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REVIEW

Beyond the genomes of *Fulvia fulva* (syn. *Cladosporium fulvum*) and *Dothistroma septosporum*: New insights into how these fungal pathogens interact with their host plants

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Abstract

Fulvia fulva and *Dothistroma septosporum* are closely related apoplastic pathogens with similar lifestyles but different hosts: *F. fulva* is a pathogen of tomato, whilst *D. septosporum* is a pathogen of pine trees. In 2012, the first genome sequences of these pathogens were published, with *F. fulva* and *D. septosporum* having highly fragmented and near-complete assemblies, respectively. Since then, significant advances have been made in unravelling their genome architectures. For instance, the genome of *F. fulva* has now been assembled into 14 chromosomes, 13 of which have synteny with the 14 chromosomes of *D. septosporum*, suggesting these pathogens are even more closely related than originally thought. Considerable advances have also been made in the identification and functional characterization of virulence factors (e.g., effector proteins and secondary metabolites) from these pathogens, thereby providing new insights into how they promote host colonization or activate plant defence responses.

For example, it has now been established that effector proteins from both *F. fulva* and *D. septosporum* interact with cell-surface immune receptors and co-receptors to activate the plant immune system. Progress has also been made in understanding how *F. fulva* and *D. septosporum* have evolved with their host plants, whilst intensive research into pandemics of Dothistroma needle blight in the Northern Hemisphere has shed light on the origins, migration, and genetic diversity of the global *D. septosporum* population. In this review, we specifically summarize advances made in our understanding of the *F. fulva*–tomato and *D. septosporum*–pine pathosystems over the last 10 years.

KEYWORDS
Dothistroma needle blight (DNB), effector proteins, genome sequences, host susceptibility and resistance, pathogen diversity, secondary metabolites, tomato leaf mould

1 | INTRODUCTION

The year 2022 marked 10 years since the first genome sequences of the dothideomycete pathogens *Fulvia fulva* and *Dothistroma septosporum* were published and compared (de Wit et al., 2012). The nomenclature of *F. fulva* has been the subject of debate for decades, with *Cladosporium fulvum*, *F. fulva*, and *Passalora fulva* being proposed (reviewed in Thomma et al., 2005). However, recent multilocus-based DNA barcoding has shown that *F. fulva* is the currently accepted name (Videira et al., 2017). Indeed, this fungus clusters more closely with *Dothistroma* than with members of the *Cladosporium* or *Passalora* genera. *F. fulva* and *D. septosporum* were an unusual choice for a comparative genome analysis as, although they have similar lifestyles, they infect very different hosts. Specifically, *F. fulva* is considered a hemibiotrophic pathogen of tomato, an angiosperm (Hane et al., 2020; Thomma et al., 2005), whilst *D. septosporum*, previously called *Dothistroma pini* (Barnes et al., 2004), is a hemibiotrophic pathogen of gymnosperm conifers, such as pine trees (Kabir et al., 2015b). Here, hemibiotrophs are defined as those pathogens that start infection as a biotroph and then switch to necrotrophy (de Wit et al., 2012; Hane et al., 2020). Both species invade their hosts through stomata and then colonize the apoplastic environment as part of a long latent asymptomatic phase, which is also observed for other closely related hemibiotrophs like *Zymoseptoria tritici* (Brennan et al., 2019). However, whilst *F. fulva* sporulates out of stomata, *D. septosporum* erupts out of needle tissue during sporulation (Kabir et al., 2015b; Thomma et al., 2005). Like *Z. tritici*, but not *F. fulva*, cell death is required for sporulation by *D. septosporum* (Kabir et al., 2015b; Sánchez-Vallet et al., 2015).

In 2012, *F. fulva* was already a well-established model for studying molecular plant–pathogen interactions and was the species from which the first fungal avirulence (*Avr*) effector gene, *CfAvr9*, was cloned (van Kan et al., 1991). In contrast, *D. septosporum* was relatively unknown, except as the causal agent of a forest disease epidemic that was suspected to be associated with climate change (Woods et al., 2005), and for its ability to secrete a secondary metabolite toxin, dothistromin (Bradshaw et al., 2006). The two species were compared because they are phylogenetically close relatives, as evident in similarities in their genome sequences (de Wit et al., 2012; Ohm et al., 2012). The comparative analysis revealed specific differences in gene and repeat content, but also identified many intriguing similarities such as orthologous effector and dothistromin biosynthetic genes (de Wit et al., 2012).

Since 2012, several additional genomic, transcriptomic, and proteomic resources for *F. fulva* and *D. septosporum* have been made available (Table 1) (Bradshaw et al., 2016; Mesarich et al., 2014, 2018; Zaccaron et al., 2022). In combination with the original genome sequences, these resources have greatly advanced our understanding of how these two pathogens interact with, and have evolved with, their host plants. In this review, we specifically highlight the advances that have taken place since the publication of the original *F. fulva* and *D. septosporum* genome sequences. A glossary of the full gene/protein names mentioned in this review can be found in Table S1.

2 | COMPLETION OF THE *F. FULVA* AND *D. SEPTOSPORUM* GENOME SEQUENCES

The first draft genome sequences of *F. fulva* isolate 0WU and *D. septosporum* isolate NZE10 were described by de Wit et al. (2012). In the case of *D. septosporum* NZE10, the genome was assembled into 14 chromosomes, while for *F fulva* 0WU a chromosome-level genome assembly could not be obtained. Instead, there was a high degree of fragmentation due mainly to the much higher repetitive DNA content in *F. fulva* 0WU (47.2%) compared to *D. septosporum* NZE10 (3.2%) (de Wit et al., 2012).

Recently, by combining long-read sequencing with a chromatin conformation capture technique, a chromosome-level genome assembly for *F. fulva* isolate Race 5 was obtained (Table 2) (Zaccaron et al., 2022). Like *D. septosporum* NZE10, the genome of *F. fulva* Race 5 is predicted to contain 14 chromosomes (Chr), of which nine show a one-to-one match to those from *D. septosporum* NZE10, while a further four match two or

TABLE 1 Genomic, transcriptomic, and proteomic resources available for *Fulvia fulva* and *Dothistroma septosporum*.

Species and isolate	Sequence resource	NCBI BioProject accession or JGI link[a]	Reference
F. fulva			
0WU (CBS 131901)	Genome	PRJNA86753 Clafu1	de Wit et al. (2012)
0WU	Transcriptome (on *Solanum lycopersicum*; time course)	PRJNA86753	Mesarich et al. (2014)
0WU	Transcriptome (in culture; nutrient-rich and nutrient-poor media)	PRJNA86753	Mesarich et al. (2014)
Race 5 (5 Kim, IPO 1979)	Genome	PRJNA565804	Zaccaron et al. (2022)
Race 5	Transcriptome (on *S. lycopersicum*; single time point)	PRJNA86753	Mesarich et al. (2014)
Race 5	Transcriptome (in culture; nutrient-rich and nutrient-poor media)	PRJNA565804	Zaccaron et al. (2022)
Four global isolates, including 0WU and Race 5	Proteome (mixed collection of small, secreted proteins in apoplastic washing fluid from *S. lycopersicum* at different time points)		Mesarich et al. (2018)
D. septosporum			
NZE10	Genome	PRJNA74753 Dotse1	de Wit et al. (2012)
NZE10	Transcriptome (on *Pinus radiata*; time course)	Dotse1	Bradshaw et al. (2016)
NZE10	Transcriptome (in culture; pine extract media[b])	PRJNA584643	de Wit et al. (2012)
NZE10	Transcriptome (in culture; nutrient-rich media[b])	PRJNA584708	de Wit et al. (2012)
NZE10 and *DsLaeA* mutant	Transcriptome (in culture; nutrient-rich and nutrient-poor media)	PRJNA422893	Ozturk et al. (2019)
18 global isolates	Genome	PRJNA381823	Bradshaw, Sim, et al. (2019)
12 New Zealand isolates	Genome	PRJNA426106	Bradshaw, Ormond, et al. (2019)
25 Scottish isolates	Genome	PRJEB14366	Ennos et al. (2020)

[a]NCBI, National Center for Biotechnological Information (https://www.ncbi.nlm.nih.gov/); JGI, Joint Genome Institute (https://mycocosm.jgi.doe.gov/mycocosm/home).

[b]Unreplicated expressed sequence tags (ESTs) used at the JGI for genome annotation.

more chromosomes (Figure 1a). The remaining chromosome, Chr14, is dispensable, as it is absent from 19 out of 24 *F. fulva* isolates examined to date (but is present in isolate 0WU) and has no matches to any of the chromosomes from *D. septosporum* NZE10 (Zaccaron et al., 2022).

Further comparative analysis of the chromosomes from *F. fulva* Race 5 and *D. septosporum* NZE10 revealed a clear pattern of mesosynteny, consisting of a few interchromosomal rearrangements but many intrachromosomal rearrangements (Figure 1a) (Zaccaron et al., 2022). In addition, large differences in repetitive DNA content meant that while the chromosomes of *D. septosporum* NZE10 are almost entirely composed of gene-dense regions with a high GC content, those of *F. fulva* Race 5 contain long repeat-rich regions with a low GC content that intersperse the gene-dense regions (Figure 1b). Such structural differences account for the large difference in size between matching chromosomes of *F. fulva* Race 5 and *D. septosporum* NZE10 and, consequently, in the genome sizes of these two species (Zaccaron et al., 2022).

Structural differences among isolates of the same species were also noted. In a recent study, the genomes of 18 *D. septosporum* isolates from a worldwide collection were sequenced, resulting in the identification of large-scale structural variants that included cases of aneuploidy and a reciprocal translocation between Chr5 and Chr13 in isolate NZE10, compared to isolates from other continents (Figure 1a) (Bradshaw, Sim, et al., 2019). Consequently, while Chr3 of *F. fulva* Race 5 matches Chr5 and Chr13 of *D. septosporum* NZE10, this same chromosome matches a single scaffold in the genome assembly of *D. septosporum* isolate SLV1 (Figure 1a).

Comparative analysis of gene content revealed that *F. fulva* Race 5 has a considerably larger number of predicted genes than *D. septosporum* NZE10 (Table 3), which is reflected in the functional gene categories of these two species (de Wit et al., 2012; Zaccaron et al., 2022) and is discussed more thoroughly in the next sections of this review.

3 | EFFECTORS AND OTHER SECRETED PROTEINS OF *F. FULVA* AND *D. septosporum*

Like all plant-pathogenic fungi, *F. fulva* and *D. septosporum* secrete effectors and other proteins into their hosts during infection. In

TABLE 2 Comparison of the genome assemblies of *Fulvia fulva* isolate Race 5 and *Dothistroma septosporum* isolate NZE10.

Assembly statistics	*F. fulva* Race 5	*D. septosporum* NZE10
Genome size (bp)	67,169,167	30,209,431
Number of scaffolds[a]	14	14
Longest scaffold (bp)	11,362,290	5,111,597
Scaffold N_{50} (Mb)	5,777,465	2,595,548
Scaffold L_{50}	5	5
Scaffold N_{90} (Mb)	3.3	1.5
Scaffold L_{90}	10	11
GC content (%)	48.9	53.1
Complete chromosomes[b]	14	11

[a]Scaffolds longer than 50 kb.

[b]Complete chromosomes refer to the number of assembled scaffolds containing telomeric repeats at both ends.

susceptible plants, these proteins promote everything from host colonization to pathogen lifecycle completion (Bradley et al., 2022; Lo Presti et al., 2015; Rocafort et al., 2020). However, in resistant plants, one or more of these proteins are recognized as avirulence determinants (Avr proteins) by corresponding extracellular or intracellular immune receptors that trigger immune responses (Cook et al., 2015; van der Burgh & Joosten, 2019). These immune responses include the production of antifungal phytoalexins, reactive oxygen species (ROS), and pathogenesis-related (PR) proteins (e.g., chitinases, glucanases, and proteases), and are often accompanied by a hypersensitive cell death response (HR) (de Wit, 2016; de Wit et al., 2009).

3.1 | Effector proteins of *F. fulva*

Prior to 2012, a number of effectors from *F. fulva* had already been identified and functionally characterized, including up to eight Avr proteins that are recognized by corresponding Cf (resistance to *C. fulvum*) immune receptors in tomato (de Wit, 2016; de Wit et al., 2009). Since this time, one additional Avr protein, *Cf*Avr5, recognized by the extracellular Cf-5 immune receptor of tomato (Dixon et al., 1998), has been identified (Mesarich et al., 2014). To identify *Cf*Avr5, an expressed transcript sequence comparison between 44 in planta-induced (candidate) effector genes of *F. fulva* isolates 0WU and Race 5 (the latter being able to overcome Cf-5-mediated resistance) was carried out. This revealed that one of these genes has a frameshift mutation in Race 5, resulting in a premature stop codon. Through a complementation assay, in which the wild-type (WT) version of this gene from isolate 0WU was introduced into isolate Race 5, it was subsequently shown that avirulence could be restored to isolate Race 5 on Cf-5 tomato, confirming identification of *Cf*Avr5. Notably, this complementation also led to an increase in fungal biomass during infection of susceptible tomato, as compared to WT Race 5, thus highlighting a yet unknown role for *Cf*Avr5 in *F. fulva* virulence (Mesarich et al., 2014).

Shortly after the identification of *Cf*Avr5, significant progress was made towards defining an inventory of all (candidate) effector proteins secreted by *F. fulva* into the leaf apoplast of susceptible tomato plants during infection (Mesarich et al., 2018). Using proteomics, a total of 75 *F. fulva* small, secreted proteins (SSPs) were identified in apoplastic washing fluid (AWF) samples collected from interactions between the fungus (including isolates 0WU and Race 5) and susceptible tomato. Crucially, this comprised all *F. fulva* effectors characterized to date, as well as most of the candidate effectors (33 out of 44) encoded by genes examined in the *Cf*Avr5 study. Based on the transcriptome sequencing data of Mesarich et al. (2014), 70 of the 75 SSP-encoding genes were shown to be up-regulated during host colonization (Mesarich et al., 2018).

Following on from this proteomics work, 41 of the 70 in planta-induced SSPs were screened for their ability to trigger a hypersensitive response (HR) across a collection of 25 predominantly wild resistant accessions of tomato, using an approach based on the potato virus X (PVX) expression system (Mesarich et al., 2018). Of these, nine SSPs (*Cf*Ecp8–16) triggered an HR in one or more accessions, suggesting that they are Avr proteins recognized by as yet uncharacterized Cf immune receptors. These accessions now represent a valuable resource for the development of new commercial tomato varieties with resistance against leaf mould disease (Mesarich et al., 2018).

One of the HR-eliciting SSPs, *Cf*Ecp11-1, has sequence similarity to the AvrLm3 and AvrLm5-9 Avr proteins from *Leptosphaeria maculans* (Ghanbarnia et al., 2018; Mesarich et al., 2018; Plissonneau et al., 2016, 2018; van de Wouw et al., 2014). While the function of *Cf*Ecp11-1 is not yet known, sequence and structural analyses determined that all three proteins, as well as two other Avr proteins from *L. maculans*, AvrLm4-7 and AvrLmS-Lep2 (Parlange et al., 2009; Xiang Neik et al., 2022), belong to a conserved group of fungal effectors, termed the "*Leptosphaeria* avirulence and suppressing" (LARS) family (Lazar et al., 2022). This family is characterized by a conserved WR(F/L/V)(R/K) motif that, when mapped onto the crystal structures of *Cf*Ecp11-1 (Figure 2a), AvrLm4-7, and AvrLm5-9, is predominantly surface-exposed, suggesting that it may play a role in functional interactions with host proteins. AvrLm4-7 is of particular interest, as it suppresses the recognition of AvrLm3 and AvrLm5-9 by the corresponding immune receptors Rlm3 and Rlm9 of *Brassica* spp., respectively (Ghanbarnia et al., 2018; Plissonneau et al., 2016). Strikingly, transformants of *L. maculans* expressing *Cf*Ecp11-1 also trigger Rlm3-mediated immunity in *Brassica* spp. Furthermore, as for AvrLm3 and AvrLm5-9, this recognition is suppressed by AvrLm4-7. These findings suggest that *Cf*Ecp11-1 is sufficiently similar to AvrLm3 to be directly or indirectly recognized by Rlm3 (Lazar et al., 2022).

In addition to *Cf*Ecp11-1, the crystal structures of the *Cf*Avr4 and *Cf*Ecp6 effectors from *F. fulva* have been solved (Hurlburt et al., 2018; Sanchez-Vallet et al., 2013), while for many other effectors and effector candidates of this fungus, tertiary structures have

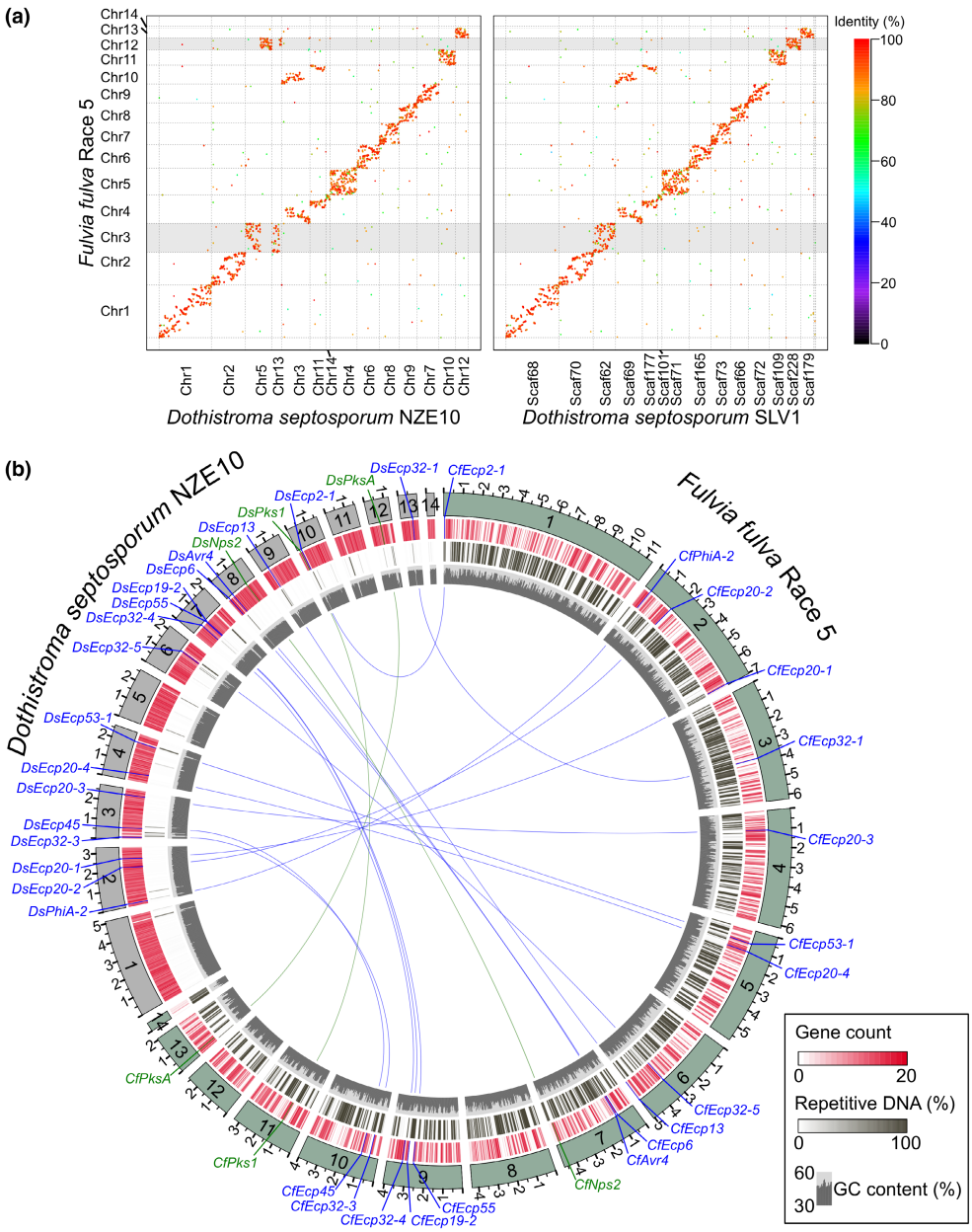

FIGURE 1 Whole-genome comparison between *Fulvia fulva* isolate Race 5 and *Dothistroma septosporum* isolate NZE10. (a) Dot plots showing whole genome synteny between *F. fulva* and *D. septosporum*. The plots show a small number of interchromosomal translocations and many intrachromosomal rearrangements. Evidence of a reciprocal translocation between Chr5 and Chr13 in *D. septosporum* is highlighted in grey. (b) Circos plot showing the chromosomes of *D. septosporum* and *F. fulva* with tick marks indicating million base pairs. The other three outermost to innermost tracks represent gene density, repetitive DNA content, and GC content, respectively, within a sliding window of 30 kb. Ribbons connect 17 genes encoding candidate effectors and three genes encoding key enzymes for secondary metabolism conserved in both genomes.

been predicted (Karimi-Jashni et al., 2022; Mesarich et al., 2018; Seong & Krasileva, 2022; Tarallo et al., 2022). *Cf*Avr4, which contains a carbohydrate-binding module family 14 (CBM14) domain (van den Burg et al., 2004) and triggers immunity in tomato in the presence of the extracellular Cf-4 immune receptor (Joosten et al., 1997), binds to chitin present in the cell walls of invading *F. fulva* hyphae to protect them against hydrolysis by plant-derived chitinases (Joosten et al., 1994; van den Burg et al., 2003, 2006). The crystal structure of *Cf*Avr4 revealed a dimeric assembly in which two molecules of *Cf*Avr4 interact to form a three-dimensional molecular sandwich that encapsulates two chitohexaose molecules (Figure 2b) (Hurlburt et al., 2018).

Like *Cf*Avr4, *Cf*Ecp6 is a chitin-binding effector; however, *Cf*Ecp6 has three lysin motif (LysM) domains that sequester chito-oligosaccharides released from the cell walls of invading *F. fulva* hyphae to prevent their recognition by extracellular chitin-binding tomato immune receptors. This, in turn, prevents activation of chitin-triggered immune responses in tomato (Bolton et al., 2008;

de Jonge et al., 2010). Based on the crystal structure of *Cf*Ecp6 in complex with chitotetraose (Figure 2c), it was shown that this sequestration is mediated through the intrachain dimerization of LysM domains 1 and 3. Although not involved in sequestration, LysM domain 2 can also bind chitin, albeit with lower affinity, and has been shown to perturb chitin-triggered immune responses, potentially through interference with the chitin/immune receptor complex (Sanchez-Vallet et al., 2013).

Despite the progress made over the last 10 years in understanding the tertiary structure and function of specific *F. fulva* effectors, very little progress has been made on understanding the regulatory mechanisms underpinning *F. fulva* effector gene expression in planta. One study that attempted to understand this regulation focused on the transcription factor (TF) *Cf*Wor1 (Ökmen et al., 2014), as its homologues in some other plant-pathogenic fungi were shown to regulate effector gene expression (Michielse et al., 2009). However, functional analyses, involving gene deletion and constitutive expression mutants, showed that *Cf*Wor1 is a global TF that regulates *F. fulva* development (Ökmen et al., 2014).

TABLE 3 Comparison of the genome annotation of *Fulvia fulva* isolate Race 5 and *Dothistroma septosporum* isolate NZE10.

Gene annotation statistics[a]	*F. fulva* Race 5	*D. septosporum* NZE10
Number of genes	14,690	12,415
Mean gene length (bp)	1284	1233
Mean exon length (bp)	598	585
Mean intron length (bp)	79	92
Mean exons per gene	2.1	2.1
Mean introns per gene	1.1	1.1
Total coding sequence length (bp)	18,870,333	15,318,053
Total intron length (bp)	1,341,392	1,270,355
BUSCO[b] completeness (%)	98.9	97.9
BUSCO duplicated (%)	0.1	0.1
BUSCO fragmented (%)	0.3	1.4
BUSCO missing (%)	0.8	0.5

[a]Untranslated regions were not considered when calculating the statistics.

[b]BUSCO, Benchmarking Universal Single-Copy Orthologs.

3.2 | Effector proteins of *D. septosporum*

Although *D. septosporum* and *F. fulva* have fairly dissimilar repertoires of (candidate) effector genes, many are still shared between the two species, consistent with their close phylogenetic relationship (Figure 1b) (de Wit et al., 2012; Mesarich et al., 2018; Stergiopoulos et al., 2012; Tarallo et al., 2022; Zaccaron et al., 2022). Comparison of their chromosomal locations showed that, in many cases, orthologous (candidate) effector gene pairs are present on homologous chromosomes in conserved locations or in locations where interchromosomal rearrangements have occurred (Figure 1b) (Zaccaron et al., 2022). It is likely that many of these orthologous genes encode "core" effectors with conserved biological functions in promoting host colonization (de Wit et al., 2012; Stergiopoulos et al., 2010). In support of this hypothesis, it has been shown that *Ds*Avr4, the *D. septosporum* orthologue of *Cf*Avr4, specifically binds to chitin and is also recognized by the Cf-4 immune receptor of tomato (de Wit et al., 2012), illustrating that *Ds*Avr4 and *Cf*Avr4 share a conserved biological function (Mesarich et al., 2016).

Another likely core effector from *D. septosporum* is *Ds*Ecp2-1, which is orthologous to *Cf*Ecp2-1 from *F. fulva* (de Wit et al., 2012; van den Ackerveken et al., 1993). Both *Cf*Ecp2-1 and *Ds*Ecp2-1 were previously shown to trigger an HR in the presence of the Cf-Ecp2 immune receptor of tomato, which has not yet been identified (de Wit et al., 2012; Laugé et al., 2000; Stergiopoulos et al., 2010). Likewise, similar to *Cf*Ecp2-1, which triggers cell death in several *Nicotiana* species (de Kock et al., 2004; Laugé et al., 2000), *Ds*Ecp2-1 was shown to trigger cell death in *Nicotiana tabacum* (Guo et al., 2020; Hunziker et al., 2021), suggesting that, in these nonhost plants, both of these proteins are recognized by an as yet uncharacterized

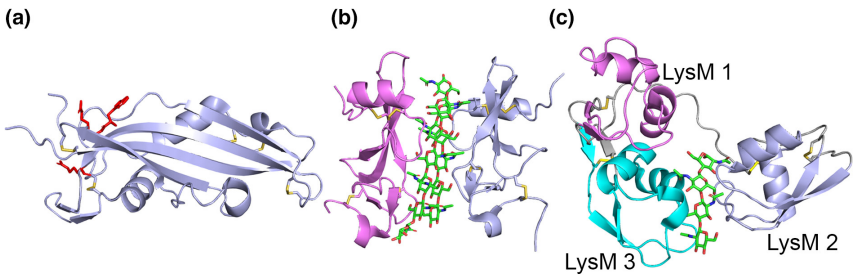

FIGURE 2 Solved tertiary structures of effector proteins from *Fulvia fulva*. Structures are visualized as cartoons using PyMol software (DeLano, 2002). Disulphide bonds are shown in yellow. (a) Crystal structure of the *Cf*Ecp11-1 effector protein (RCSB Protein Data Bank (PDB) ID: 6ZUS (Lazar et al., 2022)). The surface-exposed residues of the WRVR motif are shown as red sticks. (b) Crystal structure of the *Cf*Avr4 effector protein (dimer) in complex with chitohexaose (PDB ID: 6BN0 (Hurlburt et al., 2018)). *Cf*Avr4 molecules are coloured pink (left) and light blue (right), while chitohexaose is shown as green sticks. (c) Crystal structure of the *Cf*Ecp6 effector protein in complex with chitotetraose (PDB ID: 4B8V; Sanchez-Vallet et al., 2013). Lysin motif (LysM) domains 1, 2 and 3 of *Cf*Ecp6 are numbered and are coloured pink, light blue and cyan, respectively, while chitotetraose is shown as green sticks.

immune receptor. In *F. fulva*, *Cf*Ecp2-1 is a virulence factor of unknown function, with deletion or disruption of the encoding gene resulting in reduced virulence (Laugé et al., 1997). Like *CfEcp2-1* (Wubben et al., 1994), *DsEcp2-1* is highly expressed in planta, suggesting that this effector may also function as a virulence factor during infection of pine trees (Guo et al., 2020). But strikingly, deletion of *DsEcp2-1* results in the production of larger necrotic lesions on pine needles by *D. septosporum*, concomitant with increased colonization, suggesting that *Ds*Ecp2-1 is recognized as an Avr protein in pine by an as yet uncharacterized immune receptor, leading to partial resistance (Guo et al., 2020).

Recently, a set of 30 candidate apoplastic effector proteins, encoded by genes that are highly expressed and/or up-regulated during infection of pine seedlings by *D. septosporum* (Bradshaw et al., 2016), was identified (Hunziker et al., 2016, 2021). *Agrobacterium tumefaciens*-mediated transient transformation assays revealed that, in addition to *Ds*Ecp2-1, five of these proteins (*Ds*Ecp20-3, *Ds*Ecp32-3, *Ds*71487, *Ds*131885, and *Ds*74283) trigger cell death in *Nicotiana benthamiana* and/or in *N. tabacum* (Hunziker et al., 2021). As an extension of this work, additional members of the Ecp20 and Ecp32 families from *D. septosporum* (and their orthologues from *F. fulva*) were also screened and found to differ in their ability to trigger cell death in these two *Nicotiana* species (Tarallo et al., 2022). In cases where cell death was observed, this response was dependent on the secretion of these proteins to the apoplast, suggesting that they are recognized by extracellular immune receptors in these nonhost plants (Hunziker et al., 2021; Tarallo et al., 2022). In line with this hypothesis, cell death triggered by *Ds*Ecp20-3 (and both *Cf*Ecp20-3 and *Cf*Ecp32-3) was compromised in *N. benthamiana* plants silenced for *BAK1* or *SOBIR1*, which encode extracellular co-receptors involved in transducing defence response signals following apoplastic effector recognition (Tarallo et al., 2022). Notably, using a protein infiltration method involving clonal microshoots, it was shown that *Ds*Ecp20-3 and *Ds*Ecp32-3 also trigger cell death in three genotypes of the host plant *Pinus radiata*, suggesting that these proteins are recognized by extracellular immune receptors in this species (Hunziker et al., 2021; Tarallo et al., 2022). While not yet performed in *D. septosporum*, deletion of the *CfEcp20-2* gene in *F. fulva* isolate 0WU resulted in reduced virulence on tomato, indicating that members of the Ecp20 family are virulence factors (Karimi-Jashni et al., 2022).

3.3 | Glycoside hydrolases of *F. fulva* and *D. septosporum*

Fungal pathogens produce an array of carbohydrate-active enzymes (CAZymes) that are involved in the synthesis, metabolism, and recognition of complex carbohydrates (Bradley et al., 2022). Interestingly, it has recently been shown that fungal pathogens can be classified into different trophic lifestyles based on their complement of CAZyme-encoding genes (Hane et al., 2020). This is of particular relevance to *F. fulva*, which, although previously thought to be a biotroph (de Wit et al., 2012), is now considered to be a hemibiotroph (Hane et al., 2020; Ökmen et al., 2019). Indeed, the complement of CAZyme-encoding genes in *F. fulva* is more similar to those of hemibiotrophs than biotrophs (de Wit et al., 2012; Hane et al., 2020). Such a classification is further supported by the observation that tomato leaves often become necrotic at the late stages of infection by *F. fulva* (Thomma et al., 2005).

Among the CAZymes, the secreted glycoside hydrolases (GHs) are central to understanding interactions between fungal pathogens and plants, with characterized or anticipated roles in fungal morphogenesis, nutrient acquisition, and host colonization (Bradley et al., 2022). Secreted GHs, however, can also be immunogenic in that they, or the products of their hydrolytic activity, called damage-associated molecular patterns (DAMPs), are recognized by extracellular plant immune receptors to activate the plant immune system (Bradley et al., 2022).

Over the past 10 years, only a handful of secreted GH proteins from *F. fulva* and *D. septosporum* have been studied. The first of these is *Cf*Tom1, a GH10 enzyme from *F. fulva* with no close homologues in *D. septosporum* (de Wit et al., 2012; Ökmen et al., 2013). *CfTom1* is poorly expressed during the early stages of tomato infection but is significantly up-regulated late in the infection cycle. Functional analysis showed that *Cf*Tom1 is a tomatinase that detoxifies α-tomatine, an antifungal glycoalkaloid present in the leaf apoplast of tomato, to the nontoxic compound tomatidine by removing the tetrasaccharide lycotetraose. Consistent with this role, *CfTom1* deletion mutants were more sensitive to α-tomatine and produced less biomass during infection, indicating that *Cf*Tom1 is required for full virulence of *F. fulva* on tomato plants (Ökmen et al., 2013).

Another characterized GH protein from *F. fulva* is *Cf*GH17-1, an apoplastic GH17 enzyme with 1,3-β-glucanase activity (Ökmen et al., 2019). Expression studies showed that *CfGH17-1* is down-regulated during the early stages of biotrophic infection by *F. fulva*, but is up-regulated late in the infection cycle, when the fungus switches to its necrotrophic state. Notably, *Cf*GH17-1 elicited cell death in several near-isogenic lines of tomato, but this cell death-eliciting activity was abolished by site-directed mutagenesis of the putative active-site residues present in this enzyme. These results suggest that *Cf*GH17-1 itself is not immunogenic but, rather, that *Cf*GH17-1 releases an immunogenic oligosaccharide from a complex polysaccharide or glycoprotein of the plant cell wall that is subsequently recognized as a DAMP by a yet unknown extracellular immune receptor present in tomato plants. In line with this hypothesis, deletion of the *CfGH17-1* gene does not affect symptom development by *F. fulva*, whereas overexpression results in reduced disease symptoms. One possibility is that *Cf*GH17-1 plays a role in nutrition, releasing sugar molecules from the host cell wall to support the growth and reproduction of *F. fulva* during the late stages of infection when the host tissue is necrotic and no longer capable of recognizing and responding to DAMPs. Interestingly, a paralogue from *F. fulva* (*Cf*GH17-5), as well as the orthologue of *Cf*GH17-1 from *D. septosporum* (*Ds*GH17-1), also trigger cell death when heterologously expressed in tomato

plants, suggesting that they might play a similar role in promoting host colonization (Ökmen et al., 2019).

Aside from *Ds*GH17-1, other GH proteins from *D. septosporum* are also candidate virulence factors. One example is *Ds*GH11 (*Ds*137959), a GH11 enzyme orthologous to an endo-1,4-β-xylanase from *Z. tritici* (ZtXyl1) that has been suggested to play a role in lesion development during colonization of wheat (Bradshaw et al., 2016; Siah et al., 2010). In *D. septosporum*, *DsGH11* is up-regulated in planta at the stage when disease lesions start to appear on infected pine needles, consistent with a role in lesion development and the switch from biotrophy to necrotrophy (Bradshaw et al., 2016).

3.4 | Proteases of *F. fulva* and *D. septosporum*

Proteases of *F. fulva* and *D. septosporum* are also anticipated to play a role in promoting host colonization. The number of protease-encoding genes present in the genome of *F. fulva* is once again more comparable to that of hemibiotrophs than biotrophs (Karimi Jashni et al., 2020). However, protease-encoding genes are more frequently pseudogenized in *F. fulva* than in *D. septosporum* (van der Burgt et al., 2014). Secreted proteases have not yet been studied in detail in *D. septosporum*, but in the *F. fulva* genome 59 of the 147 encoded proteases are predicted to be secreted (Karimi Jashni et al., 2020). Of these, 18 are expressed both in culture and in planta, suggesting that a specific role in virulence for these 18 proteases is unlikely. However, a few genes, such as those encoding the predicted serine proteases *Cf*Pro41 and *Cf*Pro54, are specifically induced during host infection, while another gene encoding the predicted metalloprotease *Cf*Pro19 is more highly expressed in planta than in culture (Karimi Jashni et al., 2020). One possibility is that these proteases promote virulence by removing the chitin-binding domain from tomato chitinases, which decreases their antifungal activity, as was shown for a synergistically acting metallo-/serine-protease pair, *FoMep1*/*FoSep1*, from *Fusarium oxysporum* f. sp. *lycopersici* (Jashni et al., 2015).

4 | SECONDARY METABOLISM

4.1 | Core secondary metabolite genes of *F. fulva* and *D. septosporum*

Fungal secondary metabolites (SMs) play key roles in development, communication, microbial antagonism, and plant disease (Macheleidt et al., 2016). SMs are classified into chemical groups based on their backbone structure, the biosynthesis of which is catalysed by core enzymes, including polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs), hybrid PKS-NRPSs, terpene cyclases (TCs), and dimethylallyltryptophan synthases (DMATs) (Macheleidt et al., 2016). Efforts to identify and characterize SM genes in *F. fulva* and *D. septosporum* were greatly accelerated by analysis of their genomes (de Wit et al., 2012). However, growth of *F. fulva* or *D. septosporum*, either in planta or in culture, yields only one main detectable SM from each species, namely cladofulvin and dothistromin, respectively. The genome of *F. fulva* 0WU initially suggested a large chemical diversity with 10 *PKS*, 10 *NRPS*, two hybrid *PKS-NRPS*, and one *DMAT* core genes, but seven of these genes are truncated or pseudogenized. The *D. septosporum* NZE10 genome contains only five *PKS*, three *NRPS*, two hybrid *PKS-NRPS*, and one *DMAT* core genes, of which two are truncated or pseudogenized (de Wit et al., 2012). The only three core SM genes shared between *F. fulva* Race 5 and *D. septosporum* NZE10 are located on homologous chromosomes (Figure 1b). These shared genes are *Cf/DsPks1* involved in the biosynthesis of DHN melanin, *Cf/DsNps2* involved in siderophore production, and *Cf/DsPksA* involved in dothistromin biosynthesis (Bradshaw et al., 2006; Collemare et al., 2014; de Wit et al., 2012; Ozturk et al., 2017, 2019). Finally, expression analysis showed that, in both species, only a few SM core genes are expressed in planta or under diverse conditions in culture (Bradshaw et al., 2016; Collemare et al., 2014; Griffiths et al., 2015; Ozturk et al., 2019), with *CfPks1* and *DsPks1* being expressed in both species (Collemare et al., 2014; de Wit et al., 2012; Ozturk et al., 2017). Three additional SM core genes are expressed in *D. septosporum* (*DsPksA*, *DsPks2*, and *DsNps3*) (Bradshaw et al., 2016; Ozturk et al., 2017, 2019) and five in *F. fulva* (*CfNps4*, *CfNps8*, *CfNps9*, *CfPks5*, and *CfPks6*) (Table 4) (Collemare et al., 2014; de Wit et al., 2012).

4.2 | Functional analysis of SM genes

Functional analysis has been carried out for selected SM genes in *F. fulva* and *D. septosporum* to determine their roles. Deletion studies showed that neither of the core *CfPks1* and *DsPks1* dihydroxynapthalene (DHN) melanin biosynthesis genes are pathogenicity or virulence factors, consistent with their low expression in planta (Bradshaw et al., 2016; Griffiths, Cox, et al., 2018; Ozturk et al., 2017). Further studies revealed that *D. septosporum* appears to produce DHN melanin mostly through an alternative PKS-independent L-3,4-dihydroxyphenylalanine (DOPA) pathway (Ozturk et al., 2017), whilst in *F. fulva*, DHN melanin biosynthesis required *Cf*Pks1 (Griffiths, Cox, et al., 2018).

In *F. fulva*, deletion of the *CfPks6* gene that is required for cladofulvin biosynthesis yielded mutants that were unaltered in pathogenicity or virulence on tomato plants (Griffiths, Mesarich, et al., 2018). *CfPks6* is normally only lowly expressed during growth of the fungus inside the apoplast. However, transformants overexpressing the presumed transcriptional activator (*CfClaE*) from the *CfPks6* cluster induced rapid necrosis of tomato leaves and were arrested in growth. Together, these results suggest that *F. fulva* must repress cladofulvin production during early host colonization to sustain biotrophic growth prior to a necrotrophic switch (Griffiths, Mesarich, et al., 2018). The cladofulvin pathway of *F. fulva* was eventually deduced by analysis of metabolites produced by transformants of *Aspergillus oryzae* expressing *CfPks6*

Core gene	Predicted SM or family	In planta expression[a]	Virulence function	Reference
F. fulva				
CfPks1	Melanin; DHN	Late	No	Griffiths, Cox, et al. (2018)
CfPks5	T-toxin	No	NT	Collemare et al. (2014)
CfPks6	Cladofulvin	Early and late	No[b]	Collemare et al. (2014); Griffiths, Mesarich, et al. (2018)
CfNps4	"EAS (Euascomycete) clade"	No	NT	Collemare et al. (2014)
CfNps8	Siderophore	No	NT	Collemare et al. (2014)
CfNps9	Siderophore	Early	NT	Collemare et al. (2014)
D. septosporum				
DsPksA	Dothistromin	Mid-late	Yes	Kabir et al. (2015a)
DsPks1	Melanin; DHN	Late	No	Ozturk et al. (2017)
DsPks2	Squalestatin-like	Late	No	Ozturk et al. (2017)
DsNps3	Beauvericin-like	Early	Yes	Ozturk et al. (2019)

Abbreviations: DHN, dihydroxynapthalene; NT, not tested.

[a]In *D. septosporum*, highest gene expression occurred during early (asymptomatic), mid (early lesion) or late (sporulating lesion) stages of disease on *Pinus radiata* needles. In *F. fulva*, highest gene expression was during early (surface growth of runner hyphae 0–4 days after inoculation) or late (conidiation 10–16 days after inoculation) stages on tomato.

[b]Gene deletion mutants were not obviously altered in virulence, but *CfClaE* overexpression transformants were hypervirulent.

TABLE 4 Secondary metabolite (SM) core genes expressed in *Fulvia fulva* and *Dothistroma septosporum* in culture or in planta.

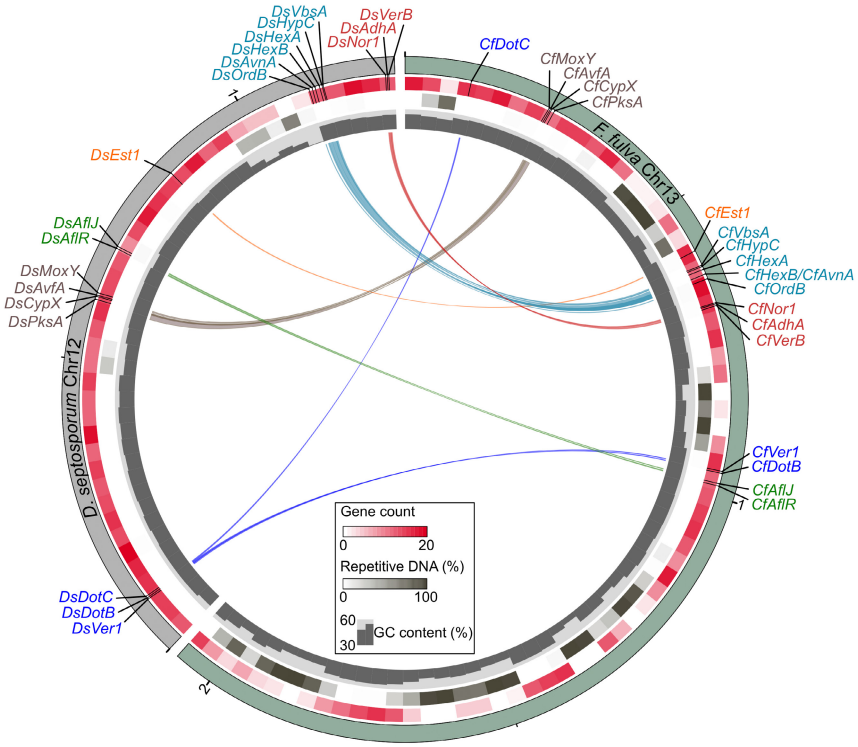

FIGURE 3 Locations of predicted dothistromin biosynthetic pathway genes in *Fulvia fulva* isolate Race 5 and *Dothistroma septosporum* isolate NZE10. Circos plot showing Chr12 of *D. septosporum* and Chr13 of *F. fulva*. From outermost to innermost, tracks represent the chromosomes, gene density, repetitive DNA content, and GC content within a sliding window of 30 kb. Ribbons connect 19 predicted dothistromin genes, which are colour-coded based on their respective subcluster (numbered 1 to 6) in *D. septosporum* (Chettri et al., 2013). All predicted homologues of the dothistromin gene cluster in *F. fulva* are located on Chr13, with all subclusters conserved. The only exception is for *CfDotC*, which is physically distant from *CfVer1* and *CfDotB*. The genes *CfHexB* and *CfAvnA* are fused in the same predicted gene model.

along with the early (*CfClaF* and *CfClaH*) or middle (*CfClaB* and *CfClaC*)-acting genes, as well as by analysis of a *F. fulva* deletion mutant (Δ*CfClaM*) that was blocked in the final step of cladofulvin biosynthesis (specifically, the dimerization of the monomeric anthraquinone, nataloe-emodin) (Griffiths et al., 2016).

In *D. septosporum*, deletion of the *DsNps3* (beauvericin-like) gene led to reduced virulence on *P. radiata* but further work is needed to elucidate the role of this potential virulence factor (Ozturk et al., 2019). Deletion of the *DsPks2* (squalestatin-like) gene, however, did not affect virulence (Ozturk et al., 2017).

4.3 | Genetics and evolution of dothistromin biosynthesis

The *D. septosporum DsPksA* gene is required for the production of dothistromin, an aflatoxin-like anthraquinone (Bradshaw et al., 2006) that was confirmed to be required for full virulence on pine trees (Kabir et al., 2015a). Whilst other genes involved in the biosynthesis of an SM are frequently clustered around the core gene (Macheleidt et al., 2016), the chromosome-level assembly of the *D. septosporum* NZE10 genome sequence revealed that dothistromin pathway genes are in six subclusters spread across chromosome 12 (de Wit et al., 2012). The complete set of dothistromin genes is conserved in *F. fulva* Race 5 (Zaccaron et al., 2022) and is similarly fragmented into subclusters throughout a single chromosome, Chr 13, but with some rearrangements (Figure 3). Dothistromin was not detected in *F. fulva* and genes in the pathway are barely expressed (de Wit et al., 2012). Further analysis revealed that dothistromin biosynthesis in this species is blocked by pseudogenization of early pathway genes, *CfHexA* and *CfNor1* (Chettri et al., 2013). In *D. septosporum*, dothistromin biosynthesis is concomitant with the start of the rapid increase in fungal biomass that occurs during the transition from biotrophic to necrotrophic growth, consistent with its role in expansion of disease lesions (Kabir et al., 2015a, 2015b).

Orthologues of some dothistromin genes are also present in other fungi, including *Rhytidhysteron rufulum*, a saprophytic dothideomycete (Bradshaw et al., 2013; Ohm et al., 2012), and some eurotiomycete species such as *Aspergillus flavus* and *Aspergillus nidulans*, in which the dothistromin orthologues are involved in aflatoxin and sterigmatocystin biosynthesis, respectively (Bradshaw et al., 2013). A comparative genomics and population genetics study of dothistromin and aflatoxin-like genes in these two fungal classes suggested that the dispersed arrangement of dothistromin genes in *D. septosporum* evolved from an ancestral gene cluster, leading to the suggestion that cluster fragmentation might facilitate chemical diversity and provide an adaptive advantage (Bradshaw et al., 2013).

4.4 | Global regulation of SM gene expression

Due to the large number of cryptic SM pathways in *F. fulva* and *D. septosporum*, there has been considerable interest in the regulation of SM gene expression. Attempts to activate cryptic SM pathways in *F. fulva* by chemical induction (trichostatin A and 5-azacytidine), or by culturing under different conditions, were unsuccessful (Griffiths et al., 2015). Manipulation of conserved global transcriptional regulators known to affect SM gene expression in other ascomycetes provided another avenue for exploration because these regulators coordinate many gene networks and their manipulation can yield phenotypes that are useful for discovering new SMs or understanding their functions. Both *F. fulva* and *D. septosporum* were previously shown to have genes encoding the global regulators *Cf*/*Ds*HdaA (histone deacetylase), *Cf*/*Ds*VeA (velvet complex scaffold), and *Cf*/*Ds*LaeA (methyltransferase), among others (de Wit et al., 2012). In *F. fulva*, deletion mutants of the transcriptional regulator genes *CfLaeA* and *CfVeA* were impaired in conidiation but still produced cladofulvin. However, the loss of hyphal pigmentation associated with melanin in Δ*CfLaeA* mutants suggested that *Cf*LaeA positively regulates melanin production (Griffiths et al., 2015). In contrast, *CfHdaA* mutants do not produce cladofulvin (Griffiths et al., 2015).

In *D. septosporum*, *Ds*HdaA, *Ds*VeA, and *Ds*LaeA, as well as the pathway regulator *Ds*AflR, were each shown to play a role in the regulation of dothistromin biosynthesis (Chettri & Bradshaw, 2016; Chettri et al., 2012, 2013, 2018). Gene clustering has been proposed to facilitate coordinated gene expression through control of chromatin structure (Macheleidt et al., 2016). However, co-expression of dothistromin genes in the fragmented dothistromin gene cluster is achieved by both direct chromatin-level H3K27me3 control of the dispersed genes as well as indirect H3K9me3/ac control via *Ds*AflR (Chettri et al., 2018).

5 | INDUCTION AND CIRCUMVENTION OF DEFENCE RESPONSES IN TOMATO BY *F. FULVA*

5.1 | Interactions of *F. fulva* effectors with Cf immune receptors and host virulence targets

Resistance to *F. fulva* in tomato is mediated by cell surface-associated receptor-like proteins (RLPs), referred to as Cf immune receptors. The extracellular domain of Cf immune receptors mostly consists of leucine-rich repeats (LRRs), which are implicated in protein–protein interactions. These RLPs allow tomato plants that are resistant to *F. fulva* to recognize specific secreted effector proteins from the fungus, thereby rendering it an Avr protein. Whether this recognition takes place directly, or indirectly through perception of the manipulated virulence target of the host by the effector, is still an area of intense study.

One of the best-studied Avr proteins from *F. fulva* is *Cf*Avr2, a protease inhibitor that interacts with the secreted papain-like cysteine protease of tomato, Rcr3. In susceptible host genotypes, *Cf*Avr2 binds to, and inhibits, Rcr3 to promote host colonization. However, in resistant tomato genotypes, the *Cf*Avr2/Rcr3 complex is recognized by the Cf-2 immune receptor to elicit plant immune responses (Rooney et al., 2005). This indirect recognition of *Cf*Avr2 through the guarded virulence target Rcr3 is an example of the guard hypothesis, in which the immune receptor (the guard, Cf-2) senses manipulation of a virulence target (the guardee, Rcr3) by an effector (Dangl & Jones, 2001; van der Biezen & Jones, 1998). *Cf*Avr2 behaves as a noncompetitive inhibitor of Rcr3. Affinity of *Cf*Avr2 mutants for Rcr3 is positively correlated with their ability to trigger a Cf-2-mediated HR (Van't Klooster et al., 2011).

Immune receptor genes are continuously subjected to selective pressure, resulting in high genetic variation and novel effector recognition specificities. Interestingly, the Rcr3 protease that is guarded by Cf-2 is also subject to selective pressure. Indeed, natural

variants of this protease have been identified, with these variants exhibiting differences in their interactions with *Cf*Avr2 that correlate with variation in immune output (Hörger et al., 2012). In addition, the evolution of Rcr3 is further impacted by its interaction with the Cf-2 immune receptor, and three amino acid polymorphisms in Rcr3 appear to have a major role in co-evolution between this guardee and the Cf-2 guard. Based on such observations, it was concluded that, as a result of natural selection, there is a subtle balance between the initiation of a Cf-2-triggered resistance response in the presence of *Cf*Avr2 and the prevention of spontaneous Rcr3/Cf-2-triggered auto-immune responses in the absence of *Cf*Avr2 (Hörger et al., 2012).

In addition to Rcr3, tomato secretes a very closely related protease, Pip1. Notably, *Cf*Avr2 can inhibit both proteases but only Rcr3 is guarded by Cf-2 (Ilyas et al., 2015). Pip1 is up to 10-fold more abundant in tomato than Rcr3 and tomato plants with reduced *Pip1* expression are hypersusceptible to a broad range of pathogens, suggesting that Pip1 is an important protease mediating basal immunity. Strikingly, *Rcr3* knock-out lines do not show significantly higher levels of infection by bacteria and fungi but do show increased susceptibility to the oomycete *Phytophthora infestans*. It has been proposed that gene duplication resulted in Rcr3 and Pip1 being functionally diversified and that co-evolution between Cf-2 and Rcr3 resulted in the indirect detection of Rcr3 inhibitors, such as *Cf*Avr2 (Ilyas et al., 2015). This work suggests that Rcr3 acts as a guarded "decoy" that betrays strains of *F. fulva* secreting Avr2 on their ingress into leaves of Cf-2-containing tomato plants.

A further study revealed that Rcr3 evolved more than 50 million years ago, whereas Cf-2 is evolutionarily much younger (Kourelis et al., 2020). In various solanaceous plants, there are ancient homologues of Rcr3 that can be inhibited by *Cf*Avr2, with four residues in these proteins that vary among the solanaceous species being able to promote this inhibition. Strikingly, the isoform of Rcr3 that is guarded by Cf-2 lacks three of these residues, which suggests that Rcr3 is employed as a type of co-receptor by Cf-2 that is inferior in binding to *Cf*Avr2 (Kourelis et al., 2020).

Interestingly, the P69 protease family of tomato consists of a set of subtilases (SBTs) that proteolytically activate Rcr3 (Paulus et al., 2020; Reichardt et al., 2018). It was observed that Rcr3 is processed by secreted P69B and other SBTs in solanaceous plants. SBTs are aspartic acid-selective proteases, meaning that they cleave amino acid chains in Asp-rich regions. Asp residues are abundantly present in the region where Rcr3-like proteases are cleaved, which suggests that in solanaceous plants the maturation and activation of immune proteases by subtilases generally occurs and supports immunity in the extracellular spaces, contributing to the first line of defence against pathogens such as *F. fulva* (Paulus et al., 2020).

Another well characterized Avr protein of *F. fulva* is *Cf*Avr4, which, as mentioned above, binds to chitin in the cell walls of invading hyphae of the fungus to protect them against hydrolysis by secreted tomato chitinases (van den Burg et al., 2006). As *Cf*Avr4 does not seem to have a virulence target in the plant, a direct interaction between *Cf*Avr4 and the LRRs of Cf-4 is expected. In addition to *Ds*Avr4 from *D. septosporum*, several orthologues of *Cf*Avr4 have been identified from plant-pathogenic dothideomycete fungi, including *Ca*Avr4 from *Cercospora apii* and *Cb*Avr4 from *Cercospora beticola*. While each of these orthologues binds chitin, only *Ds*Avr4 appears to be recognized by Cf-4. Using protein chimeras and site-directed mutants involving *Cf*Avr4, *Ds*Avr4, *Ca*Avr4, and *Cb*Avr4, a particular conserved proline residue was shown to be required for Avr4 proteins to initiate a Cf-4-dependent HR (Mesarich et al., 2016). The mechanism behind this proline requirement is not known; however, subsequent work suggested that the proline residue itself is not essential, as long as a small aliphatic amino acid residue is present at this position (Hurlburt et al., 2018). In general, mutations in *Cf*Avr4 appear to reduce its stability in the apoplastic environment. It is therefore likely that the small aliphatic residue is required to maintain the structural integrity of the protein, and that this structural integrity is needed for Cf-4 to perceive the globular fold of *Cf*Avr4 and thereby activate host immunity. Crucially, *Cf*Avr4 recognition by Cf-4 is not linked with its chitin-binding properties, meaning that the virulence function of *Cf*Avr4 is not coupled to its ability to be recognized by Cf-4 (Hurlburt et al., 2018).

5.2 | Characterization and mapping of novel Cf immune receptor genes

As broad-spectrum resistance against leaf mould disease, to our knowledge, remains elusive in commercial tomato varieties, the last decade has seen significant efforts made in the mapping, identification, and characterization of additional Cf immune receptor genes (Chai et al., 2020; Iakovidis et al., 2020; Jiang et al., 2022; Liu et al., 2018, 2019, 2022; Xu et al., 2018; Xue et al., 2017; Zhang et al., 2020; Zhao et al., 2015, 2016, 2019). For example, the Cf-*3* and Cf-*11* immune receptor genes, which correspond to the yet-unidentified *CfAvr3* and *CfAvr11 Avr* genes of *F. fulva*, have recently been mapped to a narrow region on chromosome 11 and have been shown to be closely linked (Iakovidis et al., 2020). Interestingly, the target region seems to be devoid of genes encoding RLPs, which suggests that Cf-*3* and Cf-*11* mediate resistance using a different class of immune receptors.

Little is known about the distribution and presence of Cf immune receptor genes across wild populations of tomato species. Previously, it was shown that homologues of Cf-*4* and Cf-*9* (*Hcr9*s) are found across the *Solanum* genus and probably pre-date speciation (Kruijt et al., 2005). More recently, *Solanum chilense* accessions originating from the southern part of Peru to the northern part of Chile were screened for their ability to recognize *F. fulva* effectors by infiltrating AWF collected from infected plants (Kahlon et al., 2020). This showed that recognition is mostly lost in southern accessions but retained in northern accessions. Interestingly, whilst Cf-*4* and Cf-*9* were previously thought to be mutually exclusive alleles (Parniske et al., 1997; Thomas et al., 1997), some *S. chilense* accessions possessed loci associated with both Cf-*4* and Cf-*9* and consequently recognized both *Cf*Avr4 and *Cf*Avr9 (Kahlon et al., 2020). Such

accessions could prove valuable for introgression into commercial varieties, as the simultaneous presence of both Cf-*4* and Cf-*9* was previously not thought possible. Finally, in several accessions that do not respond to *Cf*Avr9, there is still expression of Cf-*9*. This lack of recognition might be explained by inactive polymorphic versions of Cf co-receptors such as BAK1 and SOBIR1. The impact of these inactive versions on *Cf*Avr9 recognition, however, remains to be determined (Kahlon et al., 2020).

More recently, a study on the distribution of the Cf-*Ecp5* immune receptor gene (corresponding to the *Cf*Ecp5 effector of *F. fulva*) among accessions from various *Solanum* species revealed at least three distinct loci on chromosomes 1, 7, and 12 in four different accessions that harbour functional Cf-*Ecp5* alleles (Iakovidis et al., 2020). This includes the previously identified Milky Way cluster on chromosome 1 (Haanstra et al., 2000). Unexpectedly, a seemingly unlinked genetic suppression of *Cf*Ecp5 recognition was observed in some biparental crosses with accessions carrying distinct Cf-*Ecp5* loci (Iakovidis et al., 2020). This suppression could possibly also be caused by mutations in unlinked genes coding for co-receptors of Cf-Ecp5 or by other pleiotropic effects.

5.3 | Immune signalling in response to *F. fulva* effector recognition

In resistant tomato, effector perception triggers an immune response that is typically associated with an HR. In contrast to RLKs, RLPs lack a cytoplasmic kinase domain that can act as a signalling moiety. Therefore, to enable signalling, Cf immune receptors (and many other RLPs) constitutively interact with the regulatory RLK SOBIR1 (Liebrand et al., 2013), which provides the RLP with a cytoplasmic kinase domain. In this way, a bipartite RLK is generated that, on effector perception by the apoplastic LRRs of the RLP, can pass on the immune signal to the cytoplasm (Liebrand et al., 2014).

Notably, as is the case for LRR-RLKs involved in immunity, the bipartite Cf/SOBIR1 complex specifically recruits the LRR-RLK BAK1 on its activation by an Avr effector matching the Cf immune receptor (Postma et al., 2016). When Cf-4 is challenged with *Cf*Avr4, the immune response triggered on formation of the Cf-4/SOBIR1/BAK1 heterotrimer is associated with rapid endocytosis of the complex (Postma et al., 2016) and *trans*-phosphorylation events between BAK1 and SOBIR1 (van der Burgh et al., 2019). This observation, along with the requirement of SOBIR1 kinase activity for Cf-4-mediated HR (Liebrand et al., 2013), suggests that, on perception of *Cf*Avr4, SOBIR1 also facilitates the first steps in downstream signalling. The ultimate proof that SOBIR1 is essential for functionality of Cf-4, and probably all LRR-RLPs in general that are involved in plant immunity, was obtained by knocking-out the *SOBIR1* gene in stable Cf-*4*-expressing *N. benthamiana* plants, using CRISPR/Cas technology (Huang et al., 2021). A swift ROS burst is a typical reflection of the Cf-4-triggered immune output and, strikingly, this response was completely absent in Cf-*4*-expressing *N. benthamiana* plants lacking a functional SOBIR1 protein. Furthermore, the HR was absent in these *sobir1* knockout plants on challenge with *Cf*Avr4 (Huang et al., 2021).

Information on signalling candidates that function directly downstream of the active Cf-4/SOBIR1/BAK1 complex is scarce. However, Cf-9 requires ACIK1, a class VII tomato receptor-like cytoplasmic kinase (RLCK), for mediating full resistance against *Cf*Avr9-secreting isolates of *F. fulva* (Rowland et al., 2005). Current studies are focusing on whether class VII RLCKs also play a role in propagating the immune response downstream of Cf-4.

Earlier work revealed that the "helper NLR" NRC1 is involved in mounting the Cf-mediated immune response in tomato, as this protein is required for full resistance of Cf-4 tomato to *Cf*Avr4-secreting isolates of *F. fulva* (Gabriëls et al., 2007). The mechanism by which NRC1 contributes to immunity initiated by Cf-4 remained enigmatic until recently when a close homologue of NRC1 in *N. benthamiana*, NRC3, was shown to actually mediate the HR. NRC3 contains an N-terminal coiled-coil (CC) domain, including a so-called "MADA" motif, which is essential for assembly of a resistosome that has the capacity to trigger cell death (Kourelis et al., 2022).

5.4 | Circumvention of Cf-mediated resistance in tomato by *F. fulva*

Since 2012, a number of *F. fulva* races have been collected from around the world, including Argentina, China, Croatia, Cuba, Korea, Turkey, and the United States, that have overcome resistance provided by one or more of the Cf-*2*, Cf-*4*, Cf-*4E*, Cf-*5*, or Cf-*9* immune receptor genes in commercial tomato varieties (Altin, 2016; Bernal-Cabrera et al., 2021; Iida et al., 2015; Kubota et al., 2015; Lee et al., 2013; Li et al., 2015; Lucentini et al., 2021; Medina et al., 2015; Mesarich et al., 2014; Novak et al., 2021; Rollan et al., 2013; Sudermann et al., 2022; Yoshida et al., 2021). Analysis of the allelic variation present in the matching *Avr* genes has revealed many new mutations, in addition to mutations that had previously been characterized (Stergiopoulos, De Kock, et al., 2007), that enable these effectors to escape recognition by their cognate immune receptors (Table 5). Such information will enable the presence and migration of *F. fulva* races to be tracked, which may ultimately play an important role in cultivar selection and deployment.

Recently, the classification system for defining the resistance spectrum of tomato varieties against *F. fulva* has been updated by the International Seed Federation (ISF), and the differential set of tomato lines harbouring the Cf-*2*, Cf-*4*, Cf-*5*, or Cf-*9* gene or combinations thereof has been extended with a line harbouring the Cf-*6* immune receptor gene, corresponding to the yet unidentified *CfAvr6 Avr* gene of *F. fulva* (Grushetskaya et al., 2007). This inclusion suggests that breeding companies have (recently) started to deploy Cf-*6* in newly developed tomato varieties. More interestingly, a new race of *F. fulva* originating from South Korea has now been included in the differential set of isolates used for classifying the resistance

TABLE 5 Recently identified allelic variation that alters the sequence of avirulence (Avr) proteins from *Fulvia fulva*.

Avr gene (accession number[a])	Polymorphism at DNA level	Polymorphism at protein level[b]	Loss of Cf-mediated HR	Country	Reference
CfAvr2 (AJ421628)	Gene deletion	No protein	Yes	Argentina, USA	Medina et al. (2015); Sudermann et al. (2022)
	c.1A>G	LRG_199p1:p.Met1?	Yes	Japan	Iida et al. (2015)
	c.2 T>C	**LRG_199p1:p.Met1?**	Yes	USA	Sudermann et al. (2022)
	c.28T>G	p.Trp10Gly	No	Argentina	Lucentini et al. (2021)
	c.29G>T	p.Trp10Leu	No	Argentina	Lucentini et al. (2021)
	c.46del.G	p.Glu16Lysfs*2	Yes	USA	Sudermann et al. (2022)
	c.50ins.T	p.Ile18Asnfs*25	Yes	Japan	Iida et al. (2015)
	c.52A>C	p.Ile18Leu	No	Japan	Iida et al. (2015)
	c.56del.CAGCAGCCAA	p.Ala19Glufs*13	Yes	Japan	Iida et al. (2015)
	c.(64–69)del.A	**p.Lys23Asnfs*12**	Yes	USA	Sudermann et al. (2022)
	c.(64–69)ins.A	**p.Leu24Thrfs*19**	Yes	Japan	Iida et al. (2015)
	c.(117_119)ins.A	**p.Tyr41Valfs*2**	Yes	Croatia	Novak et al. (2021)
	c.121_122del.TA	p.Tyr41Ilefs*1	Yes	USA	Sudermann et al. (2022)
	c.209ins.A	p.His70Glnfs*?	Yes	USA	Sudermann et al. (2022)
	c.188G>T	p.Cys63Phe	Yes	Japan	Iida et al. (2015)
	Transposon insertion	No protein	Yes	Japan	Iida et al. (2015); Yoshida et al. (2021)
	Large del. after c.37	del. after p.Leu12	Yes	USA	Sudermann et al. (2022)
CfAvr4 (Y08356)	c.118T>C	p.Cys40Arg	Yes	Japan	Iida et al. (2015)
	c.191G>T	p.Cys64Phe	Yes	Japan	Iida et al. (2015)
	c.191G>C	p.Cys64Ser	Yes	Japan	Iida et al. (2015); Yoshida et al. (2021)
	c.191G>A	**p.Cys64Tyr**	Yes	Japan	Iida et al. (2015)
	c.318del.G	p.Ser107Valfs*5	Yes	Japan	Iida et al. (2015)
CfAvr4E (AY546102)	**Gene deletion**	**No protein**	Yes	Japan	Yoshida et al. (2021)
	c.244T>C	**p.Phe82Leu**	Yes	Japan, Argentina, USA	Iida et al. (2015); Lucentini et al. (2021); Sudermann et al. (2022)
	c.278T>C	**p.Met93Thr**	Yes	Japan, Argentina, USA	Iida et al. (2015); Lucentini et al. (2021); Sudermann et al. (2022)
CfAvr5 (KJ452245)	Gene deletion	No protein	Yes	Japan	Iida et al. (2015)
	c.11ins.TC	p.Ile5Leufs*3	Yes	France	Mesarich et al. (2014)
	c.268G>C	p.Gly90Arg	Yes	Japan	Iida et al. (2015)
CfAvr9 (X60284)	**Gene deletion**	**No protein**	Yes	Japan, Cuba	Bernal-Cabrera et al. (2021); Yoshida et al. (2021)
	c.23T>C	**p.Val8Ala**	No	Japan, USA	Sudermann et al. (2022)

Abbreviations: c, numbering based on cDNA sequence; del, deletion; fs, frameshift; HR, hypersensitive response; ins, insertion; p, numbering based on protein sequence; *, termination codon.

[a]National Center for Biotechnology (NCBI) GenBank accession number.

[b]Mutations in bold were previously described by Stergiopoulos, De Kock, et al. (2007). The description of each mutation is based on the nomenclature set forth by the human genome variation society (HGVS) (http://varnomen.hgvs.org/).

spectrum of tomato varieties, indicating that Cf-*6* has already been overcome in the field (Sangster, 2022).

6 | PROGRESS IN UNDERSTANDING *D. SEPTOSPORUM*, DOTHISTROMA NEEDLE BLIGHT, AND PINE DEFENCE

6.1 | Defence responses in pine

Although host defences in the *D. septosporum*–pine pathosystem have not been studied to the same level of molecular detail as for those for *F. fulva* and tomato, some recent progress has been made. Such studies were prompted by outbreaks of Dothistroma needle blight (DNB), such as those that caused defoliation and mortality on lodgepole pine trees (*Pinus contorta*) in north-western British Columbia in Canada (Welsh et al., 2009; Woods, 2003). In the first study of its kind to investigate the molecular resistance mechanism(s) of pine to *D. septosporum*, Lu et al. (2021) performed controlled experiments by inoculating tolerant (partially resistant) and susceptible pines with two *D. septosporum* isolates from different genetic lineages (Capron et al., 2021). Subsequent analysis of differentially expressed genes (DEGs) showed a rapid and strong transcriptomic response in the tolerant pines, and a late and weak response in the susceptible pines (Lu et al., 2021). DEGs identified from tolerant pines included those involved in extracellular and intracellular immunity, such as genes encoding calcium-dependent protein kinases, BAK1, WRKY transcription factors, and EDS1. Based on this study, the authors propose that lodgepole pine recognizes *D. septosporum* attack through immune receptors, which then trigger a series of downstream signalling responses, including a Ca^{2+} burst and the activation of a mitogen-activated protein kinase (MAPK) cascade, that lead to cell wall reinforcement, stomatal closure, and an HR. Nine candidate immune receptor genes were also identified that contained sites under positive selection. These sites are located close to regions in the genes that encode conserved domains like the NB-ARC domain, LRRs, or the coiled-coil (CC) domain. Comparison of expression profiles between inoculated and control samples showed that these candidate immune receptor genes were differentially regulated, suggesting differential responses to the pathogen (Lu et al., 2021). Further experiments are needed to identify corresponding effectors and to confirm the functions of these candidate immune receptor genes.

6.2 | Growth of a *Dothistroma* research community

Research on DNB has not only been accelerated by the availability of the *D. septosporum* genome sequence, but also by the continual improvement of molecular tools for diagnostics and population genetics (Barnes et al., 2008; Groenewald et al., 2007; Ioos et al., 2010). In response to the increased spread and severity of DNB in Europe (Drenkhan et al., 2016), an international consortium of researchers from 41 countries was developed through a European COST (Cooperation in Science and Technology) Action (2011–2015) that included training in the use of molecular tools and culminated in publication of a special issue of *Forest Pathology* on DNB (Bradshaw, 2016). The special issue contained 14 articles, including reviews on the host range and distribution of *D. septosporum* and its lesser known sister species *D. pini*, the role of climatic factors, disease management, and Pinaceae resistance mechanisms (Barnes et al., 2016; Bulman et al., 2016; Drenkhan et al., 2016; Fraser et al., 2016; Woods et al., 2016;). Many of these collaborations continue with a particular emphasis on genomic and population genetic studies.

6.3 | Genomics of other *D. septosporum* isolates

Next to the genome sequence of *D. septosporum* isolate NZE10, several additional genome sequences have been obtained from various isolates of this pathogen since 2012. Among these are isolates from the New Zealand population of *D. septosporum*, which is almost clonal, with very low diversity (Hirst et al., 1999), and is only of one mating type (Barnes et al., 2014). The availability of isolates collected in the 1960s, soon after the arrival of *D. septosporum* in New Zealand, provided a unique opportunity to determine how the pathogen co-evolved with its host over 50 years in pine plantation forests (Bradshaw, Ormond, et al., 2019). In general, genome sequences of isolates collected over the 50-year period showed low diversity, but, as expected, some diversification over time as well. However, isolates collected in the 1960s were more virulent on *P. radiata* and more potent producers of dothistromin, as compared to those collected from the 1990s onwards. This suggested adaptation towards decreased virulence over time, although no specific changes in the genome sequences could be found that correlated with this phenomenon (Bradshaw, Ormond, et al., 2019).

Genome sequences were also obtained for a global collection of 18 *D. septosporum* isolates from Europe, Africa, Australasia, and the American continent (Bradshaw, Sim, et al., 2019). Several of these isolates appeared to be aneuploid, exhibiting whole or large segmental duplications of particular chromosomes. Included in these isolates was an isolate from the German Alps, which produced significantly higher levels of dothistromin as compared to other isolates, a phenotype that was attributed to the increased copy number of many genes in chromosome duplication regions unique to this isolate (Bradshaw, Sim, et al., 2019).

Analysis of genome sequences from isolates collected from Scotland and western Canada, two regions that experienced severe epidemics of DNB (Mullett et al., 2017; Woods et al., 2005), showed the presence of at least three distinct races of *D. septosporum* in Scotland. One of the races (NPR, native pine race) was mainly associated with native Scots pine (*Pinus sylvestris*) and two others (LPR, lodgepole pine race and SR, southern race) were associated with the introduced pine species lodgepole and Corsican pine, respectively,

reflecting their likely origins (Mullett et al., 2017; Piotrowska et al., 2018). Whole-genome sequencing of 25 isolates revealed low diversity of the indigenous NPR but higher diversity and evidence of sexual reproduction within the introduced SR. The discovery of an NPR-SR hybrid, and the potential for evolution of new variants of the pathogen through intraspecies hybridization, highlighted the increased risk to native pines and the biosecurity implications of introducing more susceptible exotic pine species (Ennos et al., 2020).

In Canada, genotyping-by-sequencing of 119 *D. septosporum* isolates from both plantation and native forests also revealed genetically distinct lineages (Capron et al., 2021). The lineage associated with the Kispiox region of British Columbia, where a severe DNB epidemic occurred in the past (Woods et al., 2005), showed high genetic diversity as well as evidence for sexual reproduction, which had been reported previously in this region (Dale et al., 2011). Two lineages in other regions showed signatures of human-mediated movement and admixture, probably associated with pine breeding (Capron et al., 2021). The authors urged caution in the application of assisted gene flow policies in which pine genotypes used for reforestation are matched with predicted future climate changes. The movement of pines between regions could facilitate hybridization or recombination between genetically distinct pathogen lineages, leading to new variants and increased risk to forest health (Capron et al., 2021).

6.4 | Genetic diversity and origins of *D. septosporum*

The exact origin of *D. septosporum* remains elusive. Population genetic studies using the same set of microsatellite markers across many different studies of individual countries (e.g., Boroń et al., 2021; Jánošíková et al., 2021; Oskay et al., 2020) and intercontinental comparisons have, however, enhanced our understanding of where the pathogen is well established and/or naturalized, and where it has clearly been introduced (Adamson et al., 2018; Barnes et al., 2014; Drenkhan et al., 2013; Mullett et al., 2017). Specific pathways of migration, natural range expansions, and anthropogenic introductions are now becoming more evident.

To consolidate individual population studies, for a more global perspective, Mullett et al. (2021) studied 3800 isolates, including new isolates and those used in nine previous publications, representing the most comprehensive population study on *D. septosporum* (Mullett et al., 2021). Using an evolutionary approach, they suggested a Eurasian origin of the pathogen, with historical migration patterns moving in a westerly direction and emerging from three main clusters. The oldest cluster is represented by the East European cluster that has diverged to produce three smaller subclusters centred in Turkey, central Europe, and north-eastern Europe. The North American cluster, derived from an ancestral population in Eurasia, is exclusively found in the United States and Canada, and is the population causing disease on *P. contorta* in British Columbia (Woods et al., 2005). More detailed studies using single-nucleotide polymorphism data showed finer level population divergence (Capron et al., 2021), with a single reintroductory event in Scotland (Ennos et al., 2020). The youngest, western European cluster, which dominates most of the UK and western France, is also the one that has been introduced most dominantly throughout most of the Southern Hemisphere (Mullett et al., 2021).

The populations of *D. septosporum* in the Southern Hemisphere are mostly characterized by clonal lineages with a single mating type, MAT 1–2 (Barnes et al., 2014). This is especially true for populations in South America (Chile and Ecuador) and Australasia (New Zealand and Australia), which have had separate introductory events, most likely with *P. radiata* (Barnes et al., 2014), and coinciding with the expansion of pine plantations. Multiple introductions from other clusters (central and north-eastern Europe) have occurred in Africa (South Africa and Kenya), resulting in populations with both mating types and high diversity, reflecting the long history of pine introduction into these countries (Barnes et al., 2014; Mullett et al., 2021) and highlighting strong anthropogenic influences on global patterns of forest disease (Wingfield et al., 2015).

7 | CONCLUSIONS AND FUTURE DIRECTIONS

Although the last 10 years have seen significant advances in our understanding of how *F. fulva* and *D. septosporum* interact (and have evolved) with their host plants, there is still much to learn. For instance, through continued genome sequencing of isolates collected from around the world, and in conjunction with gene editing techniques involving CRISPR-Cas technology, it may be possible to identify a repertoire of core effector genes from *F. fulva* and *D. septosporum* that is essential for host colonization. Crucially, as a starting point for this analysis, CRISPR-Cas technology has already been applied to *D. septosporum* (McCarthy et al., 2022). Such technology may also provide insights into the role(s) of the dispensable chromosome in *F. fulva*.

Further genome sequencing will also assist in the identification of *Avr* genes from *F. fulva*, particularly those deleted or mutated in resistance-breaking strains of the fungus. This is important, as it will enable rapid identification of resistance-breaking strains in commercial greenhouse and high tunnel environments; information that can be used to inform the deployment of resistant tomato varieties. Similarly, the identification of *Avr* genes will increase the speed with which breeding and selection programmes involving cognate Cf immune receptor genes can be carried out, as they can be used as markers. Regarding whether stacked Cf immune receptor genes can provide durable resistance to *F. fulva* in tomato, a key question for the future will be whether this fungus undergoes sexual reproduction, as has been suggested by Stergiopoulos, Groenewald, et al. (2007). If sexual recombination does indeed occur, strains able to overcome stacked resistance could arise more rapidly. Finally, the identification of *Avr* genes from *F. fulva* will also assist in the mapping and cloning of corresponding Cf immune receptor genes, which

will provide new opportunities to identify host virulence targets, and to assess differences in effector recognition, downstream signalling, and immune response outputs. In any case, elucidation of the complete Cf/Avr-triggered signalling pathway leading to leaf mould resistance in tomato will be a major future focus.

Unlike in angiosperms, studies of the molecular basis of disease resistance in gymnosperm trees is in its infancy. Whilst the work described here with the *D. septosporum*–pine pathosystem and some other studies (e.g., Liu et al., 2021) suggest similarities with fungal–angiosperm pathosystems in terms of effector recognition and defence pathways, there is a lot more to discover. However, there is no doubt that the wealth of information learned from the study of how *F. fulva* interacts and evolves with its tomato host will continue to help decipher the molecular mechanisms deployed by *D. septosporum*. In turn, this will help to answer fundamental questions about the extent to which pathogen resistance mechanisms of gymnosperms function and adapt in similar ways to those of angiosperms. Future work will, however, need to rise to the challenges associated with the long lifecycles of most gymnosperm trees, their immense genomes, and the confounding influences of other factors with strong impacts on disease resistance, such as climatic conditions and complex microbiomes, including other potential pathogen species associated with forest trees (Ahmar et al., 2021; Terhonen et al., 2019; Woods et al., 2016; Zimin et al., 2014).

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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