Genes Encoding Recognition of the Cladosporium fulvum Effector Protein Ecp5 Are Encoded at Several Loci in the Tomato Genome

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ABSTRACT The molecular interactions between tomato and Cladosporium fulvum have been an important model for molecular plant pathology. Complex genetic loci on tomato chromosomes 1 and 6 harbor genes for resistance to Cladosporium fulvum, encoding receptor like-proteins that perceive distinct Cladosporium fulvum effectors and trigger plant defenses. Here, we report classical mapping strategies for loci in tomato accessions that respond to an Ecp5-induced hypersensitive response, and in four different accessions. Our mapping showed that the Ecp5-induced hypersensitive response segregated as a monogenic trait, mapping to distinct loci in the tomato genome. We identified at least three loci on chromosomes 1, 7 and 12 that harbor distinct Cf-Ecp5 genes in four different accessions. Our mapping also showed that the Cf-Ecp5 in Solanum pimpinellifolium G1.1161 is located at the Milky Way locus. The Cf-Ecp5 in Solanum pimpinellifolium LA0722 was mapped to the bottom arm of chromosome 7, while the Cf-Ecp5 genes in Solanum lycopersicum Ontario 7522 and Solanum pimpinellifolium LA2852 were mapped to the same locus on the top arm of chromosome 12. Bi-parental crosses between accessions carrying distinct Cf-Ecp5 genes revealed putative genetically unlinked suppressors of the Ecp5-induced hypersensitive response. Our mapping also showed that Cf-11 is located on chromosome 11, close to the Cf-3 locus. The Ecp5-induced hypersensitive response is widely distributed within tomato species and is variable in strength. This novel example of convergent evolution could be used for choosing different functional Cf-Ecp5 genes according to individual plant breeding needs.

KEYWORDS Cladosporium fulvum tomato plant disease resistance genes Cf-Ecp5 convergent evolution Genetics of Immunity

Plant-microbe interactions, characterized genetically by the gene-for-gene model (Flor 1951), have typically been defined by the presence or absence of a pathogen avirulence gene and it’s corresponding disease resistance (R) gene in the host which together determine the outcome of the interaction. The gene-for-gene model has been portrayed at the molecular level as being part of a larger dynamic evolutionary process known as the “zig-zag” model (Jones and Dangl 2006), where R genes and pathogen effector/avirulence genes co-evolve in perpetual ‘boom-and-bust’ cycles (Priestley 1978). To date, effector-specific R genes have been predominantly confined to a single locus. Exceptions to this have been previously reported through convergent evolution of R genes in different species (Arabidopsis RPM1 and soybean RPG1; Arabidopsis RPS5 and wheat/barley PBR1) (Ashfield et al. 2014; Carter et al. 2018 preprint), within the same species, but linked (Arabidopsis RRS1/RPS4 and RRS1B/RPS4B) (Saucet et al. 2015), and even within related species at unlinked loci (potato Rpi-mcq1 and Rpi-blb3) (Aguilera-Galvez et al. 2018).
The pathosystem of tomato (*Solanum lycopersicum*) and the leaf mold pathogen *Cladosporium fulvum* is a well-studied model of gene-for-gene interactions and plant disease resistance gene evolution (Rivas and Thomas 2005; De Wit 2016). The fungus is considered an asexual non-obligate biotroph of the *Mycosphaerella* family that infects plants via conidia that settle on the abaxial leaf side, germinating and entering the plant through open stomata, leading to reduced respiration, defoliation and even host death (Thomma et al. 2005). The fungus abundantly secretes effector proteins in the leaf apoplast and several of these effectors can be recognized by corresponding *R* genes in specific tomato accessions. The tomato *R* genes (designated *Cf* genes, for resistance genes to *Cladosporium fulvum*) encode receptor-like proteins (RLPs) that localize to the plasma membrane and contain extracellular leucine-rich repeats (eLRRs), a membrane spanning domain, and a non-signaling short cytoplasmic domain. This system has acted as a model for investigating the structure and evolution of plant disease resistance gene loci (Thomas et al. 1998; Rivas and Thomas 2005; De Wit et al. 2012; Lin et al. 2014; De Wit 2016). To date, these genes have been mapped to just two chromosomal segments in the tomato genome. The Milky Way (MW) locus on the short arm of chromosome 1, and several genetically linked loci (like ORION; OR) containing functional *Cf* genes, encode a large number of genes with distinct recognition specificities including *Cf*-4, *Hcr9*-4E, *Cf*-9, *Hcr9*-9B, *Cf*-19, *Cf*-Ep1, *Cf*-Ep2, *Cf*-Ep3, *Cf*-Ep4, *Cf*-Ep5 (Jones et al. 1994; Thomas et al. 1997; Parmisie et al. 1999; Takken et al. 1999; Haanstra et al. 2000; Panter et al. 2002; Kruijt et al. 2004; Soumpourou et al. 2007; Zhao et al. 2016). These genes were designated as *Hcr9s* (homologs of *Cladosporium* resistance gene 9). Another complex locus (*Cf*-2/*CF*-5) encoding *Cf* genes has been characterized on the short arm of chromosome 6, that includes *Cf*-2.1, *Cf*-2.2 and *Cf*-5 (Dixon et al. 1996) with their genes having been designated *Hcr2s* (homologs of *Cladosporium* resistance gene 2) respectively (Rivas and Thomas 2005). However, the chromosomal locations of several other *Cf* genes, which may comprise new complex loci, have not yet been reported (Rivas and Thomas 2005; De Wit 2016).

Here, we deployed genetic mapping to investigate the genetics of the tomato hypersensitive response-type (HR) or cell death to *C. fulvum* extracellular protein (Ecp5). Ecp5 is 115 aa long with 6 cysteine residues and is also one of the least polymorphic *C. fulvum* effectors (Haanstra et al. 2000; Stergiopoulos et al. 2007; De Wit et al. 2009). In contrast to *C. fulvum* avirulence proteins (Avrs) that are race-specific, Ecps are secreted by all *C. fulvum* strains and are known to play an essential role in infection, since their mutation or deletion results in decreased virulence of *C. fulvum* on tomato, to which Ecp5 is suspected to be no exception (Luderer et al. 2002; De Wit et al. 2009; De Wit et al. 2012; Mesarich et al. 2017). A closely related fungus to *C. fulvum*, *Dothideomycetes septosporum* contains only a pseudogenised Ecp5 homolog, despite having functional homologs of other *C. fulvum* effectors like Avr4 and Ecp2 (De Wit et al. 2012).

A single gene controlling the Ecp5-induced HR in tomato was previously designated *Cf*-Ecp5 in the line *S. lycopersicum* G1.1161 (introgressed from *S. pimpinellifolium*) and mapped at the AURORA locus (AU), proximal to MW (Haanstra et al. 2000). Additional *S. pimpinellifolium* and *S. lycopersicum* accessions appeared to also carry a single dominant *Cf*-Ecp5 gene and develop a HR following inoculation with recombinant potato virus X (PVX) strains expressing Ecp5 (Haanstra et al. 1999; Haanstra et al. 2000).

Presuming that all Ecp5-responding accessions carried a similar *Cf*-Ecp5 gene at AU, a transposon tagging strategy was deployed to isolate it from *S. lycopersicum* line Ontario 7522 (Ont7522). However, unexpected genetic ratios during initial crossing lead to further investigation and reassignment of *Cf*-Ecp5 in G1.1161 to the MW locus. Three other *Cf*-Ecp5 loci from *S. pimpinellifolium* LA0722 and LA2852, and *S. lycopersicum* Ont7522 were shown to be genetically unlinked to MW. We used AFLP bulked segregant analysis and mapped *Cf*-Ecp5 in LA0722 to the bottom arm of chromosome 7 and *Cf*-Ecp5 in LA2852 and Ont7522 to the top arm of chromosome 12. Differential cell death symptoms were also observed among the *S. pimpinellifolium* and *S. lycopersicum* *Cf*-Ecp5-carrying accessions and allelism crosses between them revealed distinct Ecp5-dependent host regulators. We screened a total of 139 domesticated and wild tomato accessions for Ecp4- and Ecp5-induced HR and identified multiple accessions carrying *Cf*-Ecp4 or *Cf*-Ecp5 genes that could be used in future studies. We found that *Cf*-Ecp5 was distributed in a wider geographical region than *Cf*-Ecp4. Lastly, we report the chromosomal locations of *Cf*-11, which maps to the top arm of chromosome 11, close to the previously reported *Cf*-3 gene (Kanwar et al. 1980). All in all, this study revealed a unique example of multiple convergently evolved tomato loci in different accessions associated with the HR to *C. fulvum* Ecp5 effector that have not been previously reported in plant-pathogen interactions.

### MATERIALS AND METHODS

#### Plant and fungal materials

Seeds from core collections of wild and domesticated tomato species were obtained from the C.M. Rick *Tomato Genetics Resource Center*, University of California, Davis, USA. These species included *Solanum arcanum*, *Solanum cheesmaniae*, *Solanum chilense*, *Solanum chilensewii*, *Solanum cornelioi molleri*, *Solanum galapagense*, *Solanum habrochaites*, *Solanum huaylasense*, *Solanum lycopersicoides*, *Solanum lycopersicum*, *Solanum noorickii*, *Solanum ochranthum*, *Solanum pennellii*, *Solanum peruvianum* and *Solanum situm* (Table S3). Additional stocks containing previously characterized *Cf*-Ecp genes were obtained from Dr M. H. A. J. Joosten and Dr P. Lindhout (University of Wageningen) including the accessions *S. pimpinellifolium* LA1683 (*Cf*-Ecp4) and *S. lycopersicum* G1.1161 containing the introgressed *S. pimpinellifolium* gene *Cf*-Ecp5 (Haanstra et al. 2000).

A number of other stocks were used in this study for the genetic mapping of *Cf*-Ecp5 genes. The following lines were supplied by The Sainsbury Laboratory, Norwich, UK; *S. lycopersicum* variety ‘Moneymaker’ (MM), which contains no *Cf* genes (C0); the FT33 line (Rommens et al. 1992), which contains a T-DNA located 3 centimorgans (cM) proximal to the MW locus on the short arm of chromosome 1 and which harbors the maize transposon Dissociation (Ds) that carries the *Escherichia coli uidA* gene (GUS); line M18 contains an insertion of *Ds* in the *Cf*-9 gene (*Ds:Cf*-9) at the MW locus (Jones et al. 1994).

Forty-nine *S. pennellii* introgression lines (ILs) in the *S. lycopersicum* M82 background (Eshed and Zamir 1994) were used for AFLP mapping of *Cf*-Ecp5 genes to defined chromosomal intervals as described previously (Thomas et al. 1995). *C. fulvum* race 3 (CAN 84) and race 2.4.5.9.11 were obtained from Dr MHA Joosten at the University of Wageningen, Netherlands. *C. fulvum* race 4 GUS (Oliver et al. 1993) and race 5 were maintained and prepared for plant infections as described previously (Hammond-Kosack and Thomas 1994). The *S. lycopersicum* stock Ont7716 and near-isogenic lines (NILs) containing single introgressed *Cf* genes were obtained from The Sainsbury Laboratory in Norwich, UK (Tigchelaar 1984).

#### DNA preparation and marker sequence analysis

DNA was prepared from individual tomato lines and bulked segregant pools as described previously (Thomas et al. 1995; Soumpourou et al. 2000).
AFLP analysis of tomato genomic DNA was performed as described previously (Thomas et al. 1995). Cleaved amplified polymorphic sequence (CAPS) and simple sequence repeat (SSR) analyses were all performed as described previously (Soumpourou et al. 2007).

DNA gel blot analysis

Two to five μg of tomato DNA was digested at 37° for 16 h, extracted with phenol-chloroform and ethanol precipitated. DNA was electrophoresed for 2 h at 2.5 V/cm in 0.8% w/v agarose gels in a vertical gel apparatus using 40 mM Tris-acetate, pH 7.9, 1 mM EDTA as running buffer. Nucleic acids were transferred to Hybond-N membrane (Amersham) and cross-linked by irradiation with UV light. Filters were hybridized with 32P-labeled probes at 65° for 16-20 h and washed four times for 15 min in 2x SSC (1x SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) and 1% w/v SDS at 65°, and for 30 min in 0.2x SSC and 0.1% w/v SDS at 65°.

Assaying Cf-Ecp5 function by infiltration with recombinant potato virus X

Cf-Ecp5 function can be assayed in several ways based on expression of the C. fulvum Ecp5 protein in plants. In a genetic test, the F1 progeny of crosses between Cf-Ecp5 containing lines and MM plants stably expressing Ecp5 exhibit a characteristic seedling lethal phenotype. Transgenic MM lines expressing the C. fulvum Ecp5 protein (MM-Ecp5) were described previously (Soumpourou et al. 2007). Alternatively, Cf-Ecp5 function can be determined by delivering ECP5 in the form of recombinant Potato Virus X, expressing ECP5 (Soumpourou et al. 2007). Plants expressing Cf-Ecp5 exhibit systemic necrosis following virus replication and spread. Stocks of A. tumefaciens GV3101 were streaked onto Luria-Broth (LB) agar plates containing 40 μg/ml kanamycin. Colonies were selected and cultured in 10 mL LB medium containing 40 μg/ml kanamycin on a shaking platform at 28° overnight. The cultures were centrifuged at 2000 x g and the pellets re-suspended in a solution containing 1x Murashige & Skoog salts and 2% w/v sucrose. To initiate the expression of vir genes, acetosyringone (3-7-8-Dimethoxy-4-hydroxy-acetophenone) was added to a final concentration of 150 μM and the bacteria were left at room temperature for 3 hr. Bacteria were infiltrated into a single cotyledon of 7-10 day old seedlings. Plants containing the corresponding Cf gene showed signs of systemic necrosis at 7-8 days post-infection (dpi) (Thomas et al. 1997; Soumpourou et al. 2007).

Marker analysis

AFLP analysis of bulked-segregant pools for identifying markers linked in trans with various Cf-Ecp5 genes was performed as described previously (Thomas et al. 1995). Generation of CAPS (Cleaved Amplified Polymorphic Sequence) markers between specific haplotypes involved PCR amplification of sequences based on the tomato EXPEN2000 genetic map (http://solgenomics.net/) and sequencing of haplotype-specific products to detect sequence polymorphisms. PCR was carried out in a total volume of 20 μL containing 10 mM Tris-HCl, pH 8.0, 1.5 mM MgCl2, 0.2 mM dNTPs and 0.2 units Taq DNA polymerase, 50 ng genomic DNA and 1 μM of each primer. PCR products were purified using Qiaspin (Qiagen) columns and were sequenced on a 3730XL sequencer. Details of primers and CAPS markers used in this study are shown in Table S1. In reference to previous studies, genetic distances were estimated by converting recombination fractions using the Haldane function.

Bioinformatic analysis to detect LRR-encoding genes in defined regions of the tomato genome

To identify candidate RLP genes for Cf-Ecp5 on tomato chromosomes 7 and 12 target regions delimited by flanking Conserved Ortholog Set II (COSII) markers were defined. For each target region protein sequences for the two markers were extracted from the TAIR website (http://www.arabidopsis.org/) and a BLASTN search was carried out against version 2.30 of the ‘Heinz 1706’ tomato genome (Tomato Genome Consortium 2012). Using these results we defined target regions on chromosomes 7 and 12 which are shown in Table S7. The nucleotide sequence for each target region was extracted and six-frame translations were generated using the EMBOSS tool TRANSEQ (Rice et al. 2000). The β-sheets of Cf proteins (and other RLP and eLRR-RK proteins) contain highly conserved leucine-rich repeat motifs (LxLxxNxLxGxIP) (Rivas and Thomas 2005). Using the six-frame translations, a Perl regular expression search was carried out to detect motifs with this canonical structure. From this search we found 13 motifs within the chromosome 7 target and 35 within the chromosome 12 target. Of the 48 sequences, 34 were unique. The unique sequences were then taken and using MEME a mixture model was created (Bailey and Elkan 1994) to form a search model for LRR motifs. Before using the model to search for unknown RLPs within the two target regions, the model was tested on a region of the tomato genome known to encode RLPS. The tomato resistance genes Cf-2 and Cf-5 are located on chromosome 6 (Jones et al. 1993) and the nucleotide sequence for Cf-5 (AF053993) was used in a BLAST analysis of the tomato genome. Two highly homologous genes were found at the predicted location of the ‘Heinz 1706’ sequence between positions 2138887 – 2142639 and 2161278 – 2167796. An 18 kb gap separates these two genes, so the target area included the gene-spanning region plus an additional 18 kb on either side was used to test our model. Using FIMO (part of the MEME Suite) (Bailey et al. 2009), we then searched for LRR-like motifs in this region with a FIMO default p-value of 1e-4. Sixty-four LRR-like motifs were identified in the target area, fifty-six within the two Cf-2/Cf-5 homologs, and eight outside it. The lowest p-value of the presumed eight false-positives outside of the Cf-2/Cf-5 homologs was 1.06e-05. When all 64 LRR-like motifs were filtered on this p-value, we were left with 49 LRR-like motifs within the two homologs, and none outside. We then used FIMO with a q-value threshold of 1.06e-05 to identify LRR-encoding motifs within the chromosome 7 and 12 target regions and regions with consecutive blocks of LRR-encoding motifs as candidate LRR-encoding genes.

Data availability

All tomato seed stocks, bacterial and fungal strains are available upon request. Supplemental material available at figshare: https://doi.org/10.25387/g3.9770507.

RESULTS

The Cf-Ecp5 gene from S. pimpinellifolium G1.1161 maps at MW

We previously reported the mapping of two other S. pimpinellifolium genes (Cf-Ecp1 and Cf-Ecp4) at MW, where the majority of Cf genes have been mapped (Soumpourou et al. 2007). We also identified recombinants between the FT33 locus (which contains a T-DNA insertion harboring Ds:GUS and located 3 cM proximal to MW) and four Cf-Ecp genes we targeted for cloning. The observed recombination frequencies between the FT33 locus and genes located at MW (Cf-Ecp1 and Cf-Ecp4), and the Orion (OR) locus (Cf-Ecp2 being
10 cM proximal to MW), appeared consistent with their reported map locations (Haanstra et al. 1999; Soumpourou et al. 2007). The Cf-Ecp5 gene in S. pimpinellifolium G1.1161 was originally mapped at the Aurora (AU) locus, 3 cM proximal to MW, and therefore close to the FT33 locus (Haanstra et al. 2000). However, in our study the observed recombination frequency between FT33 and Cf-Ecp5 was higher than expected (3.67%) (Soumpourou et al. 2007), and similar to the recombination frequencies observed between FT33 and Cf genes at MW. This result suggested that Cf-Ecp5 is either located at MW or proximal to FT33.

An allelism test was performed where G1.1161 was crossed with line M18 which contains a stable Ds:GUS-tagged allele of Cf-9 (Jones et al. 1994). The F1 plants were then test-crossed to Cf0:Ecp5, Cf0, or S. pennellii LA0716 to generate segregating populations. The resulting progeny were screened for GUS activity and either the seedling wild-type (wt) or Necrotic (N) symptoms (Figure 1A, 1B). Out of 734 progeny, no recombinants were observed between Cf-Ecp5 and the Ds::GUS-tagged Cf-9, confirming they are either allelic or very tightly linked (Table 1). This result is consistent with Cf-Ecp5 being encoded by an Hcr9 at MW in S. pimpinellifolium G1.1161 and was therefore designated Cf-Ecp5.1.

### The Cf-Ecp5 gene in S. pimpinellifolium LA2852 and S. lycopersicum Ont7522 map to chromosome 12

We assumed that Cf-Ecp5 genes previously identified in other tomato accessions (LA0722, LA1689, LA2852, Ont7522) would map to the same chromosomal location as Cf-Ecp5.1 at MW (Laugé et al. 2000). Our preliminary analysis demonstrated that Cf-Ecp5 asssorted independently of the FT33 locus in these accessions. To map these genes we identified S. pennellii markers linked in trans to each gene which could then be located on S. pennellii introgression lines (ILs) (Eshed and Zamir 1994). S. pimpinellifolium LA2852 and S. lycopersicum Ont7522 were each crossed to S. pennellii LA0716 and testcrossed to Cf0, to generate segregating populations. Testcross progeny were agro-infiltrated with PVX:Ecp5 and scored for Ecp5-induced HR. For each cross, DNA bulked segregant pools were created from the cotyledons of forty testcross plants exhibiting either wild-type (wt) or Necrotic (N) symptoms (Figure 1A, 1B). The S. pennellii markers linked in trans were similar for LA2852 and Ont7522 phenotypic bulks, suggesting the two Cf-Ecp5 genes are

| Phenotype | C0 x (G1.1161 x M18) | LA0716 x (G1.1161 x M18) | C0:Ecp5 x (G1.1161 x M18) |
|-----------|----------------------|--------------------------|---------------------------|
| GUS:wt    | 175                  | 79                       | 109                       |
| no GUS:N  | 201                  | 62                       | 108                       |
| GUS:N     | 0                    | 0                        | 0                         |
| no GUS:wt | 0                    | 0                        | 0                         |
| Recombinant fraction | 0%                  | 0%                       | 0%                        |

Table 1 The Cf-Ecp5 gene in S. pimpinellifolium G1.1161 is allelic or very tightly linked to the Cf-9 gene at MW. Test cross progeny were screened using a standard histological procedure for the presence or absence of GUS activity (GUS or no GUS) and for the presence or absence of Cf-Ecp5 (Necrotic or wild type respectively) based on the development of either a systemic necrosis phenotype following agro-infiltration with PVX:Ecp5 (crosses to Cf0 and LA0716) or a seedling lethal phenotype (crosses to C0:Ecp5).
Table 2 Unexpected suppression of Ecp5-related HR in crosses between different Cf-Ecp5-carrying accessions. Data from different populations following agro-infiltration with PVX:Ecp5. Phenotypes were recorded at 10-14 dpi. G1.1161 carries a Cf-Ecp5 gene on chromosome 1, Ont7522 and LA2852 carry a Cf-Ecp5 on chromosome 12, while Cfo and LA0716 lack Cf-Ecp5. Population B851 lacked Cf-Ecp5.12p, while B852 and B853 carried it. In this study, LA2852 was usually crossed to Cfo initially to form a bridged cross due to difficulties in direct crosses to other accessions. The cross, phenotypes (wild-type or Necrotic), expected ratios and p-values are shown (p-values were calculated in each cross based on the expected ratio if there was no suppression for dominant traits; ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗∗ ∗*
Figure 2. The Cf-Ecp5 gene in S. pimpinellifolium LA0722 maps on the bottom arm of chromosome 7 in tomato. (A) Mapping of S. pennellii AFLP marker 91R30-M88 linked in trans with Cf-Ecp5 in LA0722 alongside bulks of ILs that represent entire tomato chromosomes. Each phenotypic bulk is shown under the “wt” and “N” labels for each accession (“wt” = wild type, “N” = Nontoxic). Tomato chromosome numbers (1-12) for IL bulks are shown for each lane. (B) AFLP mapping of S. pennellii marker 91R30-M88 linked in trans with Cf-Ecp5 in LA0722 in the chromosomal interval IL7-4. S. pennellii bulk IL for chromosome 7 (IL ch 7) and individual ILs (7-1 to 7-5) covering the entire chromosome 7 are shown. (C) Genetic map of the chromosome 7 region at the Cf-Ecp5 locus in LA0722 (green box). The interval was delimited by genotyping 58 wild-type Cf0 x LA0722 F2 plants with four markers: C2_At5g14520 (34/116 recombinant gametes), C2_At1g78620 (7/116 recombinant gametes), C2_At3g15290 (11/116 recombinant gametes), and C2_At4g26750 (19/116 recombinant gametes). Arrows indicate AFLP markers linked to trans to Cf-Ecp5 in LA0722. Distances are shown in megabases (Mb) and the location of each marker on the chromosome is also shown as Mb coordinates. The recombination fraction (%) between the CE locus (black box) and each marker is shown.

The tomato Cf-11 and Cf-3 genes are genetically linked on the top arm of chromosome 11

S. lycopersicum line Ont7716 carries the Cf-11 gene, introgressed from S. pimpinellifolium, and its location has not been previously reported. Ont7716 is known to carry a copy of Cf-4 as well (Enya et al. 2009), so any mapping effort required an Ont7716 progeny line carrying Cf-11 only to facilitate disease resistance phenotyping and subsequent mapping. To this end, 91 plants from a (Cf0 x Ont7716) x Cf0 population were inoculated with C. fulvum race 4 GUS, which can overcome resistance conferred by Cf-4, but not Cf-11 (Oliver et al. 1993). Results confirmed the presence of Cf-11 (43:48 Resistant:susceptible, \(p\)-value(\(x^2\)) \(= 0.711\)) and resistant plants were randomly selected to be selfed. Seven selfed populations were infected with C. fulvum race 5 that cannot overcome resistance to either Cf-4 or Cf-11 (Table S4), thus any population segregating phenotypically in a 3:1 ratio would carry the Cf-11 gene only. Four F2 families were identified that carried Cf-11 only and further selfing and progeny tests (J476) resulted in the identification of a Cf-11 homozygous plant, designated ‘Cf11N’ that was used as parent for mapping. However, preliminary attempts to identify molecular markers linked to Cf-11 using Ont7716 x S. pennellii F2 population were unsuccessful due to challenges in disease phenotyping. Thus, to select a parent that will be polymorphic to Ont7716 for AFLP analysis and subsequent mapping of Cf-11, Ont7716 was analyzed by DNA gel blots with probes that detect Hcr9s and Hcr2s (Figure S4A, S4B). From the results the Cf2 haplotype was chosen as a polymorphic parental line to be crossed with Ont7716. Resistant and susceptible F2 bulks from a (Cf2 x Ont7716) x Cf0 cross were analyzed by AFLP analysis with 264 primer combinations and one marker (M1) linked in cis to Cf-11 was identified. BLAST analysis on the M1 sequence showed that the marker is located at 1.232 Mb on chromosome 11 (based on the S. pennellii genome sequence) (File S1). To construct a genetic map, 938 progeny from J476 (Cf0 x Ont7716) background, segregating for Cf-11, were infected with C. fulvum race 4 GUS and 214 susceptible plants were genotyped with markers M1 and SSR136 (704:234 resistant:susceptible, \(p\)-value(\(x^2\)) \(= 0.979\)) (Table S5). The results positioned the locus between the two markers (M1 and SSR136), while a subsequent screen with markers 0124F20 and 136SP6 confirmed this and further delimited Cf-11 to a 535 kb region (1.322-1.857 Mb) (Figure 4).

The tomato genetic map indicated the S. lycopersicum Cf-3 gene is also located on the top arm of chromosome 11 (Kanwar et al. 1980). Preliminary marker analysis showed that the Cf3 haplotype was similar to Cf11N at the Cf-11 locus and therefore, these genes may be linked, or possibly allelic. The progeny of a (Cf3 x Cf11N) x Cf0 cross were inoculated with C. fulvum race 4 GUS that is restricted by
either gene. If Cf-3 and Cf-11 are allelic, no susceptible progeny should have been recovered from this cross. However, a number of progeny (6 out of 288) were scored as susceptible, showing similar levels of fungal sporulation to Cf0 control plants at 14 dpi. Molecular analysis of these individuals confirmed they contained markers of the Cf-3/Cf-11 haplotype, hence Cf-3 and Cf-11 appear to be closely linked (Figure 4). The chromosomal region where both Cf-3 and Cf-11 reside appears to be devoid of RLPS in the *Solanum lycopersicum* genome (Jupe et al. 2013; Andolfo et al. 2014; Wei et al. 2016; Kang and Yeom 2018).

**DISCUSSION**

**Expanding the Cf genetic map in tomato**

Breeding for resistance to *C. fulvum* has a long history, but breeding for durable resistance will depend on successful introduction of novel Cf genes from wild tomato species (Bailey 1947; Kerr and Bailey 1964). The *Cf* gene map has been extended in this study to include new loci on three different chromosomes (Figure 5). The genetic mapping of Cf-Ecp5.1 to MW further highlights the importance of this locus in the tomato genome to generate effector recognition specificities (Jones et al. 1994; Thomas et al. 1997; Takken et al. 1999; Panter et al. 2002; Yuan et al. 2002; Rivas and Thomas 2005; Soumpourou et al. 2007; De Wit et al. 2009; Zhao et al. 2016). The reassignment of Cf-Ecp5.1 at MW from AU is possibly due to the different functional analyses performed to assay Cf-Ecp5 function. In this study we used only PVX:Ecp5, while in the original study a combination of *C. fulvum* infections and PVX:Ecp5 were used in mapping that could have resulted in assaying two distinct, but linked fungal resistance genes (Laugé et al. 2000).

All previously characterized Cf genes on tomato chromosomes 1 and 6 encode RLPS (Wulff et al. 2001; Wulff et al. 2004). To accurately identify candidate RLP genes on the newly mapped tomato loci CE, Cf-11/Cf-3 and AN on chromosomes 7, 11 and 12 respectively, a high quality genome sequence is needed from each accession, as Cf loci are highly polymorphic (Wulff et al. 2009). In an early attempt, we analyzed the *S. lycopersicum* ‘Heinz 1706’ tomato genome sequence (Tomato Genome Consortium 2012) and the *S. pimpinellifolium* LA1589 draft genome sequence (Tomato Genome Consortium 2012) in regions delimited by the flanking markers for each locus in this study, retrieving some informative data only for ‘Heinz 1706’ (Table S7). However, recent studies have identified candidate *R* genes in the regions covering the novel *Cf* loci reported in this study (Table S7) (Jupe et al. 2013; Andolfo et al. 2014; Wei et al. 2016; Kang and Yeom 2018). The *CE* and Cf-11/Cf-3 loci, on chromosomes 7 and 11 respectively, reside in regions poor in RLPS (Kang and Yeom 2018).
while the AN locus resides in a hotspot for RLPs similar to the Milky Way locus on chromosome 1, which is confirmed by both our analysis (Table S7) and another study (Kang and Yeom 2018). It will be interesting to know whether all these novel Cf genes encode RLPs or something else.

The multiplicity of Ecp5 responses in tomato species

In some plant-microbe interactions, two or more loci may control the resistance phenotype in an extension of Flor’s gene-for-gene hypothesis (Flor 1947; Bourras et al. 2016). However, these genes do not convergeently evolve in different chromosomal locations, with the exception of the potato Rpi-b1b3 and Rpi-mcq1 genes to date (Aguilera-Galvez et al. 2018), which exhibit differential effector-specific resistance to Avr2-carrying Phytophthora infestans isolates (Aguilera-Galvez et al. 2018). This distinctiveness of these two potato R genes recognizing Avr2 has been attributed to independent evolution of the underlying recognition mechanism, since the genes originate from Mexico (Rpi-b1b3) and from Peru (Rpi-mcq1) (Aguilera-Galvez et al. 2018). Avr2 is also a highly diverse effector within Phytophthora species (Veleshouver et al. 2011), which could facilitate convergent evolution of two R genes through overlapping recognition specificities.

In this study, from the mapping attempts on only four accessions, four Cf-Ecp5 loci were mapped on three different chromosomes, exhibiting differences in HR strength in both PVX-Ecp5 agro-infiltration assays and crosses to Ecp5-expressing transgenic tomatoes (Figure S3), while allelism crosses between different Cf-Ecp5-carrying accessions revealed putative suppression elements that point to distinct evolution of effector recognition or defense activation mechanisms in tomato (Table 2). Crosses between S. pennellii LA0716 and S. pimpinellifolium LA2852 or S. lycopersicum Ont7522 resulted in suppression of the Ecp5-induced HR at the F1 generation, while the same phenotype was also observed in a S. pimpinellifolium G1.1161 x (C0 x LA2852) background (Table 2). The most interesting example of Ecp5-induced HR suppression was observed in a cross between S. lycopersicum Ont7522 and F1 progeny from C0 x S. pimpinellifolium LA2852, in which both accessions carry a Cf-Ecp5 gene on chromosome 12 at AN locus (Table 2). We genotyped 39 randomly selected wild-type plants to investigate if the Ecp5-induced HR suppression asserts independently of the AN locus on chromosome 12. The results indicated that the genetic elements responsible for this Cf-Ecp5-induced HR suppression were genetically unlinked to the AN locus and accession-specific for each Cf-Ecp5 gene (Table S6). These data suggest that these Cf-Ecp5 genes have evolved distinct HR-regulating elements.

Just like other characterized Cf genes, Cf-Ecp5s possibly also encode RLPs, which would lack any intracellular signaling domain and may interact with the receptor-like kinase (RLK) SOBIR1 (Liebrand et al. 2013), or a similar RLK that will stabilize them, and require the RLK BAK1 for downstream immune signaling (Van Der Burgh et al. 2019). However, functional tests will be required to determine if Ecp5 is perceived by functionally similar/distinct CfEcp5 proteins and whether they all require SOBIR1 and BAK1 for defense activation. The suppression phenotypes of Ecp5-dependent HR in different Cf-Ecp5-carrying accessions (Table 2) may also help to elucidate this question.

Ecp5 is the most monomorphic effector reported in C. fulvum, having only one variant with a single polymorphic nucleotide in its single intron (Stergiopoulos et al. 2007; De Wit et al. 2009), which raises an interesting question as to how these different accessions evolved genetically-distinct Ecp5 recognition specificities. All Ecp5-responding accessions originated from different geographic regions (Figure 5) (Kanwar et al. 1980; Laugé 1999; Laugé et al. 2000; Rivas and Thomas 2005; De Wit et al. 2009) and whether geographic origin plays a role in the evolution of strong responses or not remains to be seen. The geographic distribution of Ecp5-responding accessions appeared much wider than either Cf-Ecp4 or Cf-9 (Figure 3), which could be explained by the convergent evolution of multiple distinct Cf-Ecp5 loci under different environmental conditions or low selection pressure (Stergiopoulos et al. 2007; Bolton et al. 2008). Another interesting question is how many distinct Cf-Ecp5 loci and types of Ecp5-responses exist throughout tomato species that were not screened in our study (Figure 3) (Table S3). If Cf-Ecp5 genes are to be incorporated into breeding programs, their allelic and variant interactions need to be studied further in different backgrounds and with multiple C. fulvum strains.

Do effector-specific R genes emerge and evolve anywhere?

Cf genes have been repeatedly bred into cultivated tomato from wild relatives such as S. pimpinellifolium, S. hirsutum and S. peruvianum (Atherton and Rudich 1986) and according to a recent study (Kang and Yeom 2018) most of these types of genes (RLPs) are located on tomato chromosomes 1 and 12. Some regions appear to be the source of major Cf loci, like MW, which are able to generate multiple...
effector-specific RLPs (Rivas and Thomas 2005; De Wit et al. 2009). Although distinct recognition of multiple effectors by one or more R proteins is known (Mackey et al. 2002; Ma et al. 2018), the opposite has only been observed as convergent evolution in different species (Ashfield et al. 2014; Sauzet et al. 2015; Carter et al. 2018 preprint) with only one recent example in closely related species (Aguilera-Galvez et al. 2018). However, considering the monomorphic nature of Ecp5 and level of genetic complexity observed among Cf-Ecp5 genes in this study, we have to address this slight paradox in terms of evolution. There are hypotheses that might be able to explain the Cf-Ecp5 evolution, since conventional conservation of a Cf-Ecp5 allele through natural selection (Mauricio et al. 2003) is unlikely to be the source of four genetically distinct genes, nor are the putative overlapping specificities of distinct R proteins for a highly variable effector like Avr2 (Gilroy et al. 2011; Aguilera-Galvez et al. 2018), as Ecp5 is not.

The first hypothesis involves small-scale genomic duplications following unequal crossover events or other chromosomal anomalies that have allowed the dispersal of an original cluster harboring additional genes in Ecp5 and level of genetic complexity observed among Cf-Ecp5 genes in this study, we have to address this slight paradox in terms of evolution. There are hypotheses that might be able to explain the Cf-Ecp5 evolution, since conventional conservation of a Cf-Ecp5 allele through natural selection (Mauricio et al. 2003) is unlikely to be the source of four genetically distinct genes, nor are the putative overlapping specificities of distinct R proteins for a highly variable effector like Avr2 (Gilroy et al. 2011; Aguilera-Galvez et al. 2018), as Ecp5 is not.

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The candidate Cf-Ecp5 gene and Ecp5 in tobacco, N. benthamiana, Cf0, or agroinfiltration of the candidate Cf-Ecp5 gene in Cf0:Ecp5 transgenic tomatoes. Silencing SOBIR1 and BAK1 in each CfEcp5 line will provide further insights about each Cf-Ecp5 protein’s downstream function like other Cf genes or not. As for the unlinked suppressor loci, a genotyping-by-sequencing strategy of suitable phenotypic bulks (Table 2; Necrotic vs. wild type) can map and facilitate their subsequent isolation and characterization, identifying novel genes in downstream defense activation. The commercial deployment of Cf-Ecp5 can force C. fulvum to lose Ecp5 from its secretome, but Ecp5’s lack of allelic variation could reflect selective restraints by the pathogen to maintain full virulence (Mesarch et al. 2017). Furthermore, the simultaneous stacking of different variants of Cf-Ecp5 genes into one cultivar could extend the duration of resistance to C. fulvum and constitute a model for horizontal resistance in other plant pathosystems in the future (Dangl et al. 2013).

**CONCLUSIONS**

In plant pathology, it is important to consider the underlying complexity of each pathosystem and its gene-for-gene interactions. The discovery of novel Cf loci in this study and expansion of the Cf repertoire with loci of variable HR strength (Cf-Ecp5s) has potential implications for breeding plant disease resistance. Vertical resistance in tomato can be defeated quickly as strains of C. fulvum that overcame several Cf genes have been reported already (Kerr and Bailey 1964; Luderer et al. 2002; Enya et al. 2009). C. fulvum secretes approximately 70 apoplastic proteins in planta (Mesarch et al. 2017), from which a significant number act as effectors and are recognized in wild tomato accessions, making it very likely that a large number of novel Cf genes can be exploited for breeding purposes in the future. Considering that Ecp5 is a core effector in C. fulvum and has no natural variability, studying the differences between distinct Cf-Ecp5 genes in the tomato germplasm can facilitate our understanding on effector- or strain-specific recognition and defense activation, while allowing us to choose or engineer optimal Cf gene variants that have the strongest possible defense responses to different C. fulvum strains with minimal cost-to-fitness. To identify the causative genes at each locus and facilitate their introgression in elite breeding lines, functional screens will be required for any candidate gene within the genomic regions of interest that has structural similarities to RLPs (Rivas and Thomas 2005). Such screens can include co-agroinfiltration of the candidate Cf-Ecp5 gene and Ecp5 in tobacco, N. benthamiana, Cf0, or agroinfiltration of the candidate gene in Cf0:Ecp5 transgenic tomatoes. Silencing SOBIR1 and BAK1 in each CfEcp5 line will provide further insights about each Cf-Ecp5 protein’s downstream function like other Cf genes or not. As for the unlinked suppressor loci, a genotyping-by-sequencing strategy of suitable phenotypic bulks (Table 2; Necrotic vs. wild type) can map and facilitate their subsequent isolation and characterization, identifying novel genes in downstream defense activation. The commercial deployment of Cf-Ecp5 can force C. fulvum to lose Ecp5 from its secretome, but Ecp5’s lack of allelic variation could reflect selective restraints by the pathogen to maintain full virulence (Mesarch et al. 2017). Furthermore, the simultaneous stacking of different variants of Cf-Ecp5 genes into one cultivar could extend the duration of resistance to C. fulvum and constitute a model for horizontal resistance in other plant pathosystems in the future (Dangl et al. 2013).

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