encoding these co-receptors
259 and regulators of Cf proteins in *S. chilense*, we performed a BLAST search against the *S.*
260 *chilense* reference genome (Stam *et al*., 2019b). Each *SOBIR1*, *SEKR3a* and *ACIK1* yielded one
261 unequivocal best target sequence in the reference genome and visual inspection after alignment
262 confirmed that the *S. chilense* homologs are in all cases similar to the reference gene of *S.*
263 *lycopersicum. BIR2* yielded two good hits, of which we dubbed the second one *BIR2b*.
264 In order to look for polymorphisms in three plants, one from LA3111, and one each from
265 southern populations LA2932 and LA4330 (Stam, *et al*. 2019a), we obtained the genomic
266 coordinates of the genes of interest from our reference genome and called the SNPs in the region
267 of interest using samtools mpileup and bcftools call (-mv -Oz) (Li *et al*., 2009; Li, 2011). We
268 removed low quality indels and the resulting vcf files were tabulated using tabix, to allow the
269 consensus sequences for the genomic regions to be extracted using bcftools consensus. Finally,
270 we used gffread from gffutils (Pertea and Pertea 2020) to obtain the correct coding sequence for
271 each of the plants. All multiple sequence alignments were made and inspected using aliview
272 (Larsson, 2014).

273

274 **Statistical testing**

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275 ANOVA, with post hoc Tukey honest significant difference test was performed using R
276 command Tukey HSD (anova) to test the statistical significance in pathogen load detected in the
277 inoculated plants via qPCR.
278

279 **Results**

280

281 ***S. chilense* populations show differences in resistance against *C. fulvum***

282 To confirm that *C. fulvum* is capable of successfully infecting *S. chilense* and to test whether *S.*
283 *chilense* populations show differences in their resistance spectrum to *C. fulvum*, we spray-
284 inoculated randomly selected individual plants of *S. chilense* from geographically distinct
285 populations LA3111 (central) and LA4330 (southern highlands), with a conidial suspension of *C.*
286 *fulvum* race 5. Upon visual inspection and microscopic evaluation, we found that plants of
287 population LA3111 show similar phenotypes as our resistant control plant MM-Cf-9, a
288 recombinant inbred line of *S. lycopersicum cv*. MoneyMaker with introgressed *Cf-9*, thus being
289 resistant to *C. fulvum* race 5 as this race does produce Avr9. By contrast, LA4330 shows a
290 phenotype similar to our susceptible control MM-Cf-5, a *Cf-5* introgression line of *S.*
291 *lycopersicum* of which resistance is circumvented due to a loss of the Avr5 gene by race 5
292 (Mesarich et al., 2014) (Figure 1a-b).

293 Next we performed quantification of *C. fulvum* DNA load and found significant differences in
294 pathogen DNA contents between the tested plants ($p < 0.001$, ANOVA). Differences can be
295 seen between the resistant and susceptible control plants ($p = 0.05$, TukeyHSD) and LA4330
296 showed a significantly higher ($p < 0.001$, TukeyHSD) presence of *C. fulvum* DNA than LA3111
297 (Figure 1c). For this we tested five individual plants from each *S. chilense* population and three

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298 plants per control. Taken together these findings show that *C. fulvum* is able to infect *S. chilense*
299 plants and that the various populations behave differently when exposed to *C. fulvum.*
300

301 **Southern *S. chilense* populations do not recognize *C. fulvum* effectors**

302 Visible HR upon effector infiltration is an efficient and reliable proxy to test for the resistance
303 properties of tomato plants on a large scale. To test the geographical variation in *C. fulvum* race 5
304 resistance, we performed an infiltration assay with apoplastic fluid (AF) of infected susceptible
305 tomato plants, which is sufficient to trigger an HR in plants carrying matching *Cf* genes. As for
306 the inoculation a race 5 was used, the AF is anticipated to contain all secreted *C. fulvum*
307 effectors, except for Avr5. We infiltrated the AF at two sites in the leaves of 155 individuals,
308 representing 15 different populations (8-17 individuals for each population). Interestingly, we
309 did not observe any HR-associated recognition of an effector present in the AF by any of the 6
310 southern populations that we tested, e.g. those belonging to the southern highlands and southern
311 coastal groups as described by Böndel (2015) and Stam *et al*. (2019b) (Figure 2). Populations
312 from the northern and central regions do show recognition of at least one of the effectors present
313 in the AF, as HR development was taking place. Yet, we did not observe this recognition to take
314 place in all tested plants of the population. Rather, we observed differences in recognition
315 capacities within the population, with some plants being able to respond with an HR, and some
316 not. Populations from central regions showed recognition of components present in the AF,
317 ranging from 10-100% of the plants tested in the different populations (Table S1). Two northern
318 populations tested showed AF mix recognition for 75% and 80% of the plants tested (Table S1).
319 All of our infiltration experiments were performed at least three times, in leaves from the same
320 perennial plants and at the same location.
321

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322 **Differential Avr9 and Avr4 recognition patterns are present in *S. chilense***

323 Recognition of Avr9 and Avr4 has been hypothesized to be an important conserved feature in
324 wild tomato species in order to maintain *C. fulvum* resistance (Kruijt *et al*., 2005). To understand
325 the role of the recognition of these Avrs in more detail, we performed additional infiltration
326 assays, but now with the individual effectors. In order to test whether *S. chilense* plants are able
327 to specifically recognize Avr9, we infiltrated the plants with a preparation enriched for Avr9.
328 Populations from the central region showed a very large variation in the ability to recognize
329 Avr9, with the capacity to recognize Avr9 ranging from 10-80% of the plants that were tested in
330 individual populations (Table S1). The two northern populations that were tested showed Avr9
331 recognition for 20% and 37.5% of the plants tested (Figure 3).
332 We also tested the ability of *S. chilense* to recognize Avr4 in a similar fashion, using *Pichia*
333 *pastoris*-produced Avr4. In the two northern populations we observed that 12.5% and 30% of the
334 plants showed the capacity to recognize Avr4, whereas in the central populations 0-33% of the
335 plants showed recognition. Interestingly, contrary to previous reports that *Cf-4* and *Cf-9* are
336 mutually exclusive, we also found plants that were able to recognize both Avr4 and Avr9 (Figure
337 2 and Table S1).
338

339 **Presence of canonical *Cf-9*-, *9DC*- and *Cf-4*-specific region in different *S. chilense***
340 **populations does not correlate with their recognition properties**

341 It has been shown that in *S. pimpinellifolium* either *Cf-9* or *9DC* are responsible for Avr9
342 recognition (Van der Hoorn *et al*., 2001). *Cf-9* or *9DC* gene sequences in *S. chilense* have not
343 been reported to date. Putative full length *Cf-9* or *9DC* genes cannot be found in the currently
344 available reference genome sequence, possibly due to misassemblies of the complex LRR

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345 regions (Stam *et al.*, 2019a). Thus, to investigate which gene is responsible for Avr9 recognition
346 in *S. chilense* we looked into the presence of *Cf-9* and/or *9DC*, using gene-specific primer
347 sequences that were used before to identify these genes (Van der Hoorn *et al.*, 2001).
348 We performed *Cf-9* gene-specific PCR amplification on genomic DNA isolated from nine *S.*
349 *chilense* populations (9 plants per population), using the previously published primers CS5-CS1,
350 amplifying a 379bp specific region of *Cf-9* (Van der Hoorn *et al.*, 2001). *Cf-9* and *Cf-4*
351 introgression lines of *S. lycopersicum* served as positive and negative control, respectively. The
352 *Cf-9*-specific sequence was amplified from all plants, although with different efficiency from all
353 plants from the phenotyping assay. However, there was no association between presence or
354 abundance of the amplicon and the response to Avr9 (Figure 4a).
355 In a similar way, we tested the presence of *9DC* using the gene-specific primers DS1-CS1
356 (product size 507bp). LP12 and MM-Cf-9 served as positive and negative control, respectively.
357 We found complete absence of the *9DC* canonical region in our populations (Figure 4a).
358 Lastly, we also evaluated the presence of *Cf-4,* using newly designed primers (PSK047-PSK050)
359 that fall over the intron that defines the difference between *Cf-4* and *Cf-9* (Figure S2) and
360 amplify a 786bp product that is only present in *Cf-4*. We found that this *Cf-4*-specific region is
361 present in a few plants belonging to the central population, suggesting that unlike in other
362 *Solanum.* spp., *Cf-4* and *Cf-9* are not mutually exclusive in *S. chilense* (Figure 4). In addition, the
363 presence of bands corresponding to the canonical *Cf-4* region in plants non-responsive to Avr4
364 and the absence in responders, suggests that other or new recombinant gene products with
365 differences in their function exist in the different populations.
366

367 ***Cf-9* is expressed in a southern population**

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368 To test whether failed *Cf-9*(*-like*) gene expression is responsible for the complete loss of Avr9-
369 triggered HR in the southern populations; we extracted RNA from 10 individuals from the
370 LA4330 population. We used a primer pair PSK009-PSK010 that should allow us to amplify
371 cDNA originating from *Cf-9* orthologs and performed semi quantitative RT-PCR on plants
372 infiltrated with water and Avr9 (8 hours after infiltration), assuming that Avr9 recognition might
373 upregulate *Cf-9*(*-like*) gene expression.
374 Without treatment, we found a transcript expressed in all tested *S. chilense* plants, as well as in
375 our MM-Cf-9 control plant (Figure S4), indicating that steady state *Cf-9* gene expression is not
376 affected in the southern *S. chilense* populations.
377 As expected, in the MM-Cf-9 plant we see a stronger band at 8 hours after treatment, indicative
378 of the upregulation and positive feedback induced by *Cf-9,* a trait that is not visible in the
379 southern *S. chilense* population (Figure S4).
380

381 **Loss of resistance might be due to mutations in *Cf*-coreceptors the southern populations**
382 Seeing that steady state expression levels of *Cf-9* are not affected in the southern populations and
383 complete loss of resistance is unlikely to result from detrimental mutations on all individual *Cf*
384 genes. We hypothesize that the general loss of *Cf* responses likely results from changes in *Cf*
385 regulatory genes, rather than mutations in the individual *Cf* genes themselves. Several core
386 regulators or co-receptors are known to regulate the Cf protein function.
387 To test this hypothesis, we examined genome sequence data that are available for three plants,
388 which are from representative populations of the central, southern coastal and southern highlands
389 region (LA3111, LA2932 and LA4330 respectively) (Stam *et al*., 2019a). We extracted and
390 aligned the genomic sequences of the co-receptors *SERK3a* (also known as *BRI1-ASSOCIATED*

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391 *KINASE 1*, *BAK1*) and *SOBIR1*, the adaptor *ACIK1*, required for regulation of the complex, as
392 well as another regulatory co-receptor of the *BAK1*-containing complex, *BIR2* and its homolog
393 *BIR2b* (Table S2). In all cases, the extracted sequence data show polymorphisms between the
394 reference genome of *S. lycopersicum* Heinz1706 and the Avr4 and Avr9 responsive plant from
395 LA3111. In-frame indels were found for *BIR2b* between its sequence in *S. lycopersicum* and
396 those in the three *S. chilense* populations, yet, the three *S. chilense* populations show similar,
397 complete sequences. Several unique polymorphisms exist in the southern populations, e.g.
398 occurring in the plants from LA2932 and LA4330, but not in LA3111 from the central region or
399 in the *S. lycopersicum* reference genome sequence, resulting in up to 19 non-synonymous amino
400 acid changes for *SERK3a* in the plant from LA4330 (Table 1). In *ACIK1*, both the LA2932 and
401 the LA4330 plant have unique indels not present in the *S. lycopersicum* reference genome
402 sequence and in the LA3111 plant (Table 1). These results suggest that there are various possible
403 amino acid changes these receptors, co-receptors and adaptors that could be responsible for the
404 observed loss of resistance.
405

406 **Discussion**

407 Natural plant populations are hypothesized to maintain a certain durable resistance against
408 naturally co-occurring pathogens, yet little is known about the dynamics and the underlying
409 genomic diversity in these populations throughout a species range, which result in the
410 maintenance or loss of durable resistances.
411 *C. fulvum* is likely a natural pathogen of wild tomato species and known functional *Cf* resistance
412 genes have been isolated from several wild tomato species. Yet, the physiological interaction
413 between *C. fulvum* and wild tomato has not been documented. We show that *C. fulvum* race 5 is

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414 able to infect *S. chilense.* The formed intercellular hyphae resemble those observed in cultivated
415 tomato, meaning that also infection in nature will lead to severe reduction in photosynthetic
416 potential and thus will lead to loss of host fitness. We observed, both by the naked eye, as well as
417 using microscopy and staining, that after inoculation with *C. fulvum* plants from a *S. chilense*
418 population from the southern edge of the species range (LA4330) show higher susceptibility
419 when compared to plants from a population from the central part of the range (LA3111). These
420 findings were confirmed via quantification of fungal DNA. Thus, we show a compatible
421 interaction between *C. fulvum* and wild tomato species, but also clear differences in resistance
422 between the host populations.

423 The interaction between *C. fulvum* and tomato has been proven to be governed by gene-for-gene
424 interactions, in which secreted Avrs from *C. fulvum* are recognized by corresponding RLP
425 product of *Cf* gene from tomato (Joosten & de Wit, 1999; Stergiopoulos & de Wit, 2007). We
426 tested recognition of such Avrs in different populations of *S. chilense* from different
427 geographical locations with diverse climatic conditions, to understand whether the observed
428 resistance and susceptibility within *S. chilense* populations to *C. fulvum* follows a specific
429 geographical pattern. We phenotyped fifteen populations of *S. chilense* covering the whole
430 species range by infiltrating an apoplastic extract potentially containing all Avrs except for Avr5,
431 which is able to elicit immune responses that result in an HR. In northern and central
432 populations, around 70% of the plants recognized at least one Avr present in this extract.
433 Populations from the southern highlands and the southern coastal genotype groups showed no
434 Avr recognition. These results point at two important conclusions. First of all, Avr recognition in
435 the northern and central regions is not as conserved as previously hypothesized and secondly,
436 Avr recognition appears to have been completely lost at the southern edge of the species range.

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437 Previous reports suggest that Avr9 and Avr4 are the main factors in recognition of *C. fulvum* and
438 this recognition is conserved throughout all wild tomato species (Kruijt *et al*., 2005). We observe
439 Avr9 recognition to be present in 29% of the tested plants, whereas 11% of the plants recognized
440 Avr4. These findings are somewhat contrasting with earlier results. Kruijt *et al*. (2005) tested
441 only a small number of plants per population and did not report on the actual amounts of
442 responding plants. In line with our results, they showed Avr4 recognition in 16 out of 20 tested
443 populations and comparison of the accession numbers and linkage to their geographical origin,
444 confirmed that only four of the populations tested by Kruijt *et al.* belonged to the southern
445 genotype groups. Surprisingly, Avr9 recognition was not observed in all 20 accessions of *S.*
446 *chilense* tested by Kruijt *et al*. (2005), although similar Avr9 have been used. These differences
447 in results could be explained by the fact that all previous reports used young seedlings to allow
448 quick screening of the plants. In our assays we use fully mature (over 1-year-old) adult plants,
449 and we performed repeated infiltrations of the same plants. In addition, we observed differences
450 in the strength of the HR upon Avr9 infiltration in young and fully developed mature leaves
451 (Figure S3). Our finding therefore poses the first evidence of Avr9 recognition in *S. chilense* and
452 suggests that detailed testing of other species under different conditions might yield novel
453 interesting results. In about 6% of the tested plants we now show dual recognition of both Avr4
454 and Avr9, which has not been shown earlier.

455 It has been shown that Avr9 can be recognized by two *Cf-9* homologs, referred to as *Cf-9*/Hcr9-
456 9C and the recombinant *9DC*. For both of them, allelic variants with only a few nonsynonymous
457 mutations are known. We evaluated the presence of known canonical domains that define *Cf-9*
458 and *9DC* and found that the tested plants in all our populations do have the *Cf-9* domain but are
459 lacking the *9DC* domain. The sole presence of the *Cf-9* domain is an interesting contrast with the

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460 findings of Van der Hoorn *et al*. (2001), who showed the presence of *9DC* to be predominant in
461 another wild tomato species, *S. pimpinellifolium*, and a complete loss of *Cf-9* in southern
462 populations of that spoecies. Note that the southernmost *S. pimpinellifolium* populations are
463 geographically relatively close to the most northernly *S. chilense* populations that were analyzed
464 in our current study, yet they populate clearly different ecological niches (Peralta *et al.*, 2008).
465 Likewise, we also evaluated the presence of the *Cf-4* canonical region in our populations. The
466 canonical *Cf-4* domain is present in some individuals, but this does not correlate with our
467 phenotypic data on the development of an HR upon infiltration with the Avr4 protein. There can
468 be various reasons for this observation. First, the annealing sites of the *Cf-4* primers used might
469 carry crucial SNPs in *S. chilense*, resulting in failure of the PCR no detectable bands. Second, the
470 targeted region might be missing in the gene coding for the Avr4-responsive Cf protein, which
471 would not be surprising as similar mechanisms of recombination events, resulting in the
472 generation of a new gene of which the encoded protein has retained its recognition specificity,
473 have been reported earlier for other *Hcr9s,* albeit on a phylogenetic rather than population scale
474 (Van der Hoorn *et al*., 2001). Moreover, a study on *Cf-2* identified 26 different homologs in *S.*
475 *pimpinellifolium* populations and revealed possible presence/absence variation of *Cf-2* among
476 individuals (Caicedo & Schaal, 2004). Presence of canonical regions of *Cf-4* in the individual
477 with *Cf-9* canonical region confirms the dual presence of previous though allelic gene which can
478 be possibly due to heterozygosity at the locus.
479 Recombination and gene conversion have been shown to play a major role in gene family
480 evolution for RLP family as well as other resistance gene families (Paniske *et al.*, 1997;
481 Mondragon-Palomino & Gaut, 2005; Mondragon-Palomino *et al.*, 2017). Recently, it has been
482 reported that such gene conversions, or micro recombinations, of NLR genes can also be

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483 observed between accessions of another wild *Solanum* sp., leading to an alternative mechanism
484 to maintain different functional alleles (Witek *et al.,* 2020). We conclude that also the *Cf* gene
485 family is likely not conserved *sensu stricto*, and hypothesize that a large number of possibly
486 functional alleles are formed and maintained through intragenic micro recombinations, not just
487 on a phylogenetic scale, but also between or even within populations of the same species.
488 The loss of Avr recognition in southern populations could be explained by the possibility that
489 these plants recognize different Avrs which are absent in *C. fulvum* race 5. Yet, our findings
490 show that northern and central populations of *S. chilense* do possess resistance against *C. fulvum*
491 race 5. Our apoplastic extract contains at least 70 secreted effectors, which are all potential Avrs
492 (Mesarich *et al.*, 2017). The chance of losing recognition for all of these effectors by mutations
493 in the matching receptors is highly unlikely, thus hinting at general differences in the regulation
494 of Avr recognition. Semi quantitative RT PCR experiments showed that *Cf-9*-like genes appear
495 to be expressed in both Avr9-recognizing and non-recognizing plants. Single point mutations in
496 genes encoding essential co-receptors, like SOBIR1 or BAK1 have been shown to lead to loss in
497 ability to induce HR when expressed in a heterologous system (Bi *et al.*, 2016) or *Arabidopsis*
498 *thaliana* (Albert *et al*., 2019). Thus accumulation of deleterious mutations in such coreceptors or
499 contrarily, accumulation of beneficial mutations related to, for example drought adaptation, that
500 generate a trade of in resistance, are possible. BAK1/SERK3a is a co-regulator, in not only
501 defense responses, but the receptor-like kinase also plays a role in general cell regulatory
502 processes and is drought-responsive (Schwessinger *et al*., 2011). Genomic data for non-
503 responding plants from the southern populations revealed several nonsynonymous mutations, as
504 well as indels in multiple RLP/Cf co-receptor-encoding genes, when comparing them to either
505 the sequence from the LA3111 reference plant, or the *S. lycopersicum* reference Heinz 1706,

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506 thus suggesting that changes in downstream regulation mechanisms could have caused the loss
507 of Avr recognition in the south. Follow-up studies should determine which of the many altered
508 genes are causal. Since the co-receptors have multiple functions apart from Cf protein signaling,
509 such experiments might simultaneously shed light on how the different components of the
510 signaling network interact.

511 Loss of resistance over time at the population level is rather poorly understood, with some
512 theories possibly explaining this mechanism (Koskella, 2018). For instance, evolutionary loss of
513 resistance in certain populations might happen as a result of genetic drift, e.g. after a severe
514 bottle neck. However, if these populations would encounter any pathogen pressure afterwards,
515 this would be detrimental. Loss of resistance can be the outcome of random processes that are
516 triggered to take place due to loss of selection pressure, but this loss can also be the result of
517 accelerated evolution due to the, much debated, assumed fitness costs of carrying obsolete
518 resistance genes (Tian *et al*., 2003; Sheldon & Verhulst, 1996). The role of distribution of *C.*
519 *fulvum* in shaping the evolutionary distribution of *Cf-2* homologs has been proposed by Caicedo
520 (2008).

521 Giraud *et al.* (2017) point out that parasites need to develop a local adaptation to their
522 environment beyond their host. Loss of resistance, as observed in our system, strongly suggests
523 that a mechanism of ecological feedback is taking place, where the ecology of the population
524 becomes a driving force to lose resistance or to maintain it. Therefore, the absence of the *C.*
525 *fulvum* pathogen in the southern locations, due to extremely dry climatic conditions, which are
526 not suitable for infection by *C. fulvum*, would be a plausible explanation. Interestingly, higher
527 susceptibility to other pathogens also requiring relatively high humidity for successful infection,
528 like *Phytophthora infestans* and *Alternaria* sp., was already described for southern populations of

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529 *S. chilense* (Stam *et al.*, 2017). Seeing that some co-receptors potentially play a role in abiotic
530 stress responses, a third possibility would be that the loss of resistance comes as a tradeoff for
531 environmental adaptation. Yet, this is likely still intrinsically coupled to a decreased pathogen
532 pressure.
533 In conclusion, we show that *Hcr9* locus in *S. chilense* is much more complex than was thought
534 before (Parniske *et al.*, 1997; Van der Hoorn *et al.*, 2001; Kruijt *et al.*, 2004; 2005). This might
535 in part be due to the stronger niche differentiation, or the larger heterozygosity of *S. chilense*
536 specifically (Moyle, 2008). However, it is also likely that there is a lot of undiscovered diversity
537 in the *Cf* gene family present in other *Solanum* species. Furthermore, we provide an example of
538 the loss of resistance in a wild tomato species at the edge of its geographical distribution,
539 possibly explained by changes in the underlying immune receptor complexes.
540 Overall this study provides a step forward in terms of placing major gene-mediated molecular
541 defense mechanisms in an ecological context.
542

543 **Acknowledgements**

544 We like to thank Liza Keitel and Lina Muñoz for help with the experiments, Sabine Zuber,
545 Bärbel Breulmann and Anneliese Keil for maintaining the *S. chilense* populations and members
546 of SBF924 for fruitful discussions and useful feedback.
547

548 **Conflicts of interests**

549 The authors declare that no competing interests exist
550

551 **References:**

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722

723 **Author contributions**

724 Conceptualization: RS, RH, PSK and MJ; Investigation: PSK, MSS, GZ and DS; Contribution of 725 materials: MJ; Funding acquisition: RS; Writing: PSK and RS. All authors reviewed and 726 approved the manuscript.

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1 **Table**

2 Table 1: Number of non-synonymous SNPs or indels observed in the open reading frame of genes
3 encoding co-receptors or adaptors of *Cf* genes in the two southern populations LA2932 and LA4330.

Gene Name	SNPs in LA2932	SNPs in LA4330	SNPs in both LA2932 and LA4330
SOBIR1	4	1	0
SERK3a	4	19	1
ACIK1	1	3	2
BIR2	2	3	0
BIR2b	2	1	1

4

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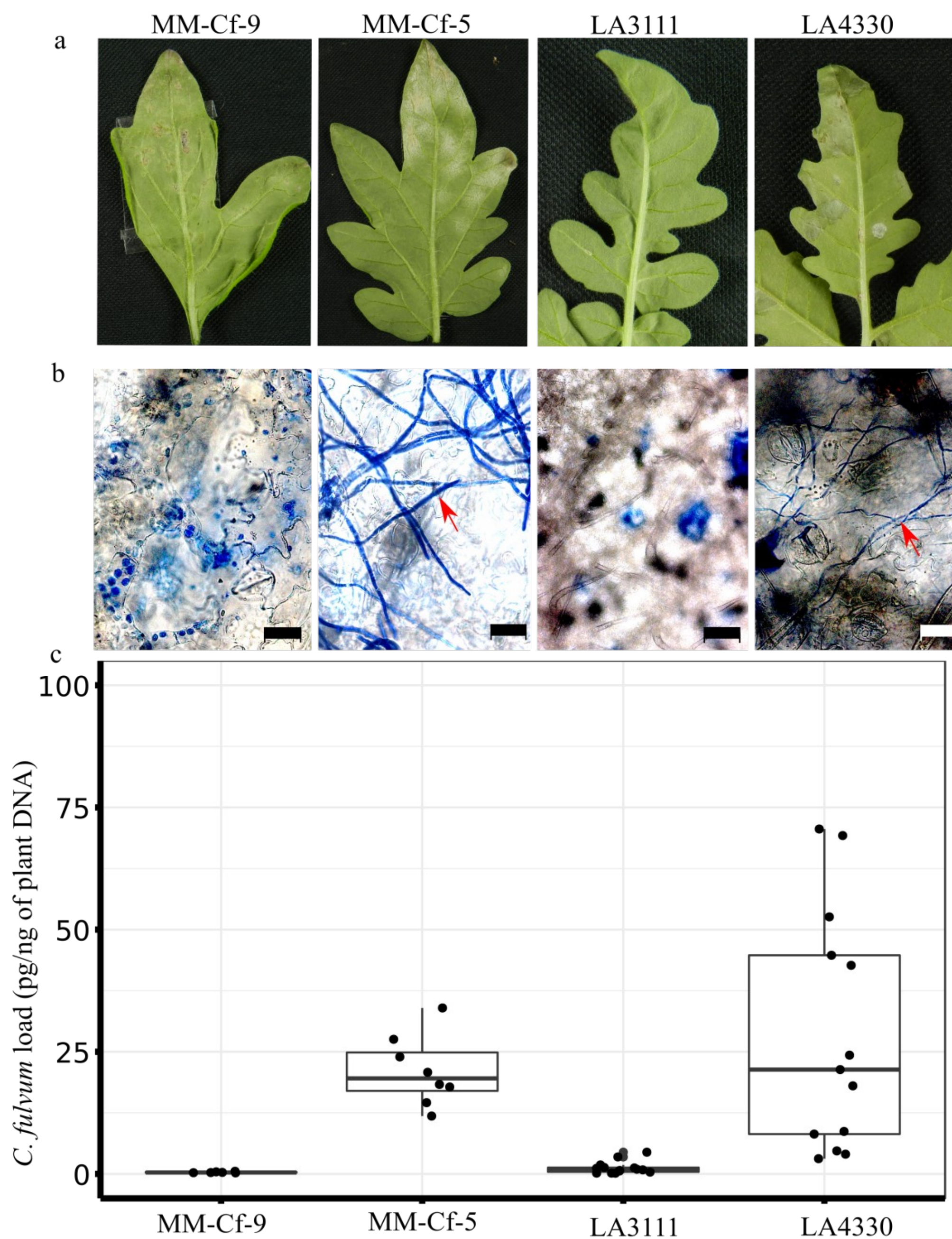
5 **Figures**



6

7 Figure 1: Tomato leaves inoculated with 2×10^4 conidia/ml. a) Left to right: inoculated leaves harvested
8 at 14 dpi of MM-Cf-9, MM-Cf-5 and *S. chilense* population LA3111 and LA4330. b) Microscopic
9 pictures of bleached leaves (scale bar = 20µm), after staining with ink (mycelium of pathogen indicated
10 with red arrows). c) Quantification of *C. fulvum* DNA load in pg/ng of plant DNA after 14 dpi of
11 inoculation in 3 plants per MM control and 5 plants per *S. chilense* population. All the plants were
12 evaluated in three technical replicates and each data point shows pathogen load per technical replicate.

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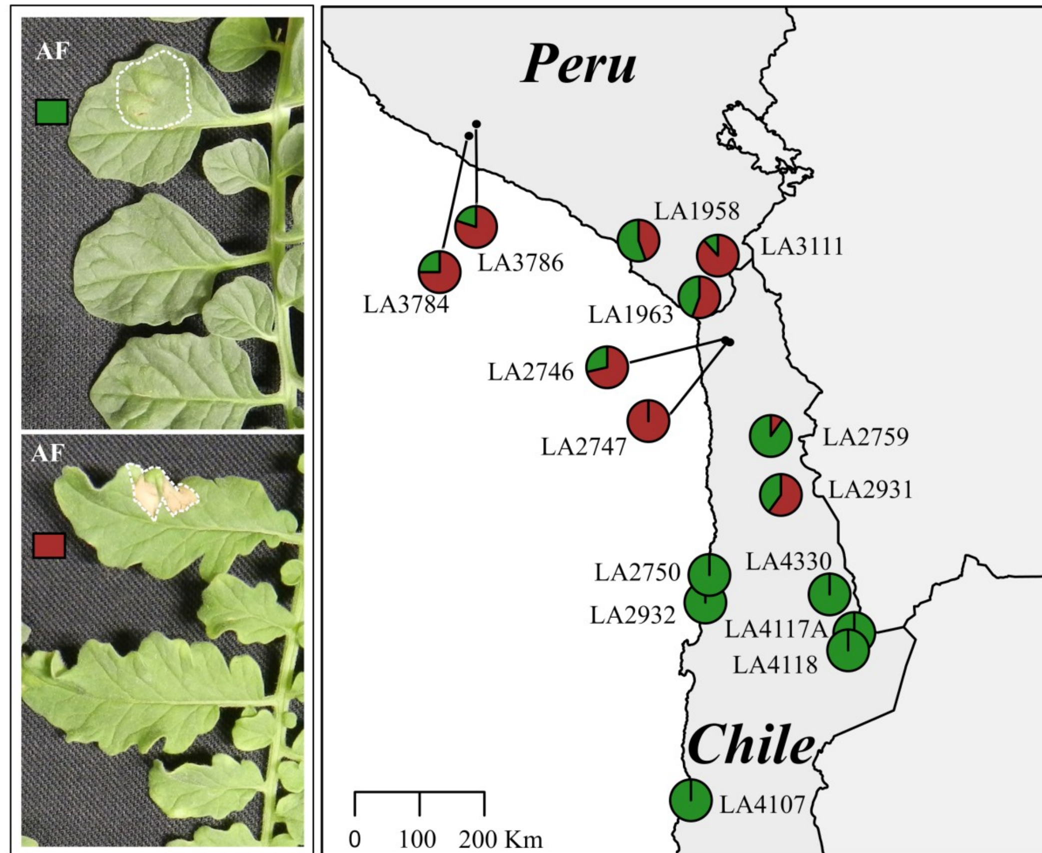

13

14 Figure 2: Infiltration of AF in populations of *S. chilense*. Leaf of an individual plant with no recognition
15 of components present in the infiltrated AF (top left) and leaf of an individual plant recognizing at least
16 one component present in the AF (bottom left). The infiltrated areas are indicated with white dotted lines.
17 The map on the right shows the geographical distribution of AF component perception in *S. chilense*. The
18 map shows the geographically distinct populations of *S. chilense* and their response to infiltration with AF
19 Each pie chart indicates one population with 8 to 17 individuals. The brick red fraction represents plants
20 that respond to *C. fulvum* AF, whereas green indicates the fraction of non-responding plants.

21

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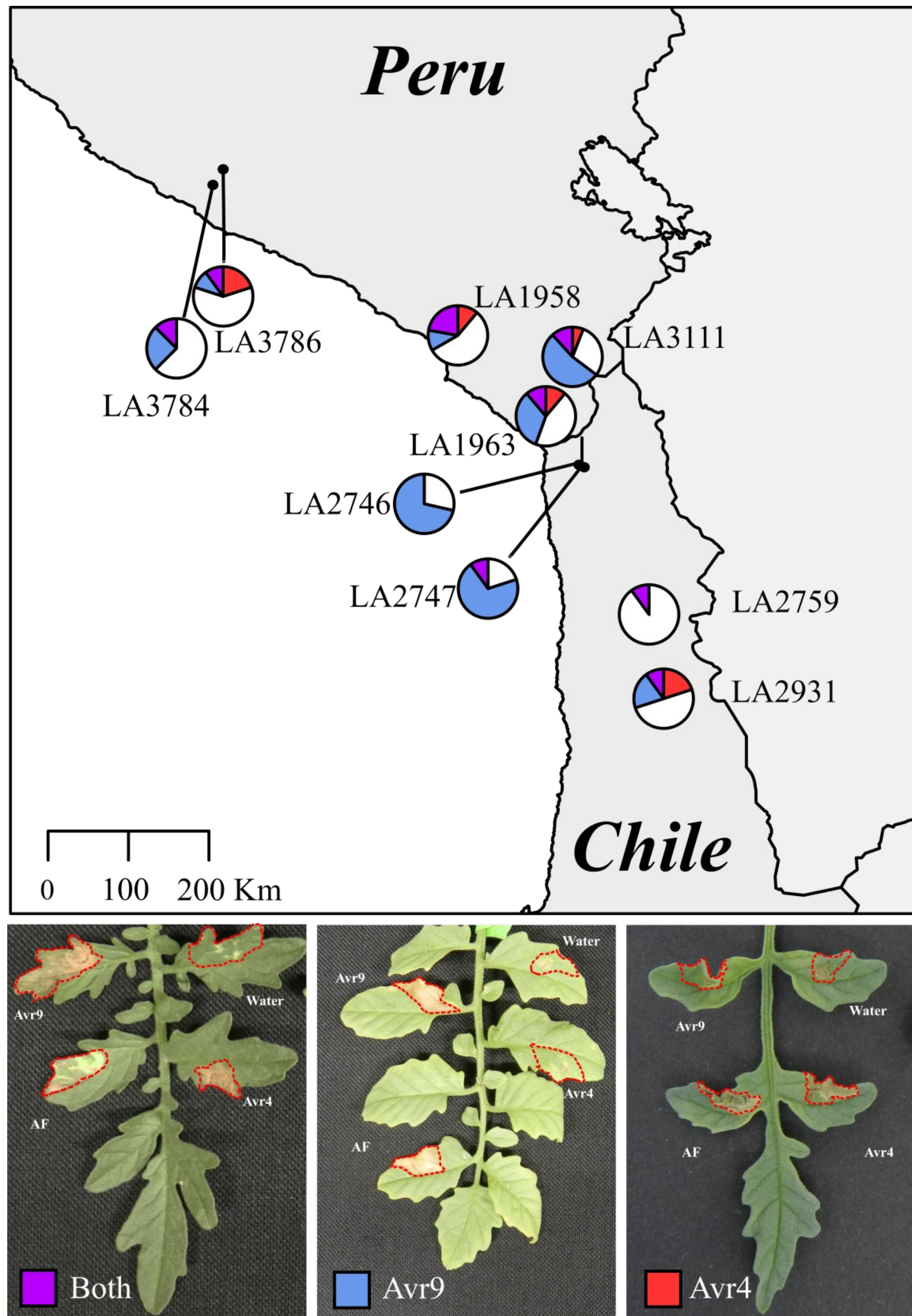

22

23 Figure 3: Geographical distribution of Avr9 and Avr4 perception in *S. chilense*. The map shows
24 populations of *S. chilense* and their response to infiltration with Avr9 and Avr4 (purple when there is an
25 HR-associated response to both), Avr9 (blue when there is an HR-associated response) and Avr4 (red
26 when there is an HR-associated response). White sectors indicate plants showing no recognition of either
27 Avr9 or Avr4. The infiltrated areas are indicated with red dotted lines. Each pie chart indicates one
28 population with 8 to 17 individuals.

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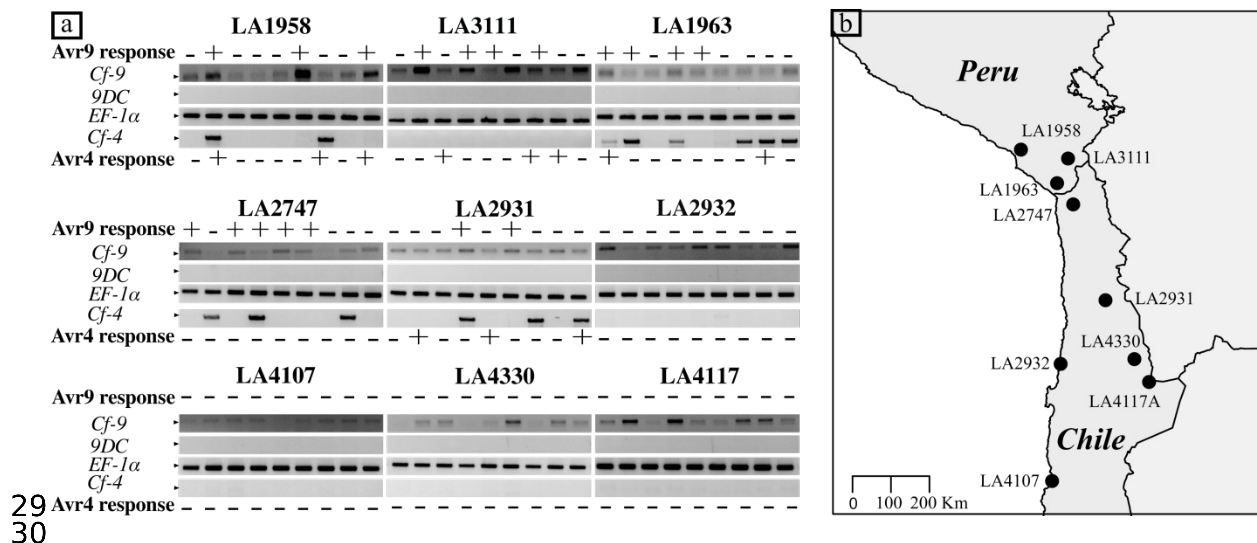

Figure 4: Amplification of the canonical *Cf-9, 9DC* and *Cf-4* region in geographically distinct populations of *S. chilense*. a) The CS5/CS1 primer pair was used to amplify a fragment of *Cf-9* (378 bp), the DS1/CS1 primer pair for *9DC* (507bp) and the PSK047/PSK050 primer pair for *Cf-4* (728bp). As a PCR control part of the coding region of *elongation factor 1 alpha (EF-1α)* was amplified using the primer pair RS003/RS004 (400 bp). MM-Cf-9 and MM-CF-4, and LP12 (*9DC*) were used as controls (Figure S2). + or – are indicative of Avr9 and Avr4 responsiveness upon infiltration of these effectors (Figure 2). b) Map shows the populations used in the analysis.