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Research

Sequential breakdown of the *Cf-9* leaf mould resistance locus in tomato by *Fulvia fulva*

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Summary

- Leaf mould, caused by *Fulvia fulva*, is a devastating disease of tomato plants. In many commercial tomato cultivars, resistance to this disease is governed by the *Cf-9* locus, which encodes five paralogous receptor-like proteins. Two of these proteins confer resistance: Cf-9C recognises the previously identified *F. fulva* effector Avr9 and provides resistance during all plant growth stages, while Cf-9B recognises the yet-unidentified *F. fulva* effector Avr9B and provides mature plant resistance only. In recent years, *F. fulva* strains have emerged that can overcome the *Cf-9* locus, with *Cf-9C* circumvented through *Avr9* deletion. To understand how *Cf-9B* is circumvented, we set out to identify *Avr9B*.
- Comparative genomics, transient expression assays and gene complementation experiments were used to identify *Avr9B*, while gene sequencing was used to assess *Avr9B* allelic variation across a world-wide strain collection.
- A strict correlation between *Avr9* deletion and resistance-breaking mutations in *Avr9B* was observed in strains recently collected from *Cf-9* cultivars, whereas *Avr9* deletion but no mutations in *Avr9B* were observed in older strains.
- This research showcases how *F. fulva* has evolved to sequentially break down the *Cf-9* locus and stresses the urgent need for commercial tomato cultivars that carry novel, stacked resistance genes active against this pathogen.

Introduction

Leaf mould, caused by *Fulvia fulva* Cooke (syn. *Cladosporium fulvum* and *Passalora fulva*) (Videira *et al.*, 2017), is a devastating fungal disease of tomato plants (*Solanum lycopersicum*), which results in severe defoliation and yield losses under humid glasshouse and high tunnel conditions (Thomma *et al.*, 2005). During infection, *F. fulva* resides in the apoplastic environment located between the leaf mesophyll cells, where it secretes an arsenal of effector proteins (virulence factors) to promote host colonisation and disease (de Wit, 2016; Rocafort *et al.*, 2020; Mesarich *et al.*, 2023). This arsenal includes at least 75 small, secreted proteins (SSPs), most of which are stabilised by intramolecular disulphide bonds that prevent their degradation by apoplastic tomato proteases (Joosten *et al.*, 1997; Luderer *et al.*, 2002a; Mesarich *et al.*, 2018).

In tomato, resistance to *F. fulva* is governed by *Cf* (resistance to *C. fulvum*) genes which, based on those cloned to date, encode cell surface-localised receptor-like proteins (RLPs) that recognise specific effectors in the apoplastic environment (Thomas *et al.*, 1998; Kang & Yeom, 2018). Since these RLPs do not have signalling capacity themselves, they must interact with receptor-like kinases (RLKs), such as SOBIR1 and BAK1, to transduce defence response signals upon effector recognition (Liebrand *et al.*, 2013, 2014; Gust & Felix, 2014; Postma *et al.*, 2016; van der Burgh *et al.*, 2019). Following these events, immune responses, such as the hypersensitive response (HR), which is a localised form of programmed cell death, are initiated that halt *F. fulva* growth (de Wit *et al.*, 2009; Huang *et al.*, 2021).

Recognised effectors are called avirulence (Avr) effectors. To date, five tomato *Cf* gene/*F. fulva Avr* gene pairs have been cloned. These are *Cf-2*/*Avr2* (Dixon *et al.*, 1996; Luderer

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et al., 2002b), *Cf-4*/*Avr4* (Joosten *et al.*, 1994; Thomas *et al.*, 1997), *Cf-4E*/*Avr4E* (Thomas *et al.*, 1997; Takken *et al.*, 1999; Westerink *et al.*, 2004), *Cf-5*/*Avr5* (Dixon *et al.*, 1998; Mesarich *et al.*, 2014), and *Cf-9C*/*Avr9* (van Kan *et al.*, 1991; van den Ackerveken *et al.*, 1992; Jones *et al.*, 1994). In these cases, strains (races) of *F. fulva* have emerged, which can overcome resistance mediated by the *Cf* gene. With regard to the corresponding *Avr* genes, circumvention has been achieved through gene deletion, indels (frame-shift mutations), transposable element insertions (disruptions) or point mutations (amino acid substitutions) (van Kan *et al.*, 1991; Joosten *et al.*, 1994, 1997; Luderer *et al.*, 2002b; Westerink *et al.*, 2004; Mesarich *et al.*, 2014).

World-wide, most commercial *F. fulva*-resistant tomato cultivars are designated Ff:A-E, Pf:A-E, or Cf:A-E, which indicates that they carry the *Cf-9* locus from wild tomato species, *Solanum pimpinellifolium* (van der Beek *et al.*, 1992; Jones *et al.*, 1993). This locus harbours five paralogous genes: *Cf-9A*, *Cf-9B*, *Cf-9C* (commonly referred to as *Cf-9*), *Cf-9D*, and *Cf-9E* (Jones *et al.*, 1994; Parniske *et al.*, 1997). Of the RLPs encoded by these five genes, two confer resistance to *F. fulva*. This first is Cf-9C, which recognises the Avr9 effector (van Kan *et al.*, 1991; van den Ackerveken *et al.*, 1992) and provides resistance during all stages of plant growth. Cf-9C is associated with an HR that generally restricts hyphal growth to within one or two epidermal cell lengths of the penetration site (Hammond-Kosack & Jones, 1994; Parniske *et al.*, 1997; Laugé *et al.*, 1998). The second is Cf-9B, which recognises the putative Avr9B effector and provides resistance in mature (flowering and fruiting) plants only (Jones *et al.*, 1994; Laugé *et al.*, 1998; Panter *et al.*, 2002). Cf-9B is not associated with an HR but, instead, leaf chlorosis and a strong accumulation of pathogenesis-related (PR) proteins that ultimately halt fungal growth after limited hyphal extension between the mesophyll cells (Parniske *et al.*, 1997; Laugé *et al.*, 1998; Panter *et al.*, 2002). Currently, the molecular mechanism governing this mature plant resistance is unknown; however, developmental control of *Cf-9B* promoter activity is not responsible (Panter *et al.*, 2002).

Perhaps unsurprisingly, given its extensive use in world-wide commercial tomato production, resistance provided by the *Cf-9* locus has been partially overcome by many *F. fulva* strains. The first evidence of this was documented in the Netherlands, Poland, and France where, following the introgression of the *Cf-9* locus into commercial tomato cultivars during the 1970s (Schouten *et al.*, 2019), race 9 (*Avr9*$^{-}$) *F. fulva* strains began to emerge, which could overcome resistance mediated by *Cf-9C* (Laterrot, 1986; Lindhout *et al.*, 1989). In another example from Japan, race 9 strains were identified in 2009, just 3 yr after commercial tomato cultivars carrying the *Cf-9* locus were introduced (Enya *et al.*, 2009; Iida *et al.*, 2010, 2015; Yoshida *et al.*, 2021). More recently, a report stated that all 36 *F. fulva* strains sampled in Cuba could overcome *Cf-9C* (Bernal-Cabrera *et al.*, 2021). So far, circumvention of *Cf-9C* appears to be exclusively the result of *Avr9* deletion (van Kan *et al.*, 1991; Stergiopoulos *et al.*, 2007b; Iida *et al.*, 2015; Bernal-Cabrera *et al.*, 2021; Yoshida *et al.*, 2021).

Interestingly, even though *Cf-9C* has been widely overcome, the resistance provided by *Cf-9B* has, until relatively recently, proven to be quite durable. However, over the last decade or so, tomato growers world-wide have reported an increase in *F. fulva* incidence on mature *Cf-9* plants. This is in addition to a study involving a New Zealand *F. fulva* strain collected in the 1980s, IPO 2679, which was found to overcome both *Cf-9C* and *Cf-9B* (Laugé *et al.*, 1998). With these points in mind, we set out to (1) identify *Avr9B*, (2) determine whether *Avr9B* has been deleted or mutated across a world-wide collection of *F. fulva* strains isolated from mature *Cf-9* plants, and (3) ascertain whether resistance mediated by *Cf-9C* and *Cf-9B* has been sequentially overcome by *F. fulva*.

Materials and Methods

General

Polymerase chain reaction (PCR) primers used in this study are listed in Supporting Information Table S1.

Fungal strains and plant material

Fulvia fulva Cooke strains used in this study are shown in Table S2. Tomato plants used in this study were wild-type (WT) *S. lycopersicum* lines 'Moneymaker' (MM)-Cf-0 (no *Cf* genes) and MM-Cf-9 (carrying the *Cf-9* locus) (Tigchelaar, 1984), as well as transgenic MM-Cf-0 lines carrying *Cf-9C* alone, or both *Cf-9A* and *Cf-9B* (2/9-75) (Parniske *et al.*, 1997). Non-host plants used in this study were WT and Δ*Sobir1 Nicotiana benthamiana* (Huang *et al.*, 2021), as well as WT *Nicotiana tabacum* cultivar Wisconsin 38.

Genome sequencing and assembly

Fulvia fulva IPO 2679 was cultured in potato dextrose broth (PDB) in the dark at 22°C with gentle orbital shaking for 2 wk. High-quality genomic DNA was extracted according to Schwessinger & McDonald (2017). A TruSeq™ Nano library was prepared and sequenced on the Illumina MiSeq™ (PE150) platform by Novogene (https://www.novogene.com). FASTP v.0.20.0 (Chen *et al.*, 2017) was used to remove low-quality bases from sequencing reads. A *de novo* genome sequence was assembled using SPADES v.3.11.1 (Bankevich *et al.*, 2012), with the final assembly generated from a set of different kmers (21, 33, 55, 77, 99, 127). The final genome assembly was assessed for quality using QUAST v.5.0.2 (Gurevich *et al.*, 2013), and screened for potential adapter contamination using a BLASTN search against the National Centre for Biotechnology Information (NCBI) UniVec database.

Bioinformatic analyses

To identify *Avr9B* candidates, sequences of previously identified *in planta*-expressed (candidate) effector genes and proteins from *F. fulva* reference strain 0WU (de Wit *et al.*, 2012; Mesarich *et al.*, 2014, 2018) were aligned to the orthologous sequences

from IPO 2679 using Geneious v.9.1.8 (Kearse *et al.*, 2012). Signal peptide, transmembrane domain, glycosylphosphatidylinositol anchor, and intrinsically disordered region (IDR) predictions were performed using SignalP v.4.1 (Nielsen, 2017), DeepTMHMM (Hallgren *et al.*, 2022), Big-PI (Eisenhaber *et al.*, 1999), and Pondr Vlxt (Romero *et al.*, 2001), respectively. Blastn/tBlastn/Blastp searches were used to identify *Avr9B*-/Avr9B-like sequences in NCBI and Joint Genome Institute (JGI) Mycocosm databases. The ColabFold (Mirdita *et al.*, 2022) implementation of AlphaFold2 (Jumper *et al.*, 2021) was used to predict the tertiary structure of the cysteine-rich region from Avr9B and was assisted with a custom multiple sequence alignment generated from the corresponding region in Avr9B-like proteins using Geneious v.9.1.8. The Avr9B tertiary structure was visualised and rendered using PyMOL (DeLano, 2002). Foldseek (van Kempen *et al.*, 2024) was used to search for structural homologs of the cysteine-rich region from Avr9B in the RCSB Protein Data Bank.

Presence/absence screens and allelic variation analysis

Genomic DNA was extracted from *F. fulva* strains according to van Kan *et al.* (1991), and *Avr9*/*Avr9B* presence or absence assessed by PCR using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA), Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), or KOD One PCR Master Mix (Toyobo, Osaka, Japan) and gel electrophoresis. To assess *Avr9*/*Avr9B* allelic variation, PCR amplicons were purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA) or ExoSAP-IT PCR Cleanup Reagent (Applied Biosystems, Waltham, MA, USA) and sequenced using Sanger technology by the Massey Genome Service (Palmerston North, New Zealand), Eurofins Scientific (Luxembourg City, Luxembourg), or Macrogen (Tokyo, Japan). To confirm *Avr9B* deletion, the missing genomic region in strain IPO 2679 was first identified through a sequence alignment to the *F. fulva* strain Race 5 genome (Zaccaron *et al.*, 2022) using Geneious v.9.1.8. PCRs were then carried out with primers flanking the missing region using GoTaq G2 DNA Polymerase (Promega, Madison, WI, USA).

Gene expression analysis

Fulvia fulva Race 5 was inoculated onto young (4-wk-old) MM-Cf-0 tomato plants and the fourth composite leaf harvested at 2, 4, 8, 12, and 16 d post inoculation (dpi). Fungal spore preparation and plant inoculations were performed according to Mesarich *et al.* (2014), with growth of inoculated plants carried out in a glasshouse without climate control under natural light conditions. *Fulvia fulva* Race 5 was also cultured in PDB, and the mycelia harvested at 4 dpi. Samples were harvested in triplicate from independent plants (leaves) or cultures (mycelia) to give three biological replicates and flash-frozen in liquid nitrogen. Total RNA was extracted, and cDNA synthesised, according to Methods S1. Real-time quantitative PCR (RT-qPCR) was performed on cDNA samples according to Mesarich *et al.* (2014), using primers designed with Primer3 (Untergasser *et al.*, 2012). Primer efficiency and specificity were determined using a dilution series of the cDNA before use. The *F. fulva actin* gene was used as a reference for normalisation of gene expression as per Mesarich *et al.* (2014), with results analysed according to the $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001). Results were the average of three biological replicates.

Transient expression assays

Generation and acquisition of vectors used in *Agrobacterium tumefaciens*-mediated transient transformation assays (ATTAs) and Potato virus X (PVX)-based expression assays are described in Methods S2. All expression vectors were sequence-verified before transformation into *A. tumefaciens* GV3101 (Holsters *et al.*, 1980). ATTAs were performed in *Nicotiana* plants according to Guo *et al.* (2020) and Tarallo *et al.* (2022) using a final bacterial OD_{600} of 0.5, with symptoms assessed at 7 d post infiltration. PVX-based expression assays were performed in tomato plants as per Mesarich *et al.* (2018), with symptoms assessed at 10–18 d post infiltration.

Protein immunoblotting

Nicotiana tabacum leaves from ATTAs were flash-frozen in liquid nitrogen at 2 d post infiltration, with total protein extracted according to Methods S3. Protein samples were loaded onto a 12% Tris-glycine gel, transferred to a PVDF membrane (Bio-Rad), and then probed with mouse anti-FLAG (1 : 5000) primary antibody (Sigma-Aldrich) and chicken anti-mouse IgG-HRP (1 : 20 000) secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Blotted membranes were incubated with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) and visualised with a C600 Gel Imaging System (Azure Biosystems, Dublin, CA, USA).

Gene complementation

Genomic DNA of *F. fulva* 0WU was extracted as per van Kan *et al.* (1991). The generation of gene complementation constructs using this genomic DNA is described in Methods S4. Sequence-verified complementation constructs were transformed into *A. tumefaciens* AGL1 (Holsters *et al.*, 1980), and then introduced into *F. fulva* IPO 2679 as per Ökmen *et al.* (2013). Genomic DNA was extracted from transformants, with complementation confirmed by PCR. Complementation of IPO 2679 with a single copy of a candidate *Avr9B* gene was confirmed by quantitative PCR in conjunction with the formula: Ratio = $((E_{target})^{\Delta Ct\ target\ (control\ -\ sample)})/((E_{reference})^{\Delta Ct\ reference\ (control\ -\ sample)})$. Here, the *F. fulva actin* gene was targeted as a reference single-copy gene.

Fulvia fulva virulence assays

Conidia from complemented and WT *F. fulva* strains were inoculated onto the four compound leaves immediately below the first flowering truss of mature (13-wk-old) tomato plants. Here,

conidia preparation, inoculation, and growth conditions were as described by Mesarich *et al.* (2014), with two exceptions. First, before inoculation, tomato plants were maintained at *c.* 100% relative humidity, with the main shoot above the first flowering truss, including the apical meristem, removed after appearance of the first inflorescence/flowering truss. Second, the lateral shoots of tomato plants were periodically removed throughout the experiment. Disease severity was assessed at 23 dpi.

Results

Genome sequencing of *F. fulva* IPO 2679 reveals two candidate *Avr9B* genes

As a starting point for *Avr9B* identification, the genome of the *Cf-9B*-breaking *F. fulva* strain, IPO 2679 (Laugé *et al.*, 1998), was sequenced (Table S3). A total of 119 previously identified (candidate) effector gene sequences from *F. fulva* strain 0WU (de Wit *et al.*, 2012; Mesarich *et al.*, 2014, 2018), which has not overcome *Cf-9B*, were then compared with the corresponding gene sequences of IPO 2679 to identify those that have been mutated or deleted (Table S4). Sequence alignments revealed that 21 of the genes have non-synonymous substitutions in IPO 2679, when compared to 0WU, while *Avr2* has a 140-base pair (bp) deletion, and a further two genes, including *Avr9*, have been deleted (Table S4).

Of these genes that have been deleted or mutated, two were of particular interest based on documented resistance-breaking mutations in other *F. fulva Avr* genes. The first is *Ecp5* (GenBank ID: EF104527.1), a previously identified candidate *Avr* gene corresponding to the *Cf-Ecp5* resistance gene of tomato (Haanstra *et al.*, 2000; Laugé *et al.*, 2000; Iakovidis *et al.*, 2020) which, like *Avr4* in many *Cf-4*-breaking strains of *F. fulva* (Joosten *et al.*, 1997), encodes a protein with a cysteine-to-tyrosine substitution (amino acid position 30 in Ecp5; Table S4). The second is *CfCE54* (GenBank ID: KX943086.1) which, like *Avr9* in *Cf-9C*-breaking strains of *F. fulva* (van Kan *et al.*, 1991), is deleted. A sequence comparison to the chromosome-level genome assembly of *F. fulva* strain Race 5 (Zaccaron *et al.*, 2022), which is expected to carry a functional *Avr9B* gene, estimated that the deleted region encompassing *CfCE54* in IPO 2679 is 5429 bp in length (Fig. S1a). Deletion of this region was subsequently confirmed by PCR (Fig. S1b).

CfCE54 is predicted to encode a 152-amino acid protein with a 21-amino acid signal peptide for secretion to the apoplastic environment, followed by a repeat-rich region made up of four direct imperfect 11-amino acid repeats and a cysteine-rich region with eight cysteine residues (Fig. 1a; Notes S1). The repeat-rich region largely overlaps with a predicted IDR (Notes S1). Like most fungal effectors, CfCE54 is not predicted to possess domains of characterised function. Hence, it was not possible to predict the biological function of CfCE54 from sequence information alone. As with *Ecp5* (Mesarich *et al.*, 2014; Zaccaron *et al.*, 2022), *CfCE54* is transcriptionally upregulated during infection of tomato, relative to growth in culture (Fig. 1b), and is adjacent to repetitive elements in the *F. fulva* genome (Fig. 1c).

CfCE54 restores avirulence of *F. fulva* IPO 2679 on mature *Cf-9* tomato plants

To determine whether *Ecp5* or *CfCE54* is *Avr9B*, complementation assays were carried out by introducing a single functional copy of *Ecp5* or *CfCE54* from strain 0WU into strain IPO 2679 and then testing the ability of these complemented strains to cause disease on mature MM-Cf-9 tomato plants carrying the *Cf-9* locus (Figs 2, S2). As expected, inoculation of mature MM-Cf-0 plants with WT IPO 2679 (*Avr9*$^{-}$/*Avr9B*$^{-}$), WT *F. fulva* strain ICMP 7320 (*Avr9*$^{-}$/*Avr9B*$^{+}$), IPO 2679 carrying the pFBTS1 empty vector (EV), or IPO 2679 complemented with *Ecp5* or *CfCE54* resulted in disease (Figs 2, S2). Likewise, as anticipated, inoculation of mature MM-Cf-9 plants with WT IPO 2679 or IPO 2679 carrying the EV, but not WT ICMP 7320, also resulted in disease (Figs 2, S2). Notably, however, inoculation of mature MM-Cf-9 plants with IPO 2679 complemented with *CfCE54*, but not *Ecp5*, resulted in resistance (Figs 2, S2). Consequently, only *CfCE54* restores avirulence of IPO 2679 on mature *Cf-9* plants, suggesting that *CfCE54*, but not *Ecp5*, is *Avr9B*.

CfCE54 triggers a Cf-9B-dependent cell death response in *N. tabacum*

To confirm that CfCE54 is Avr9B, we investigated whether CfCE54 from strain 0WU specifically triggers a Cf-9B-dependent cell death response upon co-expression with Cf-9B in *N. tabacum* using an ATTA. Here, *N. tabacum* was used, as Cf-9B alone often triggers a cell death response in *N. benthamiana* (Chakrabarti *et al.*, 2009). In this experiment, CfCE54 (or Ecp5), without its predicted endogenous signal peptide, was fused at its N-terminus to the PR1a signal peptide for secretion to the apoplastic environment in *N. tabacum*, followed by a 3xFLAG tag for detection by western blotting. As expected, co-expression of Cf-9C with Avr9 (positive control; Hammond-Kosack *et al.*, 1998) triggered a strong cell death response, while Avr9, Cf-9C, or Cf-9B alone (negative controls) did not (Figs 3a, S3). Notably, CfCE54, but not Ecp5, triggered a strong cell death response upon co-expression with Cf-9B (Figs 3a,b, S3). This response was specific to Cf-9B, as CfCE54 did not trigger this strong response upon co-expression with Cf-9C (Figs 3a, S3). Taken together, these results confirm that CfCE54 is Avr9B.

Interestingly, when Avr9B was expressed alone in *N. tabacum* or in combination with any other of the proteins tested above, chlorosis and/or a small patch of cell death was observed (Figs 3a, b, S3). Both the Cf-9B-dependent cell death response and the Cf-9B-independent chlorosis/cell death were eliminated upon expression of an Avr9B variant lacking a signal peptide (Figs 3b, S3), suggesting that secretion to the apoplastic environment, or post-translational modifications associated with the endoplasmic reticulum–Golgi secretory pathway (e.g. disulphide bond formation and/or glycosylation), are required for these responses. Interestingly, a secreted version of Avr9B with the repeat region deleted maintained the ability to trigger Cf-9B-independent

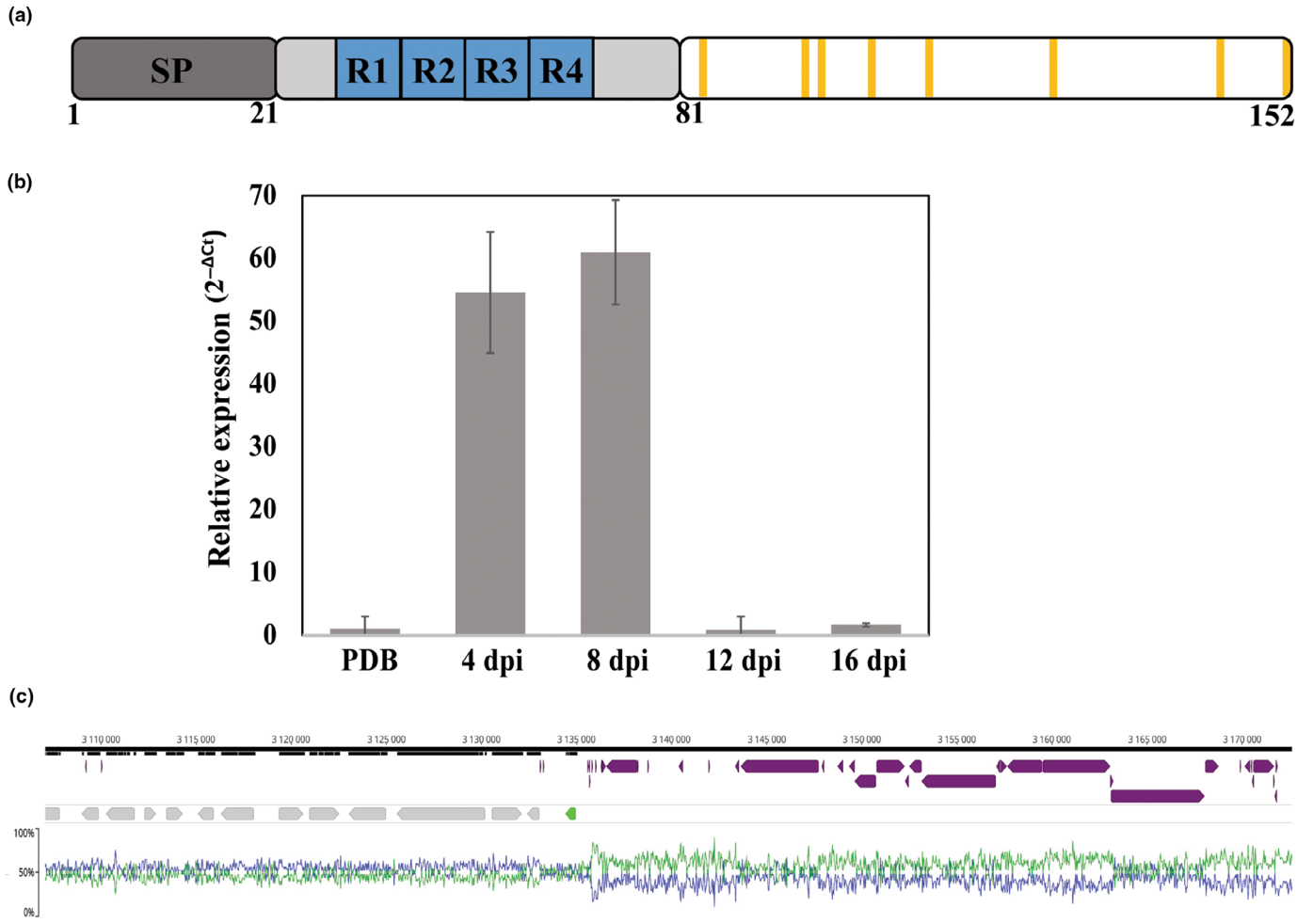

Fig. 1 *CfCE54* encodes a small, cysteine/repeat-rich secreted protein, is transcriptionally upregulated during infection of tomato by *Fulvia fulva*, relative to growth in culture, and is adjacent to repetitive elements in the *F. fulva* Race 5 genome. (a) Schematic representation of the CfCE54 protein from *F. fulva*. The predicted signal peptide is shown in dark grey (SP; amino acid residues 1–21). The predicted intrinsically disordered region is shown in light grey (amino acid residues 22–80) and contains four imperfect tandem repeats (R1–R4; blue boxes). The cysteine-rich region is shown in white (amino acid residues 81–152). Cysteine residues (eight in total) are shown by vertical yellow lines. (b) *CfCE54* expression profile. Expression was analysed during an *F. fulva* Race 5–'Moneymaker' (MM)-Cf-0 tomato interaction at 4, 8, 12, and 16 d post-inoculation (dpi), as well as in culture in potato dextrose broth at 4 dpi, using a real-time quantitative polymerase chain reaction experiment. *CfCE54* expression peaks at 4 and 8 dpi, with negligible expression at 12 dpi, 16 dpi, and in culture. Expression was normalised to the *F. fulva actin* gene according to the $2^{-\Delta Ct}$ method. Error bars represent SD across three biological replicates. (c) Location of *CfCE54* in the *F. fulva* Race 5 genome. A zoomed-in region of Chromosome 10 is shown, with *CfCE54* located at position 3134996–3134431. *CfCE54* is shown in green, while the other genes in the region are shown in grey. Repetitive elements are shown in purple. G + C content is shown by a blue line, while A + T content is shown by a green line.

chlorosis/cell death and a Cf-9B-dependent cell death response (Figs 3b, S3), indicating that the repeat region is not required for these responses in *N. tabacum*. Unfortunately, unlike Ecp5, neither Avr9B, nor any of its variants, could be detected following an ATTA by western blotting using an antibody specific to the 3xFLAG tag (Fig. S4).

To determine whether the Cf-9B-independent chlorosis/cell death triggered by Avr9B is dependent on the co-receptor SOBIR1, and thus the result of recognition by an endogenous RLP in *N. tabacum*, we transiently expressed Avr9B in WT and Δ*Sobir1 N. benthamiana* plants (Huang *et al.*, 2021) using ATTAs, and compared the responses. As expected, the Cf-9C/Avr9 pair triggered cell death in WT (positive control) but not Δ*Sobir1 N. benthamiana* plants (negative control) (Fig. S5), consistent with a previous finding that cell death triggered by the Cf-9C/Avr9 pair is SOBIR1-dependent (Huang *et al.*, 2021). In contrast to the chlorosis/weak cell death observed in *N. tabacum*, Avr9B triggered a strong cell death response when expressed alone in WT *N. benthamiana* (Fig. S5). This response was also observed in Δ*Sobir1* plants (Fig. S5), indicating that SOBIR1 is not required for the Cf-9B-independent cell death response triggered by Avr9B in *N. benthamiana*. Given this result, we anticipate that SOBIR1 is likely also not required for the Cf-9B-independent chlorosis/cell death triggered by Avr9B in *N. tabacum*. Notably, since Avr9B alone triggered cell death in both WT and Δ*Sobir1 N. benthamiana* plants, it was not possible to

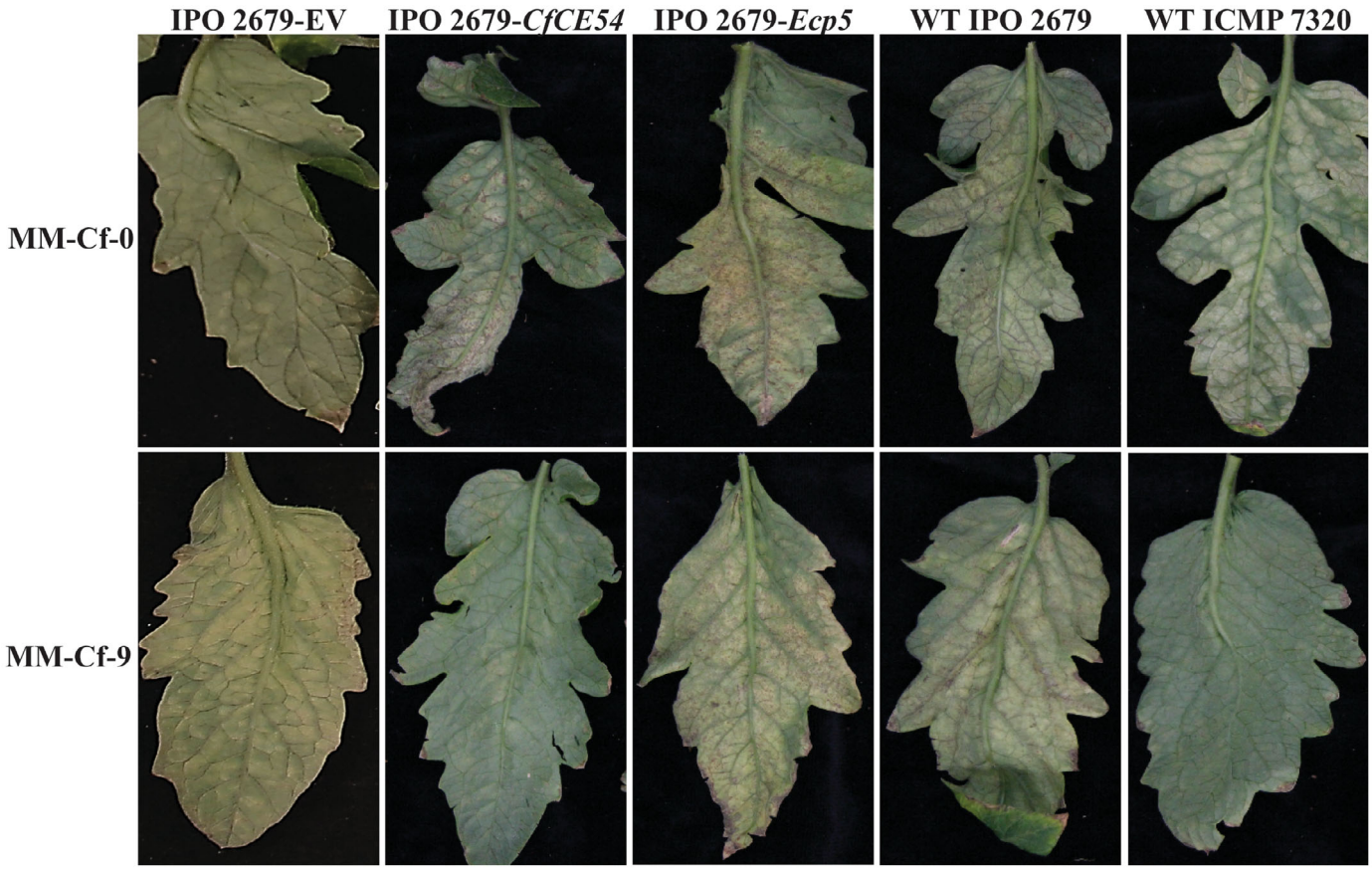

Fig. 2 *CfCE54*, but not *Ecp5*, restores avirulence of *Fulvia fulva* IPO 2679 on mature tomato plants carrying the *Cf-9* locus. *Fulvia fulva* strains were inoculated onto mature (13-wk-old) 'Moneymaker' (MM)-Cf-0 (no *Cf* genes) and MM-Cf-9 (carrying the *Cf-9* locus) tomato plants, with photographs taken at 23 d post-inoculation. IPO 2679 EV, strain IPO 2679 (*Avr9*$^{-}$/*Avr9B*$^{-}$) complemented with the pFBTS1 empty vector (EV; no insert); IPO 2679-*CfCE54*, strain IPO 2679 complemented with *CfCE54* from wild-type (WT) strain 0WU; IPO 2679-*Ecp5*, strain IPO 2679 complemented with *Ecp5* from WT strain 0WU; WT ICMP 7320, WT ICMP 7320 (*Avr9*$^{-}$/*Avr9B*$^{+}$) strain; WT IPO 2679, WT IPO 2679 strain. In total, two independent IPO 2679 EV, five independent IPO 2679-*CfCE54*, and two independent IPO 2679-*Ecp5* complementation strains were tested. Complementation strain #2 of IPO 2679 EV, #2 of IPO 2679-*CfCE54*, and #2 of IPO 2679-*Ecp5* are shown. Results are representative of all complementation strains tested.

conclude whether cell death triggered by the Cf-9B/Avr9B pair is SOBIR1-dependent (Fig. S5).

Avr9B triggers a Cf-9B-dependent HR in young *Cf-9* tomato plants, as well as chlorosis/weak necrosis and leaf curling in a wild tomato accession

The PVX-based binary expression vector, pSfinx, can be used to systemically express effectors of interest in tomato plants to a high level (Chapman *et al.*, 1992; Hammond-Kosack *et al.*, 1995; Takken *et al.*, 2000). Using this approach, an effector gene is cloned into the PVX genome harboured by pSfinx and then transferred to the genome of tomato using *A. tumefaciens* (Takken *et al.*, 2000). Upon generation of the recombinant virus in tomato plants, it spreads systemically, leading to systemic production of the effector protein. Should the effector protein then be recognised by a cognate resistance protein in tomato, a systemic, visible HR often follows, resulting in chlorosis, necrosis, stunting, or death of the plant (Mesarich *et al.*, 2018).

To determine whether the Cf-9B resistance pathway can be activated in young tomato plants, we systemically expressed Avr9B in young MM-Cf-9 plants, as well as in young transgenic MM-Cf-0 lines expressing both *Cf-9A* and *Cf-9B* (*Cf-9A* + *Cf-9B*), using the PVX-based expression system, and compared any responses with those observed in young MM-Cf-0 plants or a young transgenic MM-Cf-0 line expressing *Cf-9C*. For negative and positive controls, we also tested PVX alone (pSfinx EV) and Avr9, respectively. As expected, PVX alone did not trigger an HR in any tomato line tested (Fig. 4), whereas Avr9 alone triggered a systemic HR in MM-Cf-9 and transgenic *Cf-9C* plants, as evidenced by light necrosis on the leaves and cotyledon drop (Fig. 4). Likewise, as anticipated, Avr9B failed to trigger an HR in MM-Cf-0 or transgenic *Cf-9C* plants (Fig. 4). Interestingly, Avr9B triggered a systemic HR in both MM-Cf-9 and transgenic *Cf-9A* + *Cf-9B* plants, which was even stronger than the response triggered by Avr9 in MM-Cf-9 and transgenic *Cf-9C* plants (Fig. 4). This indicates that the Cf-9B resistance pathway can be activated by Avr9B in young tomato plants, in the absence of *F. fulva*. Curiously,

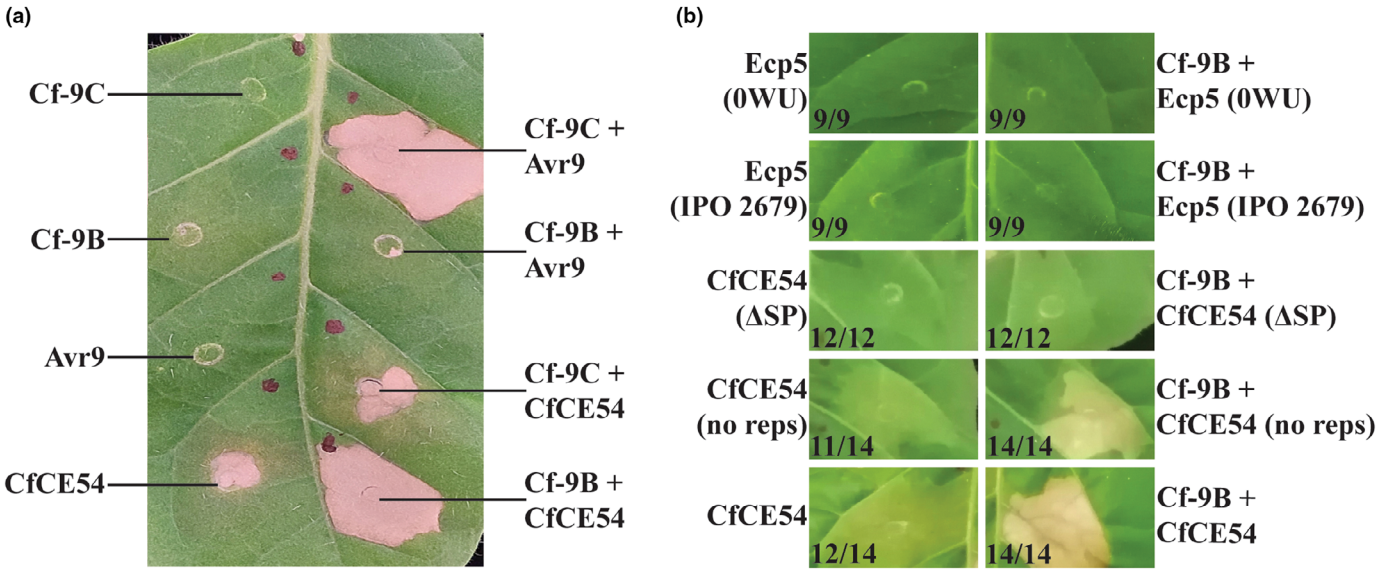

Fig. 3 CfCE54, but not Ecp5, of *Fulvia fulva* triggers a Cf-9B-dependent cell death response in *Nicotiana tabacum*. Wild-type (WT) CfCE54 from strain 0WU (a), or a variant of this protein with no signal peptide (ΔSP) or no repeat region (no reps) (see Fig. 1a for a schematic of the different protein regions in CfCE54), as well as WT Ecp5 from strain 0WU or a natural Cys30Tyr variant of this protein from strain IPO 2679 (b), were co-expressed with Cf-9B or Cf-9C from tomato in *N. tabacum* using an *Agrobacterium tumefaciens-mediated* transient transformation assay (ATTA). As a positive control for cell death in (a), Cf-9C was co-expressed with Avr9, whereas as negative controls for cell death in (a), Cf-9, Avr9 and Cf-9B were expressed alone. Please note that the ability of CfCE54 to trigger either chlorosis or a small patch of cell death when expressed alone varied from experiment to experiment (compare (a) and (b)). Leaves were photographed at 5 d post infiltration, and the results shown are representative of at least three independent ATTAs. In (b), numbers on the bottom left represent the number of times the response was observed (left) out of the number of times the infiltration was performed (right).

systemic expression of Avr9B in MM-Cf-0 plants resulted in exaggerated PVX symptoms, when compared to EV control plants (Fig. S6a). These exaggerated PVX symptoms were not observed when other characterised or candidate Avr effectors of *F. fulva* (Avr2, Avr4, Avr4E, Avr5, Avr9, Ecp4, Ecp5, and Ecp11-1) were systemically expressed in MM-Cf-0 plants (Fig. S6b).

Using the PVX-based expression system, we next sought to determine whether Avr9B triggers a systemic HR in the same collection of wild tomato accessions tested by Mesarich *et al.* (2018). Based on this experiment, only one wild tomato accession (CGN14353; *S. pimpinellifolium*) responded to Avr9B, with leaves exhibiting chlorosis, weak necrosis, and inward curling relative to EV control plants (Fig. S7a,b). However, unlike that observed in MM-Cf-0 plants, PVX symptoms did not appear to be exaggerated. This may, however, be influenced by the apparent defence responses triggered by Avr9B in this wild accession.

Avr9B-like proteins are restricted to plant-pathogenic Dothideomycete fungi

To determine whether Avr9B-like proteins exist in other fungi, a BLAST search was performed against fungal genomes and proteins present in the NCBI and JGI Mycocosm sequence databases using Avr9B (and any identified Avr9B-like proteins) as a query. This analysis revealed that genes encoding Avr9B-like proteins are exclusively present in plant-pathogenic species of the Mycosphaerellaceae and Pleosporaceae families in class Dothideomycetes, with several of these found in tomato pathogens (Fig. 5a; Notes S1). All Avr9B-like proteins are predicted to possess a signal peptide for secretion to the apoplastic environment, with many also predicted to possess an IDR (Notes S1).

Avr9B-like proteins trigger responses in *Nicotiana* species and tomato in the absence of Cf-9B

To determine whether Avr9B-like proteins from other fungi also trigger a Cf-9B-dependent cell death response, we expressed the Avr9B-like proteins from the tomato pathogens *Stemphylium lycopersici* (TW65_01570; Franco *et al.*, 2015) and *Pseudocercospora fuligena* (HII31_03919; Zaccaron & Stergiopoulos, 2020) in *N. tabacum* using ATTAs (Fig. 5b). TW65_01570 triggered a strong cell death response alone, and when co-expressed with Cf-9B or Cf-9C (Fig. 5b). Hence, it was not possible to determine whether TW65_01570 is recognised by Cf-9B in *N. tabacum*. Similar to Avr9B, HII31_03919 triggered a chlorotic response alone in *N. tabacum* (Fig. 5b). However, unlike Avr9B, a cell death response was not observed when HII31_03919 was co-expressed with Cf-9B (Fig. 5b). As recognition could have theoretically resulted in chlorosis, and because chlorosis is triggered by HII31_03919 independently of Cf-9B, it was again not possible to determine whether HII31_03919 is recognised by Cf-9B in *N. tabacum*. Unlike Avr9B, both Avr9B-like proteins could be detected by western blotting (Fig. S4). Like Avr9B, both Avr9B-like proteins triggered a strong cell death response in the absence of Cf-9B in *N. benthamiana*, with these responses not dependent on SOBIR1 (Fig. S5).

To determine whether Avr9B-like proteins can trigger a Cf-9B-dependent HR in tomato, we systemically expressed

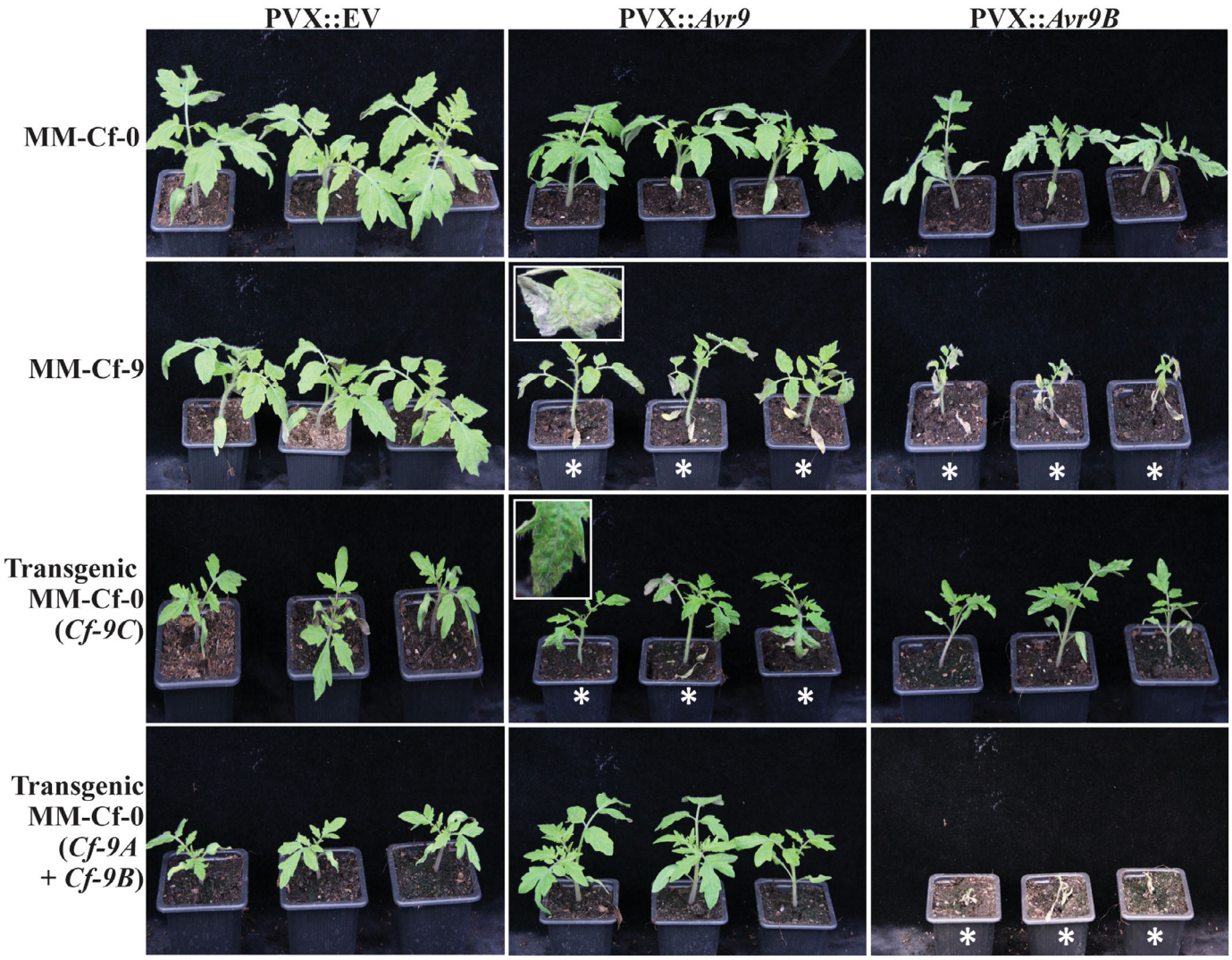

Fig. 4 Avr9B triggers a hypersensitive response (HR) in young tomato plants carrying *Cf-9B*. Avr9 and Avr9B were systemically expressed in 'Moneymaker' (MM)-Cf-9 (carrying the *Cf-9* locus), MM-Cf-0 (no *Cf* genes), and transgenic MM-Cf-0 plants carrying either *Cf-9C* or both *Cf-9A* and *Cf-9B* (*Cf-9A* + *Cf-9B*), using the Potato virus X-based expression system. Three representatives of each tomato accession were included in the experiment. White asterisks indicate plants undergoing a systemic HR, as evidenced by necrosis (insets) and cotyledon drop. Photographs were taken at 10 d post infiltration.

TW65_01570 in MM-Cf-9 plants using the PVX-based expression system and compared the responses with those observed in MM-Cf-0 plants (Fig. S8). In contrast to Avr9B, no Cf-9B-dependent HR was observed in MM-Cf-9 plants, suggesting that TW65_01570 is not recognised by Cf-9B (Fig. S8). Surprisingly, however, systemic expression of TW65_01570 consistently resulted in stunted growth regardless of the plant genotype (Fig. S8). This was most evident in the wild *S. pimpinellifolium* accession, CGN14353 (Fig. S7a).

The *Cf-9* resistance locus of tomato has been sequentially broken down by *F. fulva*

To determine whether the *Cf-9* locus of tomato has been sequentially broken down by *F. fulva*, with *Cf-9C* overcome before *Cf-9B*, the *Avr9* and *Avr9B* genes were screened for deletion or mutation across a collection of 190 geographically diverse *F. fulva* strains, which had been collected at different times, using PCR and PCR amplicon sequencing (Table S2). In total, 52 of all strains screened were isolated from *Cf-9*/Ff:A-E tomato plants (Table S2).

The PCR analysis determined or confirmed from previous studies that 93 of the strains, including all strains isolated from *Cf-9*/Ff:A-E plants, were race 9, since *Avr9* was absent (Table S2). Notably, in nine out of 11 race 9 strains collected before 1990, which lacked *Avr9*, no mutations were observed in *Avr9B* (Table S2). Strikingly, however, mutations in *Avr9B* were observed in all but one of 76 race 9 strains collected during or after 2005 (Table S2). These mutations included premature stops associated with codons 2 (p.Arg2*), 55 (p.Ser55*), 83 (p.Cys83*), and 107 (p.Cys107*), as well as a frame-shift mutation in codon 64 (p.Gly64Valfs*70) (Fig. 6a; Table S2). Other

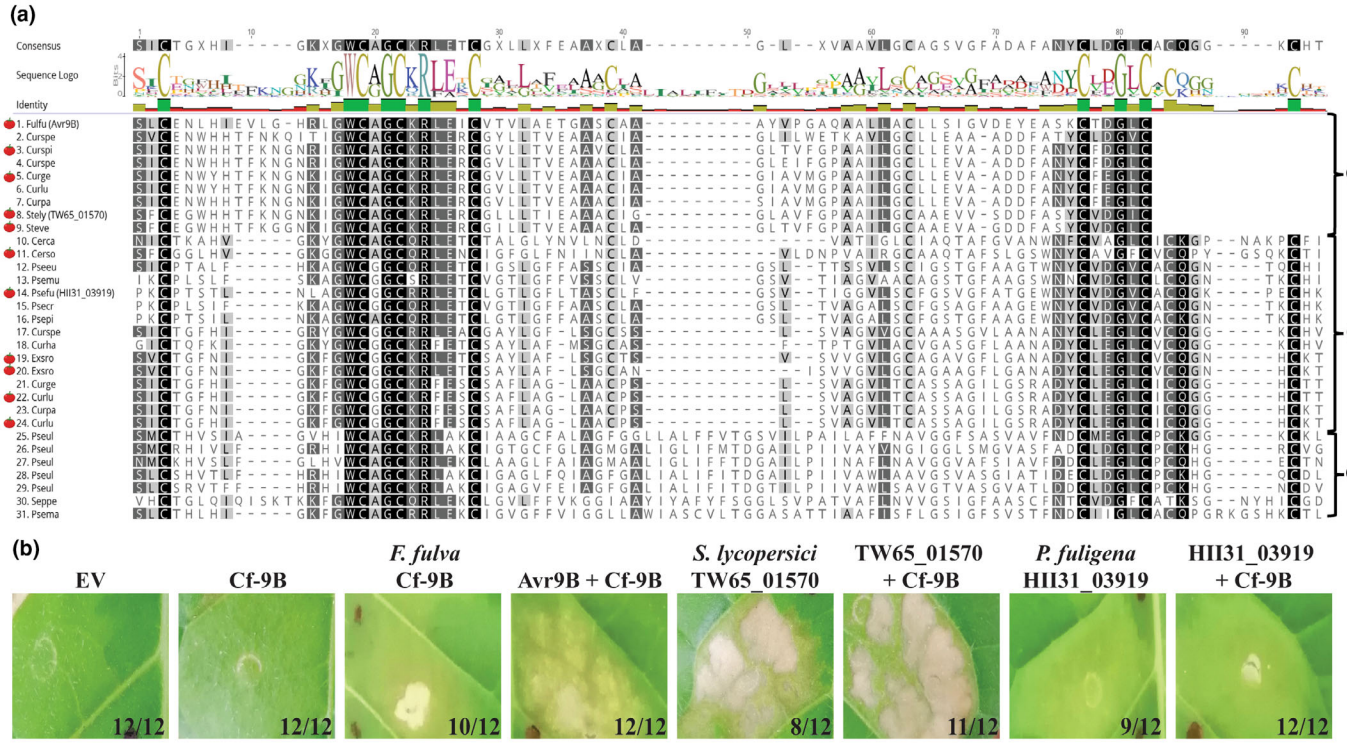

Fig. 5 Avr9B-like proteins are restricted to plant-pathogenic Dothideomycete fungi and trigger chlorosis or cell death in *Nicotiana tabacum*. (a) Alignment of Avr9B-like proteins from Dothideomycete fungi. Only the cysteine-rich region from each protein is aligned (see Fig. 1a for a schematic showing the location of the cysteine-rich region in Avr9B). Group 1 proteins (G1; sequences 1–9) are characterised by eight conserved cysteine residues. Group 2 proteins (G2; sequences 10–24) are characterised by 10 conserved cysteine residues, with the first eight cysteine residues shared with G1 proteins, and the last two cysteine residues forming part of a C-terminal extension. Group 3 proteins (G3; sequences 25–31) are characterised by eight conserved cysteine residues, with the first six cysteine residues shared with G1 and G2 proteins, and the last two cysteine residues shared with the C-terminal extension of G2 proteins. Cerca, *Cercospora canescens*; Cerso, *Cercospora sojina*; Curge, *Curvularia geniculata*; Curha, *Curvularia hawaiiensis*; Curlu, *Curvularia lunata*; Curpa, *Curvularia papendorfii*; Curspe, *Curvularia* sp. ZM96; Curspi, *Curvularia spicifera*; Exsro, *Exserohilum rostratum*; Fulfu, *Fulvia fulva*; Psecr, *Pseudocercospora cruenta*; Pseeu, *Pseudocercospora eumusae*; Psefu, *Pseudocercospora fuligena*; Psema, *Pseudocercospora macadamiae*; Psemu, *Pseudocercospora musae*; Psepi, *Pseudocercospora pini-densiflorae*; Pseul, *Pseudocercospora ulei*; Seppe, *Septoria petroselini*; Stely, *Stemphylium lycopersici*; Steve, *Stemphylium vesicarium*. Tomato pathogens are indicated by a tomato figure on the left. (b) The Avr9B-like proteins from *S. lycopersici* (TW65_01570, NCBI accession: KNG51100.1; Franco *et al*., 2015) and *P. fuligena* (HII31_03919, NCBI accession: KAF7194657.1; Zaccaron & Stergiopoulos, 2020) trigger strong cell death and chlorosis in *N. tabacum*, respectively. TW65_01570 and HII31_03919 were expressed alone or together with Cf-9B in *N. tabacum* using an *Agrobacterium tumefaciens-mediated* transient transformation assay. As positive controls for cell death, Avr9B was expressed alone (chlorosis/weak cell death) or together with Cf-9B (strong cell death). As negative controls, empty vector (pICH86988) and Cf-9B were expressed alone. Photographs were taken at 5 d post infiltration. Fractions on the bottom right represent the number of times the response was observed (left) out of the number of times the infiltration was performed (right) across at least three biological replicates.

mutations identified were an amino acid substitution associated with codon 97 (p.Trp97Cys), gene disruption through the insertion of a 289-bp miniature inverted-repeat transposable element (MITE) in codon 124 (p.Val123_Pro124ins*63) (Fig. 6a; Table S2), and an 18-bp deletion starting in codon 128, resulting in the replacement of seven amino acids (including Cys 133) with a single Val residue (p.Ala128_Leu134delinsVal) (Fig. 6a; Table S2). In the case of the MITE, a single identical copy was found *c.* 1.2 Mb away from *Avr9B* at location 1915484–1915772 on chromosome 10 in the strain Race 5 genome, suggesting it may originate from this location (Fig. S9). Remarkably, no synonymous mutations were observed in *Avr9B* (Table S2). Also remarkable, only one non-race 9 strain was predicted to possess a WT *Avr9* gene but a mutant *Avr9B* gene (Table S2). Of the mutations identified, all except p.Arg2* (identified in five countries) were restricted to one or two countries (Fig. 6b). Interestingly, deletion of *Avr9B* was restricted to New Zealand strains, with the same deletion profile observed by PCR in strain IPO 2679 collected in the 1980s as well as in strains NZ-C1, NZ-P1, and NZ-M6 collected in 2022 (Fig. S1b).

In addition to investigating *Avr9* and *Avr9B*, the mating type of each *F. fulva* strain was determined by PCR or obtained from previous studies (Stergiopoulos *et al*., 2007a,b). Based on this analysis, all mutation types were found to be restricted to strains of a particular mating type, except for p.Ser55*, which was observed in strains of both mating types collected from the Gifu prefecture of Japan (Table S2).

Taken together, the PCR and PCR amplicon sequencing results suggest that *Cf-9C* was overcome first, with most *F. fulva* strains collected before 1990 lacking *Avr9* but still carrying a functional copy of *Avr9B*, and that *Cf-9B*-mediated resistance was overcome second, with *F. fulva* strains collected during or

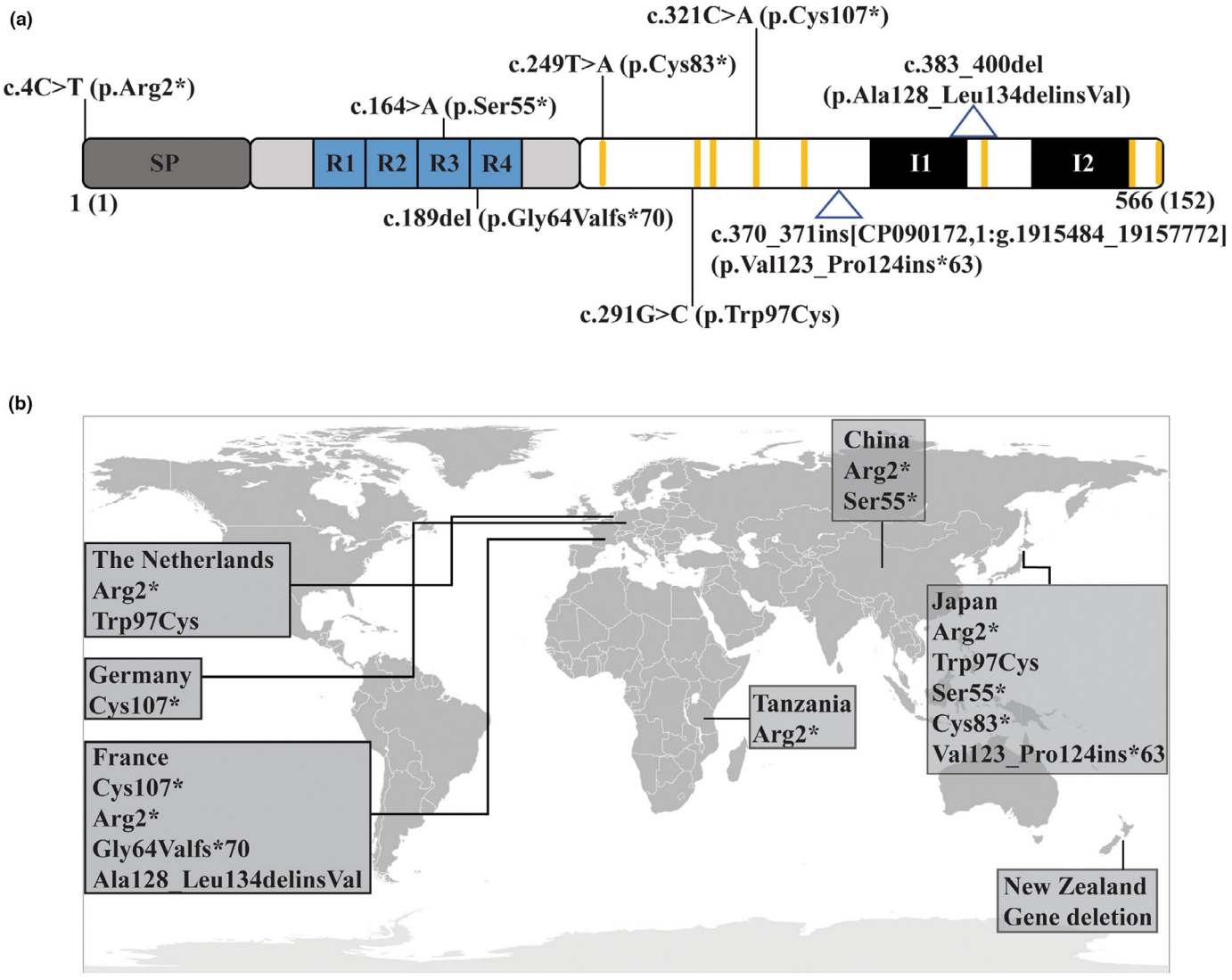

Fig. 6 Mutations identified in Avr9B and their distribution across *Fulvia fulva* strains collected from around the world. (a) Allelic variation in Avr9B of *Cf-9B*-breaking strains. The predicted signal peptide (SP) for secretion to the apoplastic environment is highlighted in dark grey. The four imperfect 11-amino acid tandem repeats (R1–R4) are shown as blue boxes. Cysteine residues are shown in vertical yellow lines. The predicted intrinsically disordered region is highlighted in light grey. Introns (I1 and I2) are shown as black boxes. c., coding sequence numbering; p., protein sequence numbering. (b) Distribution of Avr9B mutations across strains collected from around the world. The description of each mutation is based on the nomenclature set forth by the human genome variation society (HGVS; http://varnomen.hgvs.org/). CP090172.1:g.1915484_1915772 represents the likely original location of the miniature inverted-repeat transposable element on chromosome 10 of the *F. fulva* Race 5 genome (Zaccaron *et al*., 2022). del, deletion; delins, deletion–insertion; fs, frame shift; ins, insertion. Global base map generated by F. Bennet in the public domain and accessed from https://commons.wikimedia.org/wiki/File:BlankMap-FlatWorld6.svg.

after 2005 lacking *Avr9* and possessing a (putative) resistance-breaking mutation in *Avr9B*. Furthermore, the mating type data suggest that specific mutations have evolved once in *F. fulva*, with a strong correlation observed between a given mutation and a particular mating type.

The W97C mutation in Avr9B enables *F. fulva* to overcome Cf-9B-mediated resistance

Due to their deleterious nature, all mutations identified in Avr9B, except the p.Trp97Cys amino acid substitution (W97C), could be confidently assumed to result in the circumvention of Cf-9B-mediated resistance by *F. fulva* on mature *Cf-9* tomato plants. To test whether circumvention is also achieved through the W97C mutation, *F. fulva* strain P31 ($\Delta avr9/Avr9B^{W97C}$) of Japan was inoculated onto mature MM-Cf-0 and MM-Cf-9 plants and its ability to cause disease was assessed (Fig. 7a). At the same time, strain P18 ($\Delta avr9/Avr9B^{C107*}$) from Germany, which is expected to overcome both Cf-9C- and Cf-9B-mediated resistance in mature *Cf-9* plants due to deletion of *Avr9* and a p.Cys107* mutation (C107*) in Avr9B, was also tested. As expected, both strains caused disease on MM-Cf-0 plants

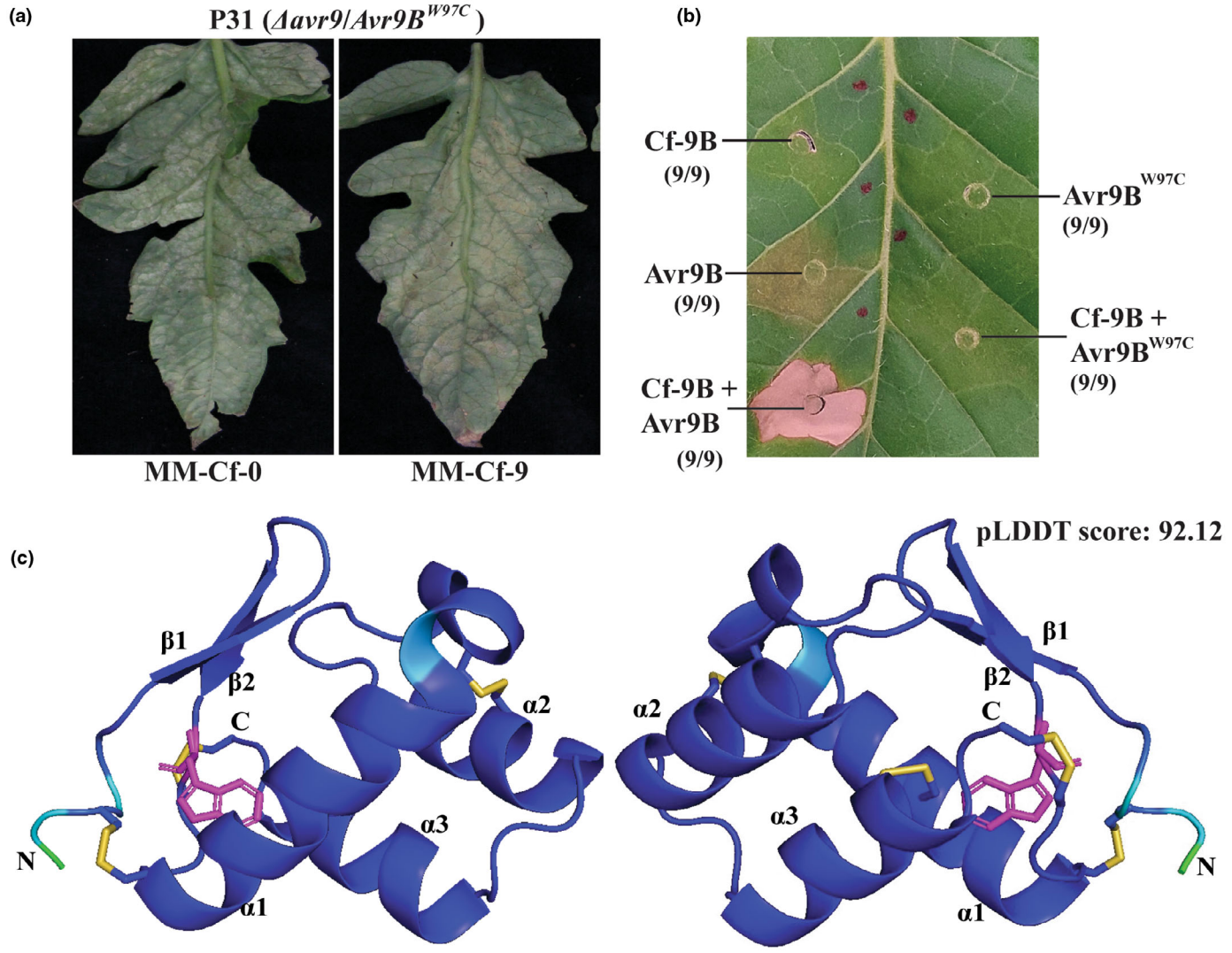

Fig. 7 W97C mutation in Avr9B results in circumvention of Cf-9B-mediated resistance in tomato and the inability to trigger both Cf-9B-dependent cell death and Cf-9B-independent chlorosis/cell death in *Nicotiana tabacum*. (a) *Fulvia fulva* strain P31 (*Δavr9/Avr9B*^{W97C}) was inoculated onto mature (13-wk-old) 'Moneymaker' (MM)-Cf-0 (no *Cf* genes) and MM-Cf-9 (carrying the *Cf-9* locus) tomato plants, with photographs taken at 23 d post inoculation. Inoculations were performed at the same time as the virulence assays shown in Fig. 2, with the results representative of three biological replicates. (b) The W97C mutant of Avr9B was co-expressed with Cf-9B in *N. tabacum* using an *Agrobacterium tumefaciens-mediated* transient transformation assay (ATTA). As positive controls for cell death, Avr9B was expressed alone (chlorosis/weak cell death) or together with Cf-9B (strong cell death). As a negative control for cell death, Cf-9B was expressed alone. The leaf was photographed 5 d post infiltration and is representative of three independent ATTAs. Numbers in parentheses represent the number of times the response was observed (left) out of the number of times the infiltration was performed (right). (c) Predicted tertiary structure of the cysteine-rich region from Avr9B, rotated 180° around its *y*-axis. The tertiary structure is coloured according to predicted Local Distance Difference Test score (confidence: green – okay, cyan – high, dark blue – very high). Tryptophan 97 is shown as sticks and is coloured magenta. β-strands and α-helices are numbered sequentially. Predicted disulphide bonds are coloured yellow (full-length protein sequence cysteine (Cys) connectivity pattern: Cys83-Cys101, Cys98-Cys152, Cys107-Cys147, Cys118-Cys133). N- and C-termini are indicated.

(Figs 7a, S10). Notably, both strains were also able to cause disease on MM-Cf-9 plants (Figs 7a, S10), indicating that, like the C107* mutation in P18, the W97C mutation enables P31 to evade recognition by Cf-9B. To support this observation, we tested the W97C mutant of Avr9B for its ability to trigger a Cf-9B-dependent cell death response in *N. tabacum* using an ATTA. Consistent with the gene complementation assay, the W97C mutant of Avr9B was unable to trigger a Cf-9B-dependent cell death response in *N. tabacum* (Fig. 7b). Interestingly, the W97C mutant of Avr9B was also unable to trigger a Cf-9B-independent chlorotic response in *N. tabacum* (Figs 7b, S11).

To gain insights into whether Trp 97 is surface-exposed, the tertiary structure of the cysteine-rich region of Avr9B was predicted (amino acid residues 81–152 in Fig. 1a). The predicted structure is characterised by three α-helices and two β-strands, stabilised by four disulphide bonds (Fig. 7c). Comparison of the predicted structure with other solved tertiary structures revealed no significant structural similarity to other proteins; hence, as

with sequence-based searches, no intrinsic virulence function for Avr9B could be inferred. Intriguingly, Trp 97 is not predicted to be surface-exposed but, rather, to occupy an internal position within the predicted Avr9B tertiary structure (Fig. 7c). This residue is conserved in all Avr9B-like proteins (Fig. 5a), suggesting that it plays an essential role in maintaining the structure of proteins with an Avr9B-type fold.

Discussion

World-wide, the *Cf-9* locus, harbouring the *Cf-9B* and *Cf-9C* resistance genes, is present in most commercially grown tomato cultivars that have resistance to *F. fulva*. While *Cf-9C*, which provides protection during all stages of plant growth, was rapidly broken down soon after deployment of the *Cf-9* locus in the 1970s (Laterrot, 1986; Lindhout *et al.*, 1989), *Cf-9B*, which provides protection to mature plants during flowering and fruiting, has proved to be more durable. However, over the last 10 yr or so, *F. fulva* incidence on fruiting and flowering plants has increased. Although *Cf-9C* breakdown has been shown to result from *Avr9* deletion in *F. fulva* (van Kan *et al.*, 1991; Stergiopoulos *et al.*, 2007b; Iida *et al.*, 2015; Bernal-Cabrera *et al.*, 2021; Yoshida *et al.*, 2021), the molecular mechanism underlying *Cf-9B* breakdown has remained unclear. To understand how *Cf-9B* has been overcome, we identified the corresponding *Avr9B* gene in *F. fulva* and inspected its level of allelic variation across the pathogen population.

Like other *Avr* genes of *F. fulva*, *Avr9B* is transcriptionally upregulated *in planta* and encodes a cysteine-rich SSP. Despite these similarities, Avr9B is unique among *F. fulva* Avr proteins in that it is predicted to have a large, repeat-rich region and, therefore, is a repeat-containing effector (Mesarich *et al.*, 2015). These repeats, however, are not necessary for recognition by Cf-9B. Notably, as with *Avr4E*, *Avr5*, and *Avr9* (Zaccaron *et al.*, 2022), *Avr9B* is closely associated with repetitive elements in the *F. fulva* genome. Furthermore, like these three *Avr* genes (van Kan *et al.*, 1991; Westerink *et al.*, 2004; Mesarich *et al.*, 2014), *Avr9B* is absent in some *F. fulva* strains. Consistent with that shown for *Avr4E*, *Avr5*, and *Avr9* (Zaccaron & Stergiopoulos, 2024), loss of *Avr9B* is anticipated to be facilitated through structural variations in the *F. fulva* genome, which are induced by repetitive elements.

Eight other *Cf-9B*-breaking mutations were identified in *Avr9B* across *F. fulva* strains collected from different geographical locations, with one of these leading to a non-synonymous amino acid substitution at position 97 (W97C). Curiously, this amino acid position is not predicted to be surface-exposed, in line with tryptophan being a large hydrophobic amino acid well-suited to the hydrophobic core of proteins. Since cysteine is a much smaller hydrophobic amino acid than tryptophan, it is possible that the W97C substitution negatively affects the folding and/or stability of Avr9B, such that it is no longer recognised by Cf-9B. Another identified mutation was the disruption of *Avr9B* though the insertion of a MITE. MITEs are a group of non-autonomous Class II transposable elements that do not encode their own transposase but, instead, commandeer transposases from other mobile genetic elements for their mobilisation (Wicker *et al.*, 2007). While MITEs have not previously been associated with *Cf* resistance breakdown, a MITE insertion in the *AvrSr35* gene of the stem rust fungus, *Puccinia graminis* f. sp. *tritici*, has been shown to enable circumvention of the *Sr35* resistance gene in wheat (Salcedo *et al.*, 2017). For *F. fulva*, the MITE in question likely originated from a nearby gene-rich region of the genome.

Several of the *Cf-9B*-breaking mutations in *Avr9B* were restricted to specific geographical locations, indicating independent mutation events around the world. For example, *Avr9B* deletion was only detected in New Zealand, with the same deletion profile detected in *F. fulva* strains collected in the 1980s and 2022. That nine independent and mostly geographically specific *Cf-9B*-breaking mutations were identified in *Avr9B* is perhaps not surprising, given the extensive deployment of *Cf-9* tomato cultivars world-wide. Remarkably, the p.Ser55* mutation was identified in Japanese strains of *F. fulva*, which carry opposite mating type genes, suggesting that this mutation has independently evolved twice or that sexual recombination has occurred. In support of the latter, the Japanese strains in question were from the same prefecture. While *F. fulva* was originally thought to be asexual (Thomma *et al.*, 2005), this information, together with allelic variation data collected from other *Avr* genes of this fungus (Stergiopoulos *et al.*, 2007a), suggests that *F. fulva* undergoes sexual reproduction, albeit rarely.

Intriguingly, when Avr9B was systemically expressed in young tomato plants using the PVX-based expression system, the Cf-9B resistance pathway was active. This is despite Cf-9B only providing resistance to *F. fulva* in mature plants at flowering and fruiting but is in line with *Cf-9B* expression not being developmentally regulated (Laugé *et al.*, 1998; Panter *et al.*, 2002). Of note, although *Avr9B* is highly expressed during infection of tomato, Avr9B was not detected by proteomic analysis in apoplastic washing fluid samples collected from young *F. fulva*-infected leaves (Mesarich *et al.*, 2018). Likewise, Avr9B could not be detected in total protein samples from *N. tabacum* ATTAs using western blotting. Together, this may suggest that Avr9B stability (and thus abundance) is compromised in young tomato plants, rendering Cf-9B ineffective. Correspondingly, in mature tomato plants, presumably due to qualitative and/or quantitative differences in the apoplastic protease profile, Avr9B is more stable (and thus more abundant), rendering Cf-9B effective. With regard to the PVX experiment described above, the observed result could then be explained by the large-scale production and secretion of Avr9B at the site of Cf-9B in the plasma membrane, above and beyond what would normally be delivered by *F. fulva* into the apoplast, thereby effectively bypassing the issue of stability/abundance. In accordance with this, Avr4 variants from *F. fulva* that naturally evade Cf-4-mediated resistance in tomato due to reduced stability in the leaf apoplast are still able to trigger a Cf-4-dependent HR in tomato plants carrying the *Cf-4* resistance gene using the PVX-based expression system (Joosten *et al.*, 1997).

Sequential breakdown of the *Cf-9* locus by *F. fulva* may therefore have occurred as follows: First, although *Cf-9B* and *Cf-9C*

are stacked in *Cf-9* tomato cultivars, the instability/low abundance of Avr9B in the leaf apoplast of young plants resulted in only Cf-9C being able to provide effective resistance. As such, there was significant selection pressure on *F. fulva* to overcome *Cf-9C* through deletion of *Avr9*. This gave *F. fulva* the ability to infect young *Cf-9* plants. Second, during the maturation of young, infected *Cf-9* plants, increasing selection pressure was exerted on *F. fulva* to overcome *Cf-9B* through deletion or mutation of *Avr9B*, concomitant with increased stability/higher abundance of Avr9B. This then gave *F. fulva* the ability to infect mature *Cf-9* plants. Of course, it could also be possible that *F. fulva* produces another effector that specifically suppresses Cf-9B-mediated resistance in young *Cf-9* plants, or that Avr9B is produced more abundantly in mature *Cf-9* plants, such that it exceeds the threshold required for the activation of Cf-9B-mediated resistance. Regardless of the hypothesis, sequential breakdown of the *Cf-9* locus is supported by our gene sequencing data, which shows that most *F. fulva* strains collected soon after the deployment of *Cf-9* cultivars have lost *Avr9* but still carry a functional copy of *Avr9B*, whereas more recently collected strains not only lack *Avr9*, but also possess a resistance-breaking mutation in *Avr9B*.

Surprisingly, Avr9B, along with the Avr9B-like proteins from the black leaf mould pathogen of tomato, *P. fuligena*, and the grey leaf spot pathogen of tomato, *S. lycopersici*, triggered chlorosis/cell death in *Nicotiana* species in the absence of Cf-9B, with these responses also triggered independently of the RLP co-receptor, SOBIR1, in *N. benthamiana*. This may suggest that these effectors interact with an endogenous resistance protein in these species that does not require SOBIR1 for transducing defence response signals following apoplastic effector recognition (e.g. an RLK). Notably, Avr9B is not the first effector of *F. fulva* to trigger cell death in non-host plants, with the candidate Avr effector Ecp2-1 also triggering cell death in several *Nicotiana* species (Laugé *et al.*, 2000; de Kock *et al.*, 2004). In this example, recognition is thought to be mediated by an endogenous resistance protein that is not homologous to Cf RLPs (de Kock *et al.*, 2004). Another possibility could be that Avr9B and the Avr9B-like proteins trigger Cf-9B/SOBIR1-independent responses by interacting with and perturbing the plant plasma membrane, as has been shown for NLP effectors of various microbial pathogens (Pirc *et al.*, 2023).

Also surprisingly, the systemic expression of the Avr9B-like protein from *S. lycopersici* in tomato resulted in stunted growth regardless of genotype, which could indicate that it has a specific function in modulating host physiology. Alternatively, it could be that this protein is recognised by an endogenous host resistance protein, but that this recognition does not trigger an HR. This may be similar to a homolog of Ecp2-1 from *P. fijiensis*, which triggers weak necrosis in tomato, independent of genotype (Stergiopoulos *et al.*, 2010). In any case, the response triggered by the systemic expression of the Avr9B-like protein in MM-Cf-0 tomato plants was different from the response triggered by Avr9B, since Avr9B did not result in stunting but, rather, exaggerated PVX symptoms. Such exaggerated PVX symptoms were not observed upon the systemic expression of other (candidate) Avr effectors from *F. fulva*, suggesting that this property is unique to Avr9B. Whether these exaggerated PVX symptoms have resulted from suppression of the plant immune system by Avr9B, enabling PVX to more effectively colonise MM-Cf-0 tomato plants, remains to be determined.

In conclusion, we have identified the *Avr9B* effector gene of *F. fulva*, corresponding to the *Cf-9B* resistance gene of tomato, and provided evidence to support the sequential breakdown of the *Cf-9* resistance locus by *F. fulva* over time. Ultimately, due to the world-wide distribution of *F. fulva* strains that can overcome both *Cf-9B* and *Cf-9C*, the *Cf-9* locus, which is present in many commercial tomato cultivars world-wide, likely now has limited value for controlling leaf mould disease. Importantly, stacking novel *Cf* resistance genes from wild tomato accessions in commercial tomato cultivars is anticipated to provide durable resistance against leaf mould disease (Mesarich *et al.*, 2018). However, our study highlights the need to first understand the dynamic relationship between Avr effectors of *F. fulva* and Cf resistance proteins of tomato to ensure that these stacked resistance genes are not sequentially overcome. In doing so, serious future outbreaks of *F. fulva* will likely be prevented.

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Competing interests

None declared.

Author contributions

CHM, SdlR, CRS, MHAJJ, YI, JKB, REB, PJGMW and YB designed the research; SdlR, CRS, ÁRP, AMH, KM, YI, MT, RJ, HGB and MR performed the research; SdlR, CRS, DJW and ÁRP carried out the data analysis, collection or interpretation; SdlR, CHM and CRS wrote the manuscript. All authors reviewed the manuscript and approved it for publication. SdlR and CRS contributed equally to this work.

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Data availability

The assembled genome sequence of *F. fulva* strain IPO 2679 has been deposited under NCBI BioProject ID PRJNA994185, BioSample accession no. SAMN36418479 and genome accession no. JAUKVN000000000.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Characterisation of the *CfCE54* genomic region in *Fulvia fulva* strains.

Fig. S2 *CfCE54*, but not *Ecp5*, restores avirulence to strain IPO 2679 of *Fulvia fulva* on mature *Solanum lycopersicum* plants carrying the *Cf-9* resistance locus.

Fig. S3 CfCE54, but not Ecp5, of *Fulvia fulva* triggers a Cf-9B-dependent cell death response in *Nicotiana tabacum*.

Fig. S4 Detection of *Fulvia fulva*, *Stemphylium lycopersici*, and *Pseudocercospora fuligena* secreted proteins by western blotting following expression in leaves of *Nicotiana tabacum* using *Agrobacterium tumefaciens*-mediated transient transformation assays.

Fig. S5 Avr9B and Avr9B-like proteins trigger chlorosis and/or cell death in *Nicotiana benthamiana* independent of SOBIR1.

Fig. S6 Avr9B from *Fulvia fulva* enhances Potato virus X symptoms in 'Moneymaker'-Cf-0 tomato plants.

Fig. S7 Avr9B from *Fulvia fulva* causes leaf curling, chlorosis, and weak cell death, whereas the Avr9B-like protein TW65_01570 from *Stemphylium lycopersici* causes stunting in the wild *Solanum pimpinellifolium* accession CGN14353.

Fig. S8 Avr9B-like protein TW65_01570 from *Stemphylium lycopersici* causes stunting in young *Solanum lycopersicum* plants.

Fig. S9 *Avr9B* gene from strain Tochigi1 of *Fulvia fulva* in Japan is disrupted by a miniature inverted-repeat transposable element.

Fig. S10 Natural *Avr9BC107** mutant of *Fulvia fulva* is virulent on *Cf-9* plants.

Fig. S11 W97C mutation in Avr9B results in the inability to trigger both Cf-9B-dependent cell death and Cf-9B-independent chlorosis/cell death in *Nicotiana tabacum*.

Methods S1 Extraction of RNA and synthesis of cDNA.

Methods S2 Generation and acquisition of vectors used in transient expression assays.

Methods S3 Extraction of total protein.

Methods S4 Generation of vectors used in the genetic complementation of *Fulvia fulva*.

Notes S1 Predicted Avr9B-like protein and Avr9B-like gene sequences in different fungi.

Table S1 Primers used in this study.

Table S2 *Fulvia fulva* strain data for Avr9 and Avr9B allelic variation and mating type genes.

Table S3 Statistics for the *Fulvia fulva* strain IPO 2679 Illumina genome assembly.

Table S4 Protein and gene coding sequences compared between *Fulvia fulva* strains 0WU and IPO 2679.

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