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Effector proteins required for virulence of *Neonectria ditissima*, a fungal pathogen of apple.

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Introduction

European canker, caused by the necrotrophic fungal pathogen *Neonectria ditissima*, is one of the most damaging apple diseases in New Zealand and north-western European countries[1,2,3]. We hypothesise that during plant colonization, *N. ditissima* secretes effectors that can be recognised by plant susceptibility proteins to activate a hypersensitive response (HR). The HR-generated dead tissue serves as an ideal nutrition source for this necrotrophic pathogen and allows for extensive plant colonization. We aim to identify and functionally characterise *N. ditissima* effector proteins by following three approaches: RNA sequencing during a time course of infection, candidate effector tertiary structure prediction and pathogenicity assessments post candidate gene disruption. Identification of effectors may ultimately lead to the understanding of the molecular virulence strategy of *N. ditissima* and formulation of novel control strategies.

Methods

- Plant infection assay: Fruit was inoculated with water control and a low- (RS305p) and high-virulence isolate (RS324p) of *N. ditissima*. At 2, 3, 4 and 5 weeks post inoculation, fruit were sampled to give four biological replicates. The low- and high-virulence isolates were also grown *in vitro* in quadruplicate.
- Total RNA from each sample was sequenced on the NovaSeq 6000 platform at AGRF (Melbourne, Australia). The most upregulated genes (*in planta* vs *in vitro*) identified in a differential gene expression (DGE) analysis were assessed for presence of classic effector-like features using the following prediction software packages: SignalP 5.0, SecretomeP 2.0, WoIfPSORT 1.0 and EffectorP-fungi 3.0.
- The selected candidate effectors were assessed with AlphaFold2 for tertiary structure prediction to gain insights into their putative function. Their structures were investigated for similarity to proteins with characterised structures using the DALI server.
- Candidate effector genes were disrupted in *N. ditissima* using CRISPR-Cas9 (CC9) gene editing. Pathogenicity of *N. ditissima* gene disruptants was assessed in apple fruit and twigs. Disease progression in fruit and twigs was measured in terms of lesion length at five and 12 different time points, respectively.

Results

RNA-seq: The percentage of reads mapping to the RS324p *N. ditissima* genome increased as the infection time course progressed. The biological replicates clustered robustly within time points, with a clear distinction of genes expressed between the low- and high-virulence isolates, and between their expression *in planta* and *in vitro* (Figure 1).

Overall, 401 RS324p genes were found differentially expressed *in planta* compared with *in vitro* (Figure 2). The genes most upregulated during fruit infection were compared with the top-30 most upregulated genes during *N. ditissima* infection of apple twig tissue[4]. Nine of the top-30 genes appear to be equally required for both apple fruit and twig infection (Figure 3A), 15 required exclusively for fruit infection and six exclusively for twig infection (Figure 3B). This suggests that *N. ditissima* gene expression can vary according to the host tissue.

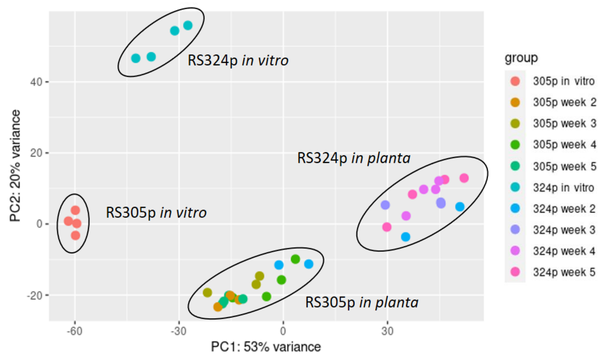


Figure 1. Principal component analysis (PCA) of RNA-seq data from *Neonectria ditissima* during infection of apple fruit. Four biological replicates were used for each *in planta* time point and *in vitro* growth condition.

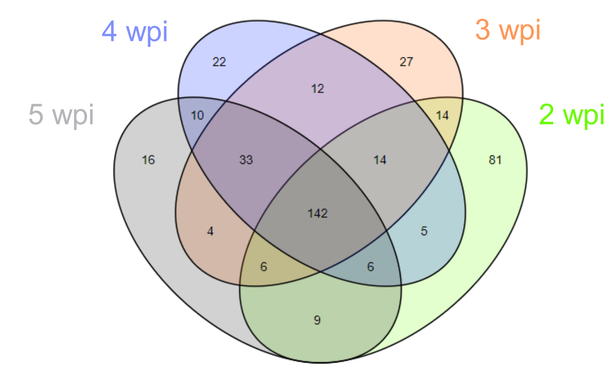


Figure 2. Venn diagram showing number of *N. ditissima* RS324p differentially expressed genes (DEGs) per comparison across *in planta* time points vs *in vitro*. wpi: weeks post inoculation.

Table 1. Candidate effector prediction software output summary.

	Secretion prediction software			
	SignalP 5.0	SecretomeP 2.0	WoLFPSORT 2.0	EffectorP-fungi 3.0
S4. g4542	Yes – 0.9901	Yes – 0.711	Yes (26)	Yes (cytoplasmic) – 0.791
S28.g5809	Yes – 0.9626	Yes – 0.785	Yes (27)	Yes (apoplastic) – 0.826
S34.g7123	Yes – 0.9887	Yes – 0.921	Yes (25)	Yes (cytoplasmic) – 0.523 Yes (apoplastic) – 0.755

Effector prediction using bioinformatics software: According to the effector prediction software, *g4542*, *g5809* and *g7123* are likely to encode effector proteins with the presence of a signal peptide (0.9626 – 0.9901), being secreted (0.711 – 0.921) and an overall effector likelihood (0.755 – 0.826, Table 1). AlphaFold2 protein prediction software and the DALI server predicted functions:

- g4542: a transporter for efflux of fungitoxic compounds released by the plant (pLDDT 74.53, Z-score 3.5, Figure 4A).
- g5809: a metalloprotease that binds to plant chitinases to stop production of chitin oligos and therefore, chitin-triggered plant immunity (pLDDT 99.21, Z-score 7.9, Figure 4B).
- g7123: an outer membrane protein for ferric ion acquisition usually required during plant colonization (pLDDT 72.66, Z-score 4.0, Figure 4C).

Virulence assessment post candidate effector gene disruption: Disrupting candidate effector genes resulted in reduction in virulence in both apple fruit and twig tissue (Figure 5). The gene disruptants were not compromised in their growth *in vitro* (data not shown). In fruit, the gene disruptants were less virulent than the wild-type high-virulence RS324p isolate causing similar symptoms to the low-virulence RS305p isolate (Figure 5A). However, in fruit, *g7123* disruptants caused smaller lesions than wild-type RS324p but the browning phenotype in the skin and flesh of the fruit was retained (Figure 5A). This result aligns with *g7123* RNA-seq data; a requirement for infection in apple twig tissue, rather than fruit. Furthermore, in twigs, the gene disruptants were less virulent than the wild-type high-virulence RS324p isolate but more virulent than the low-virulence RS305p isolate (Figure 5B). In twigs, sporodochia at the wound entry point was paired with mycelium growth outside the wound. This phenotype differed from the cankers and lesions in RS324p-infected tissue and the absence of fungal growth and symptoms in RS305p-inoculated tissue (Figure 5B).

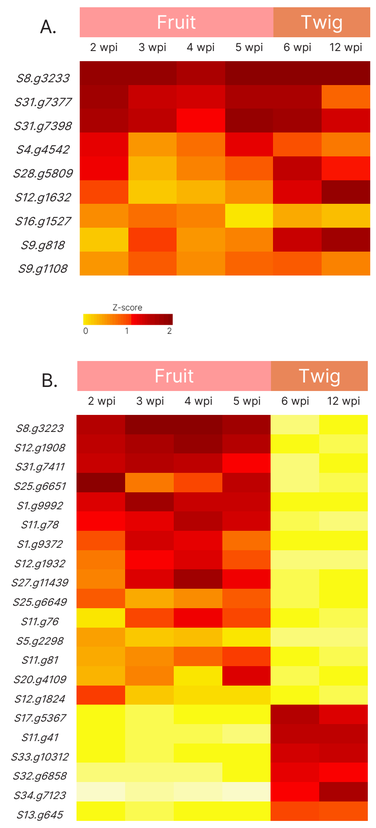
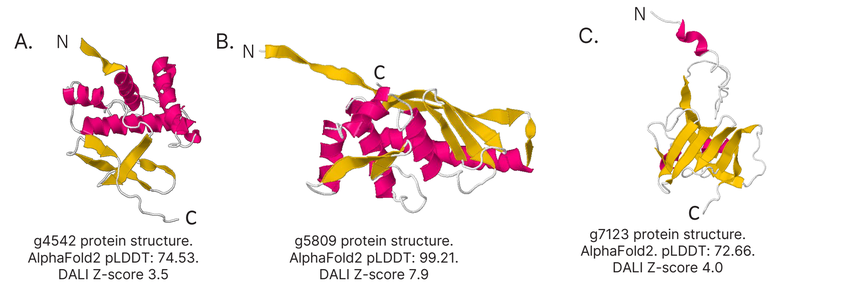


Figure 3. Heat-map of top 30 upregulated *N. ditissima* genes during plant infection compared to *in vitro* growth. A. Upregulated genes during both fruit and twig infection. B. Upregulated genes during either fruit or twig infection.



g4542 protein structure. AlphaFold2 pLDDT: 74.53. DALI Z-score 3.5

g5809 protein structure. AlphaFold2 pLDDT: 99.21. DALI Z-score 7.9

g7123 protein structure. AlphaFold2. pLDDT: 72.66. DALI Z-score 4.0

Figure 4. Predicted tertiary structures of effector candidates from *Neonectria ditissima*. a) g4542, b) g5809 and c) g7123 structures predicted by AlphaFold2. N: amino (N) terminus; C: carboxyl (C) terminus. pLDDT: predicted Local Distance Difference Test score (0–100). A pLDDT score of 70–100 is indicative of medium to high confidence. A Dali Z-score above 2 indicates 'significant similarities' between proteins.

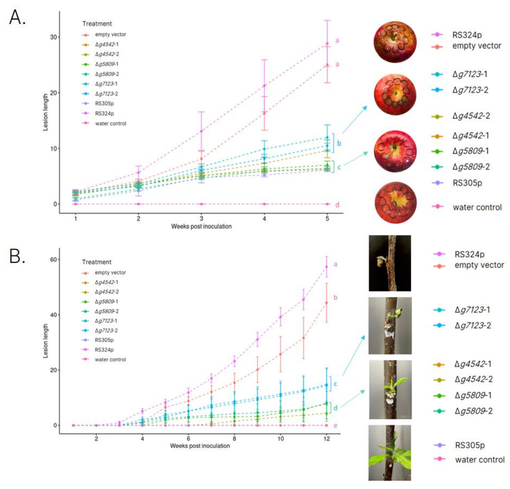


Figure 5. Candidate effector disruptant phenotype *in planta*. Δ*g4542*, Δ*g5809* and Δ*g7123* time course of infection in apple fruit (5A) and apple twigs (5B). Controls: water control, RS305p low virulence isolate, RS324p high virulence isolate, RS324p empty vector. Error bars represent standard error of the mean (SEM) among biological replicates. Letters represent significant differences ($p < 0.01$).

Conclusion

Our study identifies novel effectors in the necrotrophic pathogen *N. ditissima* and reveals their predicted function and potential interplay of their virulence role in different host tissues.

Acknowledgements

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