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

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Introduction

European canker, caused by the necrotroph pathogen *Neonectria ditissima*, is one of the most damaging apple diseases worldwide[1,2,3]. Its molecular virulence mechanisms are yet to be understood. Several candidate virulence genes (one putative protein kinase and three candidate effectors) were selected from a comparative genomics and transcriptomic analysis to be further analysed[4,5]. Homologous recombination (HR) and CRISPR-Cas9 (CC9) gene editing methodologies were used to silence these genes and assess their virulence role in apple fruit and twigs.

Methods

- Protoplast-mediated transformation (PMT) was used to transform *N. ditissima*.
- Both HR and CRISPR-Cas9 required KO vectors with the hygromycin B resistance marker and ~600-800bp flanks of the gene of interest, for HR, or the Cas9 enzyme and gene-specific guide RNA, for CC9.
- Confirmation of KOs was done through end-point PCR for the HR candidates and a high-resolution melting (HRM) curve analysis for the CC9 ones.
- The virulence of the KO mutants was assessed in apple fruit and twigs by measuring disease incidence, lesion length and symptomatology.

Results

1. CRISPR-Cas9 exhibited a higher transformation efficiency (94%) compared to HR (0.6%).

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

2. CRISPR_Cas9 gene-edited mutants confirmed through HRM (Figure 1).

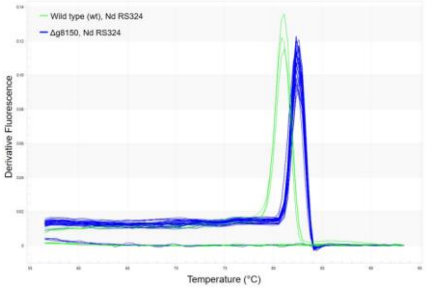

Figure 1. Screening of CRISPR-Cas9 transformants of *Neonectria ditissima* using qPCR-HRM analysis. Melting curves of g8150 amplicons between N. ditissima wild type and transformants. Melting curves were generated by Eco Software v5.0 – Illumina. The experiment is based on four technical replicates per sample.

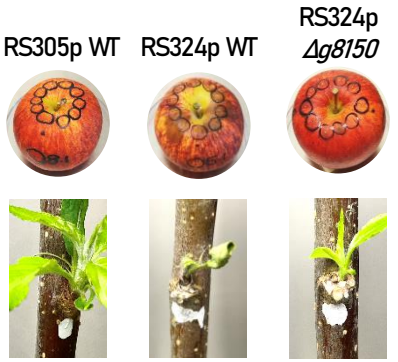

Figure 2. Reduce virulence when knocking out *g8150*. Apple fruit and twigs infected with low-virulence isolate (RS305p), high-virulence isolate (RS324p) and *Δg8150* RS324p mutant.

3. The single KO generated through HR (*Δg8150*) reduced disease incidence and symptomatology in apple twigs and produce smaller lesions in apple fruit compared to RS324p wild-type (WT, Figure 2 and 3).

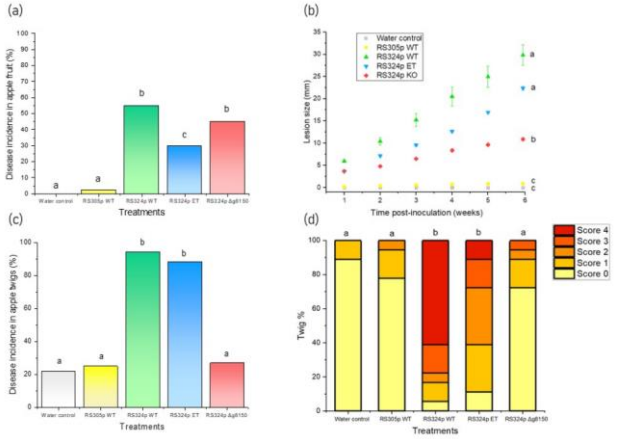

Figure 3. Disease expression and symptomtology of Δ*g8150* in apple fruit and twigs. (a) Disease incidence and (b) lesion size in apple fruit 6 weeks post inoculation with Neonectria ditissima isolates and water control. WT: wild type. ET: ectopic transformant. *Δg8150*: g8150 knockout. Error bars represent standard error of the mean (SEM) among biological replicates. Letters represent significant differences (p<0.01).

Conclusions

- *g8150* protein kinase has a potential role in *N. ditissima* virulence in apple.
- CRISPR-Cas9 and HRM were successfully applied for the first time in *N. ditissima* facilitating further studies to understand the life cycle and virulence of this fungus.

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