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CRISPR-Cas9 gene editing system to characterise virulence genes on *Neonectria ditissima*, a necrotrophic fungal pathogen of apple.

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}. A better understanding of the genetic mechanisms underlying the virulence of *N. ditissima* is required to develop durable control strategies against this disease. Initial research, comparing the genome sequences of a high and low virulence isolate of *N. ditissima* pointed towards a cluster of genes possibly involved in pathogenicity⁴. In addition, a transcriptome analysis of apple infected with *N. ditissima* helped to identify fungal candidate effectors (proteins that enhance virulence often by the suppression of host defences) recorded during the infection process⁵. However, while several *N. ditissima* candidate virulence genes have been selected, none have been functionally characterized to date. Gene deletion or mutation techniques such as homologous recombination (HR) and Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 (CRISPR-Cas9 or CC9) systems are commonly used to assess gene function. We assessed both techniques for their future use in characterising virulence genes on *N. ditissima*.

Methods

- A polyethylene glycol (PEG)-mediated protoplast transformation (PMT) protocol was developed and optimised for transformation of *N. ditissima*. Protoplasts were made using 10mg/ml disulphine and KC buffer (0.6 M KCl and 50 mM CaCl₂) and a PEG/20µg plasmid DNA mixture was used for the transformation.
- A homologous recombination knockout plasmid carrying a hygromycin B resistance marker with ~600–800bp flanks of the gene of interest was used to generate knockouts (KO). The genes of interest targeted were a putative protein kinase gene (*g8150*) and three candidate effector genes (*g4542*, *g5809* and *g7123*). End-point PCRs using transformants gDNA were carried out to confirm KO events; presence of Hyg resistance gene and absence of gene of interest.
- Cas9-HyGAMAccB-sgRNA plasmids, carrying a hygromycin B resistance marker, the Cas9 enzyme and guide RNAs were used to gene edit the candidate virulence genes. A high-resolution melting (HRM) curve analysis was performed following a qPCR amplification programme to confirm CRISPR-Cas9 mutations in the candidate transformants. Sequencing was required to confirmed HRM results and determine CC9 gene-edited mutants.
- The virulence of *N. ditissima* gene KO mutants was assessed in apple fruit and twigs. (Figure 1) Disease progression was measured in terms of disease incidence, lesion length and symptomatology score (for twigs).

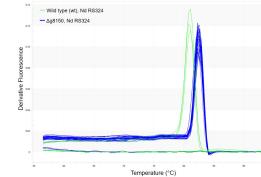


Figure 1: Pathogenicity assay set-up in fruit and twigs.

HR and CRISPR-Cas9 transformation efficiency comparison. HR and CC9 methodologies generated similar percentage of candidate transformants carrying the selectable marker, with HR (14.2%) generating slightly less candidates than CC9 (20.4%, Table 1). Within those percentages, only a single knockout was generated among the HR candidates, rendering this methodology extremely inefficient. In comparison, CC9 exhibited a high transformation efficiency where most (94%) of the candidates carrying the selectable marker also had disruptions in the gene of interest (Table 1).

Table 1: Transformation and mutation efficiencies: Homologous recombination vs. CRISPR-Cas9.

	Homologous recombination	CRISPR-Cas9
Number of colonies (NC)	1085	1480
NC with Hygromycin resistance	155	302
NC with Hyg resistance and KO mutation	1	284



CRISPR-Cas9 gene-edited mutants. Sequencing confirmed individual *N. ditissima* mutants carrying gene disruptions of either the *g8150* protein kinase gene or one of three candidate effector genes. The HRM curve analysis can efficiently identify CRISPR-Cas9 gene-edited mutants (Figure 2) and discriminate between different types of mutations (data not shown).

Figure 2: Screening of CRISPR-Cas9 transformants of *Neonectria ditissima* using qPCR-HRM curve. Melting curves of *g8150* amplicons between *N. ditissima* wild type and transfectants. Melting curves were generated by Eco Software v5.0 – Illumina. The experiment is based on four technical replicates per sample.

Virulence of *Ag8150* *N. ditissima* mutants on apple fruit and twigs. The single knockout generated through HR (*Ag8150*) was used for pathogenicity assessment. *Ag8150* disease incidence remained similar to wild type (WT) but it produced smaller lesions in apple fruit (Figure 3). In contrast, reduced disease incidence and less severe symptoms on apple twigs were observed with *Ag8150* compared to WT and an ectopic transformant (ET). *In vitro* *Ag8150* growth in rich medium was unaffected, suggesting a role in virulence.

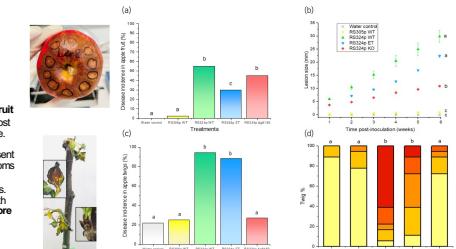


Figure 3: Disease expression and symptomatology of *Ag8150* in apple fruit and twigs. (a) Disease incidence and (b) lesion size in apple fruit 6 weeks post inoculation with *N. ditissima* isolates and water control (wild type). ET: ectopic-transformant. *Ag8150* *g8150* knockout. Error bars represent standard error of the mean (SEM) among biological replicates. Letters represent significant differences ($p<0.01$). (c) Disease incidence and (d) disease symptoms in apple twigs 8 weeks post inoculation with *N. ditissima* isolates and water control. Symptom scores were classified accordingly. Score 0 = no symptoms. Score 1 = mycelium with small spots or excreta. Score 2 = mycelium with around bud scar or leaf veins (curliness, chlorosis and spots). Score 3 = mycelium and sporodochia formation around the bud scar and leave symptoms. Score 4 = mycelium and sporodochia formation beyond bud scar (over 20mm), necrotic or dead leaves.

Conclusions

- g8150* (encoding a putative protein kinase) may have a role in *N. ditissima* virulence as loss of this gene renders the fungus less virulent on apple fruit and twigs compared to the wild type virulent isolate.
- CRISPR-Cas9 gene editing and HRM curve analysis was successfully applied for the first time to the filamentous fungal pathogen, *N. ditissima*, facilitating future studies to characterise gene functions associated with the life cycle and virulence of this fungus.

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