Final Report

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Title: Exploiting pathogenomics and resistance for the control of Fusarium wilt of lettuce

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CONTENTS

1. Industry Summary

Fusarium wilt of lettuce caused by the soilborne fungal pathogen *Fusarium oxysporum* f.sp. *lactucae* (FOL) results in severe losses in production areas globally. Four races exist, with race 1 (FOL1) being the most widespread, particularly in warmer parts of the world such as the USA and Italy. FOL was first reported in the UK in 2017 and identified as the recently emerged FOL race 4 (FOL4).

The main aims of this project were to investigate the genetics of FOL4 to identify and characterise virulence genes and study interactions between the pathogen and susceptible/resistant lettuce lines. Further work focuses on characterising new sources of FOL resistance in lettuce and screening a mapping population to better understand resistance trait segregation.

The specific project objectives were to:

1. Characterise FOL isolates from different European locations.
2. Identify putative FOL4 effector genes expressed during lettuce infection through RNAseq and confirm their roles in virulence.
3. Generate CRISPR Cas9 mediated knockouts of putative effectors in FOL4.
4. Confirm resistant and susceptible lettuce phenotypes of selected lettuce lines and examine segregation of a new lettuce mapping population.
5. Investigate the extent of root colonisation of resistant and susceptible lettuce lines by FOL4.

Objective 1 showed that all FOL1 and FOL4 isolates characterised have identical *translation elongation factor 1*α (*TEF*) gene sequences and therefore cannot be differentiated between each other by *TEF* sequencing. Moreover, the FOL1 and FOL4 *TEF* sequences differed to FOL2 and FOL3 *TEF* sequences indicating that FOL1 and FOL4 likely share a common origin whilst FOL2 and FOL3 likely have a different origin. Characterisation of isolates also included screening them for the 14 known Secreted In Xylem (*SIX*) pathogenicity genes. Results revealed that FOL1 contained *SIX9* and *SIX14* whilst FOL4 contained *SIX8*, *SIX9* and *SIX14*. This highlighted a clear difference in that all FOL4 isolates screened contained *SIX8* whilst this gene was absent in all FOL1 isolates screened. *SIX* gene sequence variability was then assessed across all FOL isolates. This revealed that there was sequence variation present within the *SIX8* gene across FOL4 isolates with the gene sequences separating into 2 clades. *SIX9* and *SIX14* sequences however showed no sequence variability across all the FOL isolates.

Objective 2 and 3 allowed for the identification of putative effector genes expressed during FOL4 infection on lettuce roots, as well as confirming the role of a putative effector in virulence. It was found that relative expression of *SIX8*, *SIX9* and *SIX14* increased between 0 and 120 hours post infection (hpi), although there was no statistically significant difference between 96 and 120 hpi for all *SIX* genes across all lettuce varieties, which could indicate a plateau effect after 96 hpi. To ensure genes expressed early during infection would not be missed, the 96h timepoint was selected for RNAseq analysis. This identified a range of highly expressed putative effector genes including *SIX8*, *SIX9* and *SIX14*. In addition, the role of *SIX8* in pathogenicity was examined using CRISPR mediated transformation, which resulted in a reduction in FOL4 pathogenicity when the gene was knocked out compared to wild type FOL4, indicating that *SIX8* likely has a role in pathogenicity of FOL4 on lettuce.

Examining resistance and susceptibility phenotypes of lettuce lines in Objective 4 revealed clear differences in the susceptibility of lettuce lines to FOL4. We identified both resistance and susceptible lines which could be used as parents of mapping populations for future genetic analyses and breeding for resistance. Following on from this, a single mapping population was screened, however further investigation to uncover resistance loci or genes is yet to be completed.

The final objective was to investigate the extent of root colonisation of FOL4 in resistant and susceptible lettuce lines. Results revealed that FOL4 was able to colonise the root tissue of both susceptible and resistant lettuce lines. Resistant lettuce lines showed no or few symptoms of vascular browning, while susceptible cultivars exhibited severe vascular browning. FOL4 was isolated from the bottom and middle locations of the taproots of all lettuce lines (including resistant lines), which indicates that growing resistant lettuce lines in the field may not reduce FOL4 soil inoculum levels overtime.

2. Introduction

2.1. Fusarium wilt of lettuce

This project focuses on Fusarium wilt of lettuce caused by *F. oxysporum* f. sp. *lactucae* (FOL) which was first identified in Japan in 1967 (Matuo & Motohashi, 1967) and has since been found in multiple lettuce producing countries worldwide. Four races (1, 2, 3 and 4) of FOL have been identified so far with race 1 being the most prominent globally, having been reported in the USA (Hubbard & Gerik, 1993), Europe (Garibaldi *et al.*, 2002), Iran (Millani *et al.*, 1999), Taiwan (Huang & Lo, 1998) and South America (Ventura & Costa, 2008, Malbrán *et al.*, 2014). FOL races 2 and 3 are currently confined to Asia (Fujinaga *et al.*, 2005, Lin *et al.*, 2014) while race 4 has only recently emerged and was first identified in the Netherlands in 2013 (Gilardi *et al.*, 2017a). FOL4 has since spread and has been reported in Belgium (Claerbout *et al.*, 2017) and the UK and Ireland (Taylor *et al.*, 2018). So far, in contrast to FOL1, FOL4 has mainly only affected protected lettuce crops. In mainland Europe and the USA, FOL is considered as one of the main limiting factors for commercial production of lettuce during the summer season (Taylor & Clarkson, 2018). Reports from France (FOL1 or FOL4) and the Netherlands (FOL4) have commonly observed 50% yield losses (Gilardi et al., 2017a, Gilardi *et al.*, 2017b) while in Italy up to 70% losses of field lettuce have been observed (AHDB, 2018).

So far, FOL4 has been restricted to protected lettuce in the UK and has not yet been identified as causing disease in the field. Control of FOL4 is challenging, as like all *F. oxysporum* f.spp., it produces chlamydospores which survive for long periods of time in the soil. This makes management using fungicides or biological control agents difficult. Currently there is limited varietal resistance widely available for the lettuce types typically grown under protection although breeding companies have begun the release of new resistant or partially resistant cultivars. Consequently, FOL4 represents a significant threat to the UK lettuce industry. Crop hygiene is therefore very important to prevent further local and regional spread.

2.2. Symptoms of Fusarium wilt of lettuce

Symptoms of Fusarium wilt of lettuce include stunting, wilting and leaf yellowing (often at leaf margins), but the key characteristic symptom of the disease is a brown, black, or red discolouration of the vascular tissue of the stem/taproot which can be observed upon longitudinal dissection of infected plants (Taylor & Clarkson, 2018; Figure 1). FOL travels through the xylem and blocks the vascular tissue, causing wilt symptoms, ultimately resulting

in plant death. One of the main modes of FOL transmission appears to be spread via infested soil on farming equipment, trays, pallets and footwear.

2.3. FOL resistant lettuce cultivars and distribution of FOL in the UK

FOL isolates in UK protected lettuce thus far have all been identified to be FOL4 (Taylor et al., 2018) with one exception of FOL1 reported in Northern Ireland in 2022 (unpublished). As previously mentioned, all outbreaks of FOL4 within the UK, Belgium and the Netherlands (from where it was first identified) have been confined to protected lettuce with none identified in outdoor production. However, there is particular concern that FOL4 may begin to affect field grown crops despite current measures in place to limit pathogen spread. Arguably the best option for control of *F. oxysporum* pathogens is the cultivation of resistant varieties (Okungbowa & Shittu, 2012). As part of the Defra-funded Vegetable Genetic Improvement Network (VeGIN) project a FOL resistance screening experiment was carried out where 54 accessions from the Warwick lettuce diversity set were screened against FOL1 and FOL4. This succeeded in identifying resistant lettuce lines that have been used as parents of mapping populations in a collaboration with Enza Zaden, with the aim of discerning the genetic nature of the resistance. Development of FOL4 resistant lettuce cultivars would be of great benefit to UK growers and consumers by reducing losses, decreasing the need for less environmentally and more costly interventions such as soil steaming / sterilisation and application of fungicides, therefore enabling year-round production.

Figure 1 Vascular browning in lettuce caused by FOL4

2.4. Aims and Objectives

The main aim of this project is to investigate the genetics of FOL4 to identify and characterise virulence genes and study interactions between the pathogen and susceptible/resistant lettuce lines. Further work will focus on characterising new sources of FOL resistance in lettuce and screen a mapping population to better understand resistance trait segregation.

1. Objective 1: Characterisation of FOL isolates from different European locations
2. Objective 2: Identify putative FOL4 effector genes expressed during lettuce infection through RNAseq and confirm their roles in virulence
3. Objective 3: CRISPR Cas9 mediated knockouts of FOL4 putative effectors
4. Objective 4: Confirm resistant and susceptible lettuce phenotypes of selected lettuce lines and examine segregation of a new lettuce mapping population
5. Objective 5: Investigate the extent of root colonisation of resistant and susceptible lettuce lines by FOL4

3. Materials and methods

3.1. Objective 1: Characterisation of FOL isolates from different European locations

3.1.1. Fungal isolate growth and maintenance

Forty-eight FOL1 and forty-one FOL4 isolates were obtained from different European locations (Table 1). Isolates were obtained as pure cultures from collaborators (Enza Zaden), in-kind donations (BASF, and G's) or isolated from diseased lettuce plants in previous work. All Fusarium isolates obtained were used to produce potato dextrose agar (PDA) slope cultures for storage at 4°C and spore suspensions in potato dextrose broth (PDB) + 20% glycerol for storage on ceramic beads at -80°C.

For production of freeze-dried material for DNA extractions isolates were sub-cultured from glycerol stocks onto fresh PDA plates by placing a ceramic bead containing frozen spore suspension using sterile tweezers onto fresh PDA plates. Plates were then stored at 25°C for approximately 2 weeks. Three 5 mm agar plugs were removed from the growing edge of each actively growing culture and used to inoculate 20 mL of 50% PDB in a Petri dish. Plates were incubated at 20°C for 5 days. PDB was then removed by centrifugation (3000 rpm for 15

minutes) and mycelium from each isolate was rinsed twice with sterile water (centrifugation at 3000 rpm for 15 min each time). The remaining mycelium was freeze-dried for 2 days.

For making sore suspensions cultures were initiated from glycerol stocks onto PDA and grown at 20°C for approximately 14 days. Spores were acquired by adding 20 mL of SDW onto PDA plates and using a sterile spreader to agitate spores into the solution. The solution was then filtered through 3 layers of Mira cloth to filter out mycelium but allow spores to pass through. The concentration of the resulting spore suspension was adjusted to 1 x 10^6 spores mL^{-1} with 0.012% Tween in SDW.

3.1.2. Molecular identification and characterisation of Fusarium isolates

All Fusarium isolates were identified and characterised through sequencing of *translation elongation factor 1*α (*TEF)* (part of the gene), *SIX8*, *SIX9* and *SIX14*; as well as by screening them against FOL1 (Pasquali *et al.*, 2007) and FOL4 (Andrew Taylor, unpublished) specific PCR assays. DNA was extracted from freeze-dried mycelium using the DNeasy plant mini kit (Qiagen, Hilden, Germany) or via a rapid DNA extraction protocol (Acme) (S. Rehner, personal communication). The DNeasy plant mini kit was used in accordance with manufacturer's protocol with a minor modification whereby the mycelium was homogenised in a lysing matrix A tube (MP Biomedicals, CA, USA) in a FastPrep-24™ machine set at 6 ms^{-1} for 40 s. For the Acme protocol, the mycelium was transferred into 2 mL tubes containing 6-10 glass beads and 0.5 g zerconia silica beads (0.1 mm, BioSpec Products) and ground three times in a FastPrep-24 ™ machine set at 5.5 ms^{-1} for 20 s. Acme DNA extraction reagent (300 µL of sodium metasilicate 2.1 g, citric acid 0.5 g, 2-butoxy ethanol 2.64 mL, 1M Tris-HCl pH 7.0 13.6 mL) was added, tubes heated for 10 minutes at 100°C, and centrifuged at 16,000 g for 5 minutes, rotated 180° and centrifuged again for 5 minutes. The supernatant (175 µL) was transferred to a clean tube and diluted 1:10 in TE buffer for use in PCR.

Identification of Fusarium isolates was carried out by PCR amplification of part of the *TEF* gene using published primers (Taylor et al., 2016) (Table 2). All PCR reactions were set up using REDTaq® ReadyMix® (Sigma-Aldrich) in 20 µL reaction volumes containing approximately 10 ng of DNA and a final concentration of 0.5 µM of each primer. Thermocycling conditions for *TEF* were: one cycle of 2 min at 94°C; 30 cycles of 45 s at 94°C, 30 s at 64°C and 1 min at 72°C, followed by one cycle of 5 min at 72°C. PCR amplicons were visualised using gel electrophoresis (1% agarose gel containing GelRed™ at 2 µL per 100 mL of gel), purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using both the

forward and reverse primer sequence to make a consensus sequence per isolate. Consensus sequences were then used to generate phylogenetic trees.

Characterisation of FOL1 and FOL4 isolates involved screening for the presence / absence of the 14 known *SIX* genes on a subset of 20 isolates by PCR amplification using primers listed in Table 2. The remaining isolates were then screened by PCR amplification of *SIX8*, *SIX9*, *SIX14*, as well as FOL1 (Pasquali et al., 2007) and FOL4 (Andrew Taylor, unpublished) specific PCR assays. All PCR reactions were set up as stated prior. Thermocycling conditions for *SIX8* were: one cycle of 2min at 94°C; 30 cycles of 45 s at 94°C, 30 s at 59°C and 1 min at 72°C, followed by one cycle of 5 min at 72°C. Thermocycling conditions for *SIX9* were: one cycle of 2min at 94°C; 30 cycles of 45 s at 94°C, 30 s at 59°C and 1 min at 72°C, followed by one cycle of 5 min at 72°C. Thermocycling conditions for *SIX14* were: one cycle of 2min at 94°C; 30 cycles of 45 s at 94°C, 30 s at 51°C and 1 min at 72°C, followed by one cycle of 5 min at 72°C. Thermocycling conditions for the FOL1 diagnostic consisted of a touchdown PCR: one cycle of 1min at 94°C; 10 cycles of 15 s at 94°C, 30 s at 66°C (the annealing temperature decreasing by 0.5°C each cycle); 25 cycles of 15 s at 94°C, 30 s at 61°C, followed by one cycle of 2 min at 72°C. Themocycling conditions for the FOL4 diagnostic were: one cycle of 2min at 94°C; 30 cycles of 45 s at 94°C, 30 s at 57°C and 1 min at 72°C, followed by one cycle of 5 min at 72°C. PCR amplicons were visualised, purified, sequenced, consensus sequences produced per isolate, and trees produced as prior.

TEF and *SIX* gene sequences from all identified FOL1 and FOL4 isolates from different European locations were then aligned and trimmed in using MEGA version 11 (Tamura *et al.*, 2021). Forty-eight FOL1 and forty-one FOL4 isolates as well as other isolate sequences downloaded from NCBI were used to construct maximum-likelihood trees. Sequences were aligned (ClustalW method; Thompson et al., 1994), concatenated using MEGA version 11 (Tamura et al., 2021) and a maximum likelihood trees constructed using the calculated best model, Kimura-2-parameter plus gamma (Kimura, 1980). Bootstrap consensus trees were inferred from 1000 replicates (Felsenstein, 1985).

3.2. Objective 2: Identify putative FOL4 effector genes expressed during lettuce infection through RNAseq and confirm their roles in virulence

3.2.1. Inoculation of lettuce seedlings in an in-vitro system

Lettuce seeds (cv. Temira) were surface sterilised by addition to FICHLOR solution (3.1g sodium dichloroisocyanurate dihydrate and 1 drop of Nonodet in 50mL deionised water) followed by gentle shaking at approximately 200 rpm for 4 minutes. Seeds were then rinsed in sterile deionised water (SDW) and 10 seeds placed across square Petri dishes (12 x 12 x 1.7 cm, Greiner Bio-One, UK) containing autoclaved ATS medium (1M KNO_3, 1M KPO_4, 1M $MgSO_4$, 1M $Ca(NO_3)_2$, 20 mM Fe-EDTA, 70 mM H_3BO_3, 14 mM $MnCl_2$, 0.5 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.2 mM Na_2MoO_4, 10 mM NaCl, 0.01 mM $CoCl_2$, 0.45% Gelrite (Duchefa Biochemie, Haarlem, The Netherlands) with the top 5cm of the gel removed with a sterile spatula. Plates were sealed with tape and cling-filmed in stacks of 7-10 after which they were incubated at 4°C in the dark for 4 days (to promote seed germination), then at 15°C in light / dark (16 h day length) for 7 days and finally at 25°C in light / dark (16 h day length) for 5 days to promote seedling growth until main tap roots began to reach the end of the plate.

A spore suspension (1.5 mL at a concentration of 1 x 10^6 spores mL^{-1}) of FOL4 isolate AJ516 was prepared as described in section 2.2.1 and then pipetted directly over the lettuce seedling roots in each plate which were then tilted from left to right to distribute the inoculum evenly. Plates were then allowed to dry under sterile air flow for approximately 15 min before sealing with tape. Plates were wrapped in cling film in treatment batches leaving the bases open, after which they were then placed in randomised block design in the incubator at 25°C (16 h photoperiod). Here, individual plates were arranged in 7 sealed packs randomly assigned to different locations over 2 shelves of the incubator with each pack containing 4 replicate plates. One plate pack was removed at 6, 12, 24, 48, 72, 96, and 120 hours post inoculation (hpi) with a 0 h time point also included (pre-inoculation). At each timepoint, the roots of all ten plants for each of the 4 plates were removed, rinsed in SDW, flash frozen in liquid nitrogen and stored at -80°C until use. This same approach was then then used for a second experiment using resistant (cv. Webbs Wonderful) and susceptible (cv. Steamboat) lettuce lines.

3.2.2. RNA extraction and cDNA synthesis

Lettuce roots were ground to a fine powder using a pestle and mortar filled with liquid nitrogen and approximately 100 mg of tissue was transferred to a 2 mL tube. Frozen root material was then ground further using a Dremel drill (model 398, with a rounded drill bit) and then RNA extracted using Trizol ® reagent (Ambion, Thermo Fisher Scientific) following the manufacturers guidelines. Extracted RNA was precipitated using 900 µL of lithium chloride to 100 µL RNA (250 µL $LiCl_2$ + 650 µL DEPC treated water) to remove contaminants. Remaining DNA was removed from samples using DNase 1 (Sigma-Aldrich). RNA samples were visualised on a 2% agarose gel (containing GelRed at 2 µL per 100 mL of gel) with the addition of loading dye (Orange G, Sigma- Aldrich), to check for degradation. First strand cDNA was synthesised using Superscript II reverse transcriptase (Invitrogen, Thermo Fisher Scientific) following the manufacturers protocol.

3.2.3. Quantitative PCR analysis of *SIX* gene expression

The expression of *SIX8*, *SIX9* and *SIX14* genes identified in FOL4 was assessed using quantitative PCR of the cDNA produced from each of the inoculated lettuce root samples at each time point. Primers (Table 2) were designed within the coding region of the gene using Primer3Plus (Untergasser *et al.*, 2007) and checked for self-hybridisation potential using Eurofins Oligo Analysis Tool (Eurofins) as well as any ability for DNA secondary structures to form (Zuker, 2003), or by manually selecting candidate primers. Expression of the (putative effector) gene targeted by the FOL4 specific diagnostic primers (Table 2) was also quantified. Reverse transcription qPCR was performed in a QuantStudio 5 Real-Time PCR machine using PowerUp SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific), following the manufacturers protocol. All primers were used at a final concentration of 0.5 µM (except FOL4 diagnostic and *TEF* primers which were used at 0.4 µM) in a final reaction volume of 19 µL per well, using the following conditions: one cycle of 95°C for 120s; 45 cycles of 95°C for 3 s, primer annealing temp (Table 2) for 30 s. A melt curve analysis (following amplification) was used to confirm the presence of a single PCR product. All samples were run in triplicate, standard curves were prepared for each gene target, by using serially diluted genomic DNA, and for analysis, the DNA concentration of each gene expressed relative to *TEF*.

3.2.4. Statistical analysis

All statistical analyses were carried out in R Studio® (Release (ldb809b8, 2022-05-16)). The concentration of DNA for each *SIX* gene and the housekeeping gene *TEF* was calculated from the standard curves. DNA concentration values for the *SIX* genes were averaged across replicates and divided by the mean DNA concentration for *TEF* for corresponding samples. These relative concentration values were then log transformed to account for increased variance across the time course. Analysis of variance (ANOVA) was then carried out using the transformed relative DNA concentration for each *SIX* gene. The overall effect of sampling time and lettuce type was observed for the expression of each gene. Significant differences between individual time points and lettuce type were compared with the least significant difference (LSD) at the 5% level.

3.2.5. RNAseq gene expression analysis of FOL4 inoculated lettuce

RNA extracted from FOL4 isolate AJ516 infected lettuce roots of each replicate at the single 96 hpi time point was used to generate RNAseq data in order to examine gene expression at the early stages of infection *in planta*. RNA was also extracted from 4 replicate PDA plates of FOL4 AJ516 mycelium grown for 2 weeks at 25°C as a control. All extracted RNA was treated with lithium chloride and DNase 1 (Sigma-Aldrich) as above before RNAseq library preparation. RNA integrity and quality was determined using an Agilent Bioanalyser and library preparation was carried out using the Illumina TruSeq RNA V2 kit with the starting amount of total RNA normalised to 300 ng (Genomics facility, University of Warwick). RNA sequencing was carried out using an Illumina NextSeq machine generating 75 bp single read data, with libraries producing approximately 400million reads per sample. Trimming and alignment of read data was carried out using command line tools (SAMtools, LiBinorm, hisat2 (Kim *et al.*, 2019). Differentially expressed gene (DEG) analysis of RNAseq data was performed in R. Quality control of raw RNAseq reads was checked using fastQC (Andrews, 2010). Reads were aligned to gene models using igv (Robinson *et al.*, 2011). A principal component analysis (PCA) was conducted in order to compare similarities and differences in sample transcripts within biological replicates and between treatments. Here, first (PC1) and second (PC2) principal components relating to the highest and second highest levels of variation within samples respectively were plotted. DESeq2 analysis (Anders & Huber, 2010, Huber *et al.*, 2015) was used to identify differentially expressed up and downregulated genes (DEGs) which showed a greater than two-fold change in expression compared to FOL4 AJ516 mycelium grown on plates. This analysis identified a list of 12471 DEGs which was then then fed through a Fusarium effector prediction pipeline (run by Jamie Pike (PhD student, University of

Warwick)), this pipeline was based on a previous published pipeline (van Dam *et al.*, 2016) whereby effectors were identified by being within 2kb of a Miniature Impala Element (MIMP). Putative effectors were further filtered for the presence of signal peptide sequence and being located on putative FOL4 pathogenicity chromosomes identified through a previous FOL4 genome analysis (Helen Bates, NIAB). This further reduced the list of expressed effector candidates to 150 genes. These genes were then sorted by RNA transcript count, DEG p value, and Effector P score (schematic; Figure 2). Finally, a BLAST search was carried out on the top 60 expressed effector genes from the sorted list and the most interesting candidates selected as shown in Table 5.

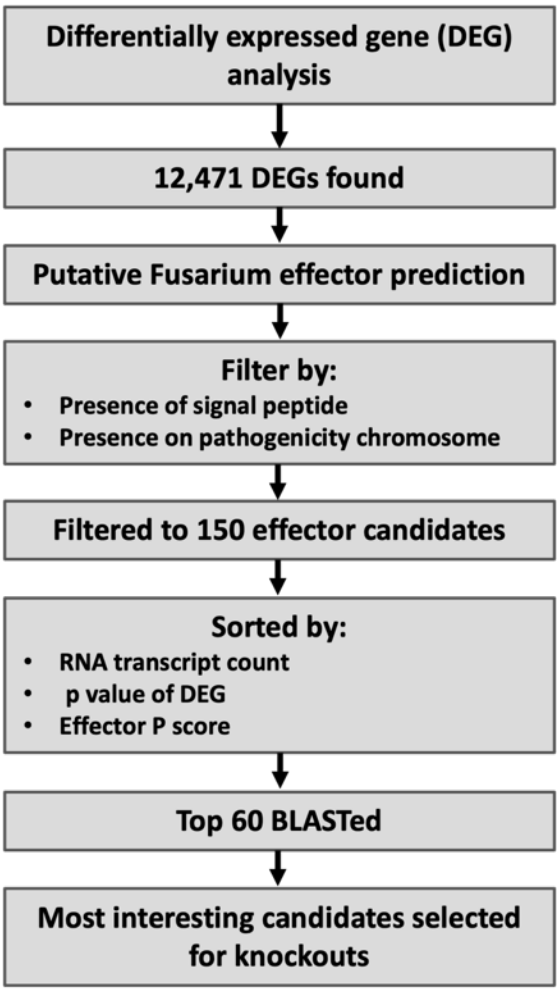

Figure 2 Flowchart outlining methods used to select candidate putative FOL4 effectors for knockout studies.

3.3. Objective 3: CRISPR Cas9 mediated knockout of *SIX8* in the FOL4 isolate AJ516

3.3.1. Design and synthesis of sgRNAs

Sequences of the consolidated list of candidate effectors (Table 5) were imported into Geneious prime (Geneious Prime® 2023.0.4 Build 2023-01-24) and the CRISPR site prediction tool used to predict CRISPR sites with protospacer and PAM sequences of $(N)_{20}$NGG in the *SIX8* coding region; if the starting nucleotide of the target sequence lacked a G, at least one G was added after the T7 promoter. Candidate sgRNAs were then sorted based on their activity score (Doench *et al.*, 2016), specificity score (Ran *et al.*, 2013) and proximity to the 5' end of the gene coding region. Off targets were scored against the FOL4 isolate AJ516 genome assembly and only sgRNAs that were 100% specific selected. *SIX8* sgRNA forward primers (T7 promoter- $(N)_{20}$-GTTTTAGAGCTAGAAATAGCAAG) and an sgRNA universal primer (Table 3) were used to generate *SIX8* sgRNAs (Table 3) using the EnGen® sgRNA synthesis kit (New England Biolabs).

3.3.2. Design of donor DNA and cloning

Construction of the *SIX8* knockout donor DNA template was carried out using NEBuilder® HiFi DNA Assembly Master Mix Kit. *SIX8* flanking regions were amplified from FOL4 genomic DNA using HiFi primers (Table 3) and assembled into the pPK2HPHGFP plasmid either side of a *hph* cassette.

3.3.3. *In vitro* cleavage assay

In vitro cleavage of *SIX8* was mediated by Cas9 fused with a H2B nuclear localisation sequence (NLS) (donated from Professor Martijn Rep; University of Amsterdam), and *SIX8* specific sgRNAs. The *SIX8* locus was amplified using genomic DNA and purified using a commercial PCR purification kit (GeneJET). sgRNA, Cas9 protein, and DNA template were added at a ratio of 1:1.25:0.25 (400 ng, 500 ng, 100 ng) respectively to 2 µL of 10x Cas9 nuclease buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl, 0.1 mM EDTA, pH 6.5) and DEPC H_2O added to a final volume of 20 µL. Cleavage was initiated by incubating at 37°C for 1hour and cleavage activity assessed by gel electrophoresis. All sgRNAs used in the study are listed in Table 3.

3.3.4. CRISPR Cas9 mediated knockout of *SIX8* in FOL4

Fungal pre-cultures of FOL4 isolate AJ516 were initiated by inoculation of Darken media (15 g L^{-1} cornsteep solids, 30 g L^{-1} sucrose, 1 g L^{-1} $(NH_4)_2SO_4$, 7 g L^{-1} $CaCO_3$) by addition of an agar plug taken from an actively growing colony on a PDA plate. 500 µL of 48–72 hour pre cultures were then used to inoculate main cultures of ICL media (80 g L^{-1} D-glucose, 1 g L^{-1} $MgSO_4$, 0.5 g L^{-1} KH_2PO_4, 2 mL L^{-1} trace elements solution, 6mM L-glutamine). Main cultures were grown for 17 hours at 25°C and fungal germlings collected, washed and treated with a mixture of 4 mg mL^{-1} Lysing enzymes (Sigma), 0.2 mg mL^{-1} Lyticase (Sigma), 0.2mg mL^{-1} Yatalase (TakaraBio), 0.2mg mL^{-1} BSA suspended in 1.2M KCl, 50uM $CaCl_2.2H_2O$ for 3-4 hours. Fungal protoplasts were then collected, separated from remaining fungal mycelium by filtering through Mira cloth, and made up to a concentration of 2 x 10^7 protoplasts/mL in 1x STC buffer (1.2M Sorbitol, 0.01M Tris/HCl (pH 7.5), 0.05M $CaCl_2$). Cas9 RNPs were assembled to a 1:1 mole ratio of Cas9:sgRNA into a final volume of 50µl composed of 20µg Cas9, 20µg sgRNA, 5µl 10x Cas9 nuclease buffer, and Diethyl pyrocarbonate (DEPC) treated water added to volume. The mixture was incubated at 37°C for 20mins. 200µl of the 2 x 10^7 protoplasts/mL solution was then mixed with 50µl of assembled RNPs and 5µl donor template (300-400ng) and incubated at RT for 20mins. Transformation was initiated by addition of 1.6 mL 50% w/v PEG solution followed by incubation at RT for 10mins. The reaction was then halted by addition of 3.2 mL 1X STC buffer. Fungal protoplasts were regenerated by pipetting 475µl of transformed protoplasts per plate onto sterile petri dishes, covering with approximately 20 mL of regeneration media (RM) (239.4 g L^{-1} Sucrose, 0.5 g L^{-1} Yeast extract, 20 g L^{-1} Bacto agar (BD Difco™)), and swirling plates to mix. Protoplasts were allowed to regenerate overnight before adding a selection layer of selective regeneration media containing 350 µg mL^{-1} hygromycin by pouring over the top of protoplast plates. Plates were then incubated at 25°C for 2-3 days. Transformants became visible 3-5 days after plating on to selection media. The individual transformants were then transferred onto Czapek Dox agar (CDA) (Thermo scientific) with 100 µg mL^{-1} hygromycin to confirm phenotype.

Four pairs of primers were used for screening of putative *SIX8* mutants (Table 3). Two pairs were confirming the presence of regions spanning the inserted cassette and genomic sections either side of the left and right homology arms. Remaining pairs were used to confirm the presence of the hygromycin phosphotransferase gene (*hph*), the absence of the *SIX8* gene and the presence of full donor DNA insert. All PCR reactions were set up into 20 µL total reaction volumes (1X PCR buffer, 0.2mM dNTP's, 0.5 µM forward and reverse primers, 0.1 µL Dream Taq (Thermo scientific), MilliQ water added to volume) containing 1 µL transformant

DNA. Thermocycling conditions were: one cycle of 30 s at 94°C; 30 cycles of 10 s at 94°C, 30 s at primer pair annealing temperature (Table 3) and 1 min 30 s at 72°C (except for presence of donor DNA insert which was 3 min at 72°C), followed by one cycle of 5 min at 72°C. PCR amplicons were visualised using gel electrophoresis (1% agarose gel containing Ethidium bromide at 2 µL per 100 mL of gel).

3.3.5. *In vitro* lettuce screen against FOL4 *SIX8* mutants

Seven FOL4 (isolate AJ516) *SIX8* knock out mutants were selected and tested for virulence against the susceptible lettuce variety cv. Steamboat using the *in vitro* lettuce seedling inoculation assay described previously (section 3.2.1), only differing in that five seeds were sown per plate. The wild type FOL4 isolate AJ516, and an uninoculated control comprising 0.012% Tween in SDW were used for positive and negative controls respectively. Lettuce seeds were surface sterilised, washed, plated onto ATS media and spore suspensions of each of the treatments added as described in section 3.2.1. Plates were wrapped in cling film in treatment batches (2 batches per treatment) leaving the bases open after which they were then placed in randomised block design in the incubator at 25°C (16 h photoperiod). Here, individual plates were arranged in 20 sealed packs randomly assigned to 20 different locations over 5 shelves of the incubator with each pack containing 4 replicate plates. Disease development was scored over a period of 28 days using a root browning score based on the percentage of total roots affected (Figure 3).

3.3.6. Growth assessment of FOL4 *SIX8* mutants

To assess whether the reduction in pathogenicity of the *SIX8* knockout mutants was due to reduced fitness due to potential deleterious effects caused by transformation, their growth on PDA was measured. A 5 mm core borer was used to isolate mycelial plugs from *SIX8* mutant isolates and wild type FOL4 plates. Plugs were subbed onto PDA plates as well as PDA plates appended with hygromycin such that each isolate treatment had four replicates on standard PDA plates and four replicates on PDA + hygromycin plates. Two lines were marked on each individual plate starting from the edge of the plug until the border of the plate. Two radii of mycelial growth were measured along each line and averaged. Measurements were taken twice a day for the standard PDA plates and once a day for PDA + hygromycin plates for 5 days.

3.3.7. Statistical analyses

All statistical analyses were carried out in R Studio® (Release (ldb809b8, 2022-05-16)), with advice and support from James Lynn, Applied Statistical Solutions. For the *in vitro* lettuce screen no significant batch effect was seen, therefore, treatment batches were pooled and the root browning scores from the different treatments were analysed at each timepoint using analysis of variance (ANOVA). Treatment comparisons were then made at each time point by comparing ANOVA means using the least significant difference (LSD) values at the 5% level. The final timepoint was further investigated to determine differences between wild type FOL4 and the other treatments using Tukey HSD and plotted.

For the growth assessment of FOL4 *SIX8* knockout mutants the final assessment at 5 days post subbing was investigated. ANOVA was used for analysis followed by a Tukey HSD post hoc test to identify significant differences between isolate comparisons in both PDA and PDA supplemented with hygromycin.

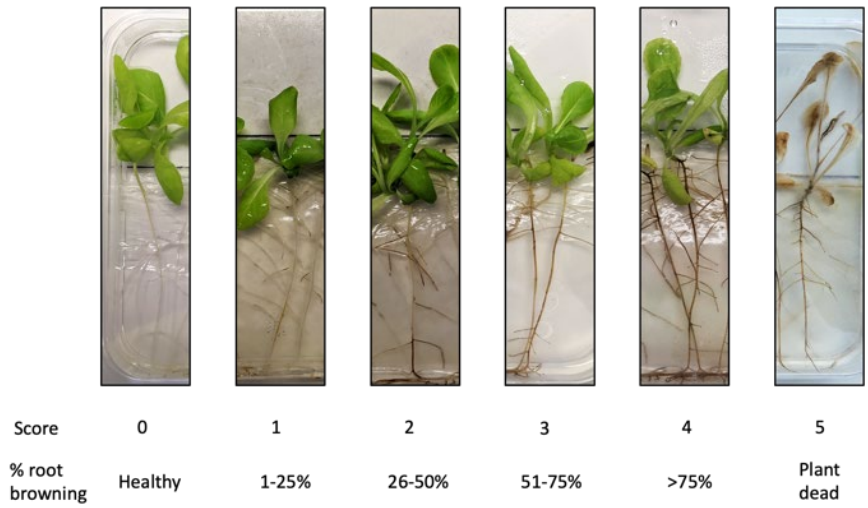

Figure 3 Images of lettuce cultivar 'Steamboat' as an example of progressive fusarium root browning caused by Fusarium oxysporum f.sp. lactucae. Root browning was scored on a scale from 0-5. A score of 0 denotes a healthy plant with no browning, scores of 1,2,3, and 4 denote % browning ranges of 1-25%, 26-50%, 51-75%, >75% respectively. A score of 5 denotes whole plant death.

3.4. Objective 4: Confirm resistant and susceptible lettuce phenotypes of selected lettuce lines and examine segregation of a new lettuce mapping population

3.4.1. Confirming resistance / susceptibility of lettuce lines by screening against FOL4

Lettuce seed of 16 lettuce lines (Table 7) were sown and plants raised in Levingtons F2 + S compost in P84 module trays for 3 weeks in the glasshouse (16 h day length, 18°C). Plants were then transplanted into a FOL4 inoculated polytunnel and arranged in 4 blocks with each block comprising 16 plots each with 8 replicate plants per treatment. Treatment plots were randomised within each block. Plants were scored for Fusarium wilt (as described in Figure 4) approximately twice a week for 37 days, then harvested and root vascular browning measured after cutting root material vertically (Figure 5).

3.4.2. Statistical analyses

All statistical analyses were carried out in R Studio® (Release ldb809b8, 2022-05-16), with advice and support from James Lynn, Applied Statistical Solutions. For wilt data an ANOVA was used for analysis of wilt scores at each individual time point. Treatment comparisons were then made at each time point by comparing ANOVA means using the least significant difference values (LSD) at the 5% level. For the vascular browning data recorded 37 days post inoculation (dpi) a non-parametric Kruskal Wallis H test was used due to the data not conforming to a normal distribution. Differences between treatments when compared to the standard susceptible control were determined using The Dunn-Bonferroni post-hoc test and plotted.

3.4.3. Assessing Fusarium resistance segregation in a mapping population screen

A lettuce mapping population generated from cv. Webbs Wonderful (resistant) and cv. Steamboat (susceptible) by Enza Zaden was screened for resistance in a FOL4 infected poly tunnel. 654 lettuce individuals from the population as well as 24 of each of the resistant and susceptible parental lines were raised in P84 modules and seedlings transplanted as described above (section 3.4.1). Parental lines were placed in 6 plots with each plot containing 4 replicate plants. Parental line plots were located in four different areas of the polytunnel to act as disease indicators. Wilt scores were recorded (as described in Figure 4) every 2 weeks

for 8 weeks. Plants were harvested at 10 weeks post transplanting and vascular browning scores recorded (Figure 5).

3.5. Objective 5: Investigate the extent of root colonisation of resistant and susceptible lettuce lines by FOL4

3.5.1. Preparing Fusarium flasks and soil inoculum

Actively growing FOL4 isolate AJ516 cultures grown on PDA were used to inoculate sterile 500 mL flasks containing an M2 compost/bran mix of 78.9% moisture content by addition of 3 PDA agar plugs (approximately $5mm^2$). Inoculated flasks were grown at 25°C for at least 5 weeks with regular manual agitation. CFU counts of the flasks were checked with serial dilution and an infested loam based compost (Bathgate John Innes No. 3) prepared to give a final concentration of 1 x 10^6 cfu/g.

3.5.2. Root colonisation assay

Eight lettuce cultivars were selected with different levels of resistance/susceptibility to FOL4 (Table 6). Lettuce seed was sown and plants raised in P84 compost modules for 3 weeks. Seedlings were then transferred into pots filled with the FOL4 inoculated compost. An untreated control of the susceptible cv. Amica was also set up. There were 10 replicate pots per treatment which were arranged in a glasshouse compartment in a randomised block design with each block containing one replicate pot of each cultivar and a non-inoculated control. Plants were maintained at 23°C day, 18°C night, 16 h photoperiod for a maximum of 31 days. Plants were assessed for Fusarium wilt (Figure 4) from 10 days post inoculation (PTI) then approximately every 3 days and treatments harvested upon reaching an average wilt score of above 3 or at 3-5 weeks after infection. At harvest, the main tap root of each harvested plant was washed, cut vertically, and a vascular browning score (Figure 5) recorded. Isolation of FOL4 was then attempted by excising pieces of tissue from three separate locations within each tap root (top, middle, bottom (Figure 22A), rinsing in SDW, surface sterilising in 70% EtOH for 20s and washing twice in SDW before placing onto PDA amended with chlorotetracycline (20µg mL^{-1}). Fungal morphologies were assessed approximately 1 week post root isolation. Fusarium like morphologies of various pigmentation were recorded and designated into 3 pigmentation groups (white, purple, and orange pigments). Exemplar samples consisting of a single plate of each present morphology type for each tap root location in each treatment were selected. DNA was isolated from these samples using the Acme DNA extraction protocol (section 3.1.2) and screened against the FOL4 diagnostic PCR test

(section 3.1.2) in order to confirm the morphologies as being FOL4. Percentage recovery of FOL4 was recorded based on presence of PCR verified FOL4 morphologies.

3.5.3. Statistical analyses

All statistical analyses were carried out in R Studio® (Release ldb809b8, 2022-05-16), with advice and support from James Lynn, Applied Statistical Solutions. For wilt data an ANOVA was used for analysis of wilt scores at each individual time point. For the vascular browning data an ANOVA was used for analysis of overall differences between treatments. Tukey's HSD was then utilised to investigate differences between treatments when compared to the uninoculated susceptible control post hoc. For the recovery data the recovery of FOL4 was converted into presence or absence counts for each treatment in each location. Count data was then compared between treatments for each location separately to investigate overall significance between treatments within each location using Fisher's Exact Test. Pairwise differences between treatments within locations was then assessed using pairwise fishers t-test taking into account multiple comparisons using false discovery rate (FDR). Overall differences between locations within lettuce treatments was investigated using Fisher's Exact Test.

Score	0	1	2	3	4	5	6	7	8
leaf wilt scores	No wilt/ healthy	1-2 leaves wilted	<10% wilt	11-25% wilt	26-50% wilt	51-75% wilt	76-99 wilt	100% wilt	Plant dead

Figure 4 Images of lettuce cultivar 'Gisella' as an example of progressive fusarium wilt caused by Fusarium oxysporum f.sp. lactucae. Lettuce leaf wilt was scored on a scale from 1-8. A wilt score of 1 denotes wilting of 1-2 leaves, wilt scores of 2,3,4,5,6 and 7 denote % wilting ranges of <10%, 10-25%, 25-50%, 50-75%, 75-99%, and 100% respectively. A score of 8 denotes plant death.

Figure 5 Vascular browning was scored on a scale from 0-4. Scores of 0,1,2,3 and 4 denote the categories of no symptoms, mild vascular browning, vascular browning, severe vascular browning, and plant death (not shown) respectively.

4. Results

4.1. Objective 1: Characterise FOL isolates from different European locations.

TEF was sequenced for 89 FOL1 and FOL4 isolates from different European locations and all isolates had identical sequences indicating a common monophyletic origin for FOL1 and FOL4 (Figure 6). Moreover, FOL2 and FOL3 *TEF* sequences (from online database) differed both from FOL1 / FOL4 and each other.

BLAST searches of the 14 known *SIX* genes revealed that FOL4 (AJ516 genome) contained *SIX8*, *SIX9* and *SIX14* whilst FOL1 (AJ520 genome) contained *SIX9* and *SIX14*. To confirm allocation of race based on *SIX* gene presence / absence, primers for the 14 known *SIX* genes were then used to screen a subset of 20 FOL isolates and it was confirmed that all FOL1 isolates were positive for *SIX9* and *SIX14* amplification only whilst FOL4 isolates were positive for *SIX8*, *SIX9* and *SIX14* amplification (Table 1). The remaining 69 FOL isolates in the collection were tested for presence of *SIX8*, *SIX9*, *SIX14*, as well as screened against FOL1 (Pasquali et al., 2007) and FOL4 (Andrew Taylor, unpublished) specific PCR assays. Results showed that FOL1 specific primers identified all isolates containing *SIX9* and *SIX14* while FOL4 specific primers identified all isolates containing *SIX8*, *SIX9* and *SIX14*, further confirming the allocation of race based on *SIX* gene presence / absence. Sequence similarity of *SIX8* within FOL4, and sequence similarity of *SIX9* and *SIX14* within each race and between races were then compared, and phylogenetic trees constructed. This showed that there were two sequence variants within *SIX8* in FOL4 isolates (Figure 7) while *SIX9* and *SIX14* sequences in both FOL1 and FOL4 were identical (Figure 8 and Figure 9).

Table 1 Results from screening European FOL isolates for presence of SIX genes (SIX1-14) and using FOL4 specific (G23490), FOL specific (G19968), FOL1 specific (Pasquali et al, 2007) primers. + symbol denotes a positive PCR result, whilst – symbol denotes a negative PCR result. NA indicates PCR tests not carried out.

Isolate ID	Supplier original ID	Race	Country	*SIX8*	*SIX9*	*SIX14*	FOL1 specific PCR	FOL4 specific PCR	Other *SIX* genes
AJ686	Fyto7067	1	SP	-	+	+	+	-	NA
AJ685	Fyto7081	1	IT	-	+	+	+	-	NA
AJ684	Fyto7083	1	IT	-	+	+	+	-	NA
AJ682	Fyto7735	1	IT	-	+	+	+	-	NA
AJ683	Fyto7736	1	IT	-	+	+	+	-	NA
AJ689	Fyto5047	1	IT	-	+	+	+	-	NA
AJ688	Fyto7079	1	IT	-	+	+	+	-	NA
AJ687	Fyto7080	1	IT	-	+	+	+	-	NA
AJ718	Fyto7211	1	FR	-	+	+	+	-	NA
AJ520	MYA 3040	1	IT	-	+	+	+	-	NA
AJ716	AT105	1	IT	-	+	+	+	-	-
AJ717	AT106	1	IT	-	+	+	+	-	-
AJ697	P142	1	SP	-	+	+	+	-	-
AJ865	PF-1	1	SP	-	+	+	+	-	NA
AJ866	PF-2	1	SP	-	+	+	+	-	NA
AJ867	PF-3	1	SP	-	+	+	+	-	NA
AJ868	PF-4	1	SP	-	+	+	+	-	NA
AJ869	PF-5	1	SP	-	+	+	+	-	NA
AJ870	PF-6A	1	SP	-	+	+	+	-	NA
AJ871	PF-6B	1	SP	-	+	+	+	-	NA
AJ873	PF-8	1	SP	-	+	+	+	-	NA
AJ874	PF-9A	1	SP	-	+	+	+	-	NA
AJ875	PF-9B	1	SP	-	+	+	+	-	NA
AJ876	PF-10A	1	SP	-	+	+	+	-	NA
AJ877	PF-10B	1	SP	-	+	+	+	-	NA
AJ878	PF-11	1	SP	-	+	+	+	-	NA
AJ879	PF-12	1	SP	-	+	+	+	-	NA
AJ882	PF-15	1	SP	-	+	+	+	-	NA
AJ883	PF-16A	1	SP	-	+	+	+	-	NA
AJ884	PF-16B	1	SP	-	+	+	+	-	NA
AJ885	PF-17A	1	SP	-	+	+	+	-	NA
AJ886	PF-17B	1	SP	-	+	+	+	-	NA
AJ888	PF-19	1	SP	-	+	+	+	-	NA
AJ889	PF-20	1	SP	-	+	+	+	-	NA
AJ892	PF-23	1	SP	-	+	+	+	-	NA
AJ893	PF-24	1	SP	-	+	+	+	-	NA

AJ895	PF-28	1	SP	-	+	+	+	-	NA
AJ896	PF-30	1	SP	-	+	+	+	-	NA
AJ897	PF-31	1	SP	-	+	+	+	-	NA
AJ898	PF-32	1	SP	-	+	+	+	-	NA
AJ899	PF-33	1	SP	-	+	+	+	-	NA
AJ900	PF-34	1	SP	-	+	+	+	-	NA
AJ881	PF-14	1	SP	-	+	+	+	-	NA
AJ872	PF-7	1	SP	-	+	+	+	-	NA
AJ880	PF-13	1	SP	-	+	+	+	-	NA
AJ887	PF-18	1	SP	-	+	+	+	-	NA
AJ891	PF-22	1	SP	-	+	+	+	-	NA
AJ894	PF-25	1	SP	-	+	+	+	-	NA
AJ694	Fyto7566	4	UK	+	+	+	-	+	NA
AJ693	Fyto7627	4	IT	+	+	+	-	+	NA
AJ690	Fyto7628	4	IT	+	+	+	-	+	NA
AJ691	Fyto7726	4	UK	+	+	+	-	+	NA
AJ692	Fyto7733	4	IT	+	+	+	-	+	NA
AJ695	Fyto7734	4	IT	+	+	+	-	+	NA
AJ696	Isolate N	4	IT	+	+	+	-	+	NA
AJ705	AD035	4	NL	+	+	+	-	+	-
AJ709	AN072	4	IE	+	+	+	-	+	-
AJ712	AN190	4	BE	+	+	+	-	+	-
AJ699	AP001	4	IE	+	+	+	-	+	-
AJ698	AP002	4	IE	+	+	+	-	+	-
AJ706	AP004	4	IT	+	+	+	-	+	-
AJ715	AR002	4	IT	+	+	+	-	+	-
AJ708	AR069	4	IT	+	+	+	-	+	-
AJ704	AR106	4	IT	+	+	+	-	+	-
AJ703	AS027	4	IT	+	+	+	-	+	-
AJ702	AS063	4	IT	+	+	+	-	+	-
AJ707	AT021	4	IT	+	+	+	-	+	-
AJ701	AT131	4	UK	+	+	+	-	+	-
AJ700	AU069	4	UK	+	+	+	-	+	-
AJ710	AU079	4	IT	+	+	+	-	+	-
AJ713	AU122	4	IT	+	+	+	-	+	-
AJ711	AU153	4	IT	+	+	+	-	+	-
AJ516	Preston 1-2	4	UK	+	+	+	-	+	NA
AJ507	Butterhead 1 (Ireland 2nd)	4	IE	+	+	+	-	+	NA
AJ510	Baby Gem 1-1 (Ireland 3rd)	4	IE	+	+	+	-	+	NA
AJ524	Lettuce no.2	4	UK	+	+	+	-	+	NA
AJ555	FS 10-1 (prep4)	4	UK	+	+	+	-	+	NA
AJ571	Butterly 1-2	4	IE	+	+	+	-	+	NA

AJ582	Carrolls 1-1	4	IE	+	+	+	-	+	NA
AJ580	L1	4	UK	+	+	+	-	+	NA
AJ563	ROG1-1	4	UK	+	+	+	-	+	NA
AJ602	GHP L1-1 (3rd)	4	UK	+	+	+	-	+	NA
AJ592	B5-1 (Cambridgeshire 2nd)	4	UK	+	+	+	-	+	NA
AJ522	015/04750896	4	NL	+	+	+	-	+	NA
AJ521	015/04750888	4	NL	+	+	+	-	+	NA
AJ624	B Sutton (PR4) Lettuce 2	4	UK	+	+	+	-	+	NA
AJ620	Red Rose salads (PR9) Lettuce TL-1	4	UK	+	+	+	-	+	NA
AJ618	D. Crook Lettuce 1 FOLR4	4	UK	+	+	+	-	+	NA
AJ616	Asda 1	4	UK	+	+	+	-	+	NA

Table 2 Primer pairs used in the characterisation of Fusarium species, SIX gene presence/absence and SIX gene expression studies, with primer name and sequence.

Gene	Primer pairs	Sequence 5'-3' (forward/reverse)	Annealing temp. (°C)	Reference
TEF	exTEF_F/FUexTEF_R	ACCCGGTTCAAGCATCCGATCTGCGA/AGCTTGCCRGACTTGATCTCACGCTC	64	1
SIX1	SIX 1 F/SIX 1 R	GTATCCCTCCGGATTTTGAGC/AATAGAGCCTGCAAAGCATG	59	2
SIX2	SIX 2 F/SIX 2 R	CAACGCCGTTTGAATAAGCA/TCTATCCGCTTTCTTCTCTC	59	2
SIX3	SIX 3 F/SIX 3 R	CCAGCCAGAAGGCCAGTTT/GGCAATTAACCACTCTGCC	59	2
SIX4	SIX 4 F/SIX 4 R	TCAGGCTTCACTTAGCATAC/GCCGACCGAAAAACCCTAA	59	2
SIX5	SIX 5 F/SIX 5 R	ACACGCTCTACTACTCTTCA/GAAAACCTCAACGCGGCAAA	59	2
SIX6	SIX 6 F/SIX 6 R	CTCTCCTGAACCATCAACTT/CAAGACCAGGTGTAGGCATT	59	2
SIX7	SIX 7 F/SIX 7 R	CATCTTTTCGCCGACTTGGT/CTTAGCACCCTTGAGTAACT	59	2
SIX8	FOL4 SIX8 F1 (PCR)/FOL4 SIX8 R1 (PCR)	CGCATCAAGAGTCCGGGTTTAC/CAGCATCCATATCCACGCCATA	59	3
SIX9	FOL SIX9 F1 (PCR)/FOL SIX9 R1 (PCR)	TCGATGCCGAGGAAATCACTTT/CAACATGCCAAGAACAGCCAAG	59	3
SIX10	Fol SIX 10 F/Fol SIX 10 R	GTTAGCAACTGCGAGACACTAGAA/AGCAACTTCCTTCCTCTTACTAGC	65	2
SIX11	Fol SIX 11 F/Fol SIX 11 R	ATTCCGGCTTCGGGTCTCGTTTAC/GAGAGCCTTTTTGGTTGATTGTAT	61	2
SIX12	Fol SIX 12 F2/Fol SIX 12 R2	CTAACGAAGTGAAAAGAAGTCCTC/GCCTCGCTGGCAAGTATTTGTT	61	2
SIX13	Fol SIX 13 F2/Fol SIX 13 R2	CCTTCATCATCGACAGTACAACG/ATCAAACCCGTAACTCAGCTCC	61	2
SIX14	FOL SIX14 F1 /FOL SIX14 R1	ATAACTGAACTTCTATTCCCA/GCATCTCCTGTTTCCTGTG	51	3
PSE1	FOL4 PSE1 F1/FOL4 PSE1 R1	CCTGCGTTCTGCCCAAC/TCGGCTTCGGAGTTAGGTTTCGG	51	3
FOL4 diagnostic	G23490 FOR3/G23490 REV	TGGTTGACAGCCAGATCATAG/GTTGACAAGCCTGCTTTAGCG	60	4
Primers used for real time reverse transcription-polymerase chain reaction (qPCR)				
TEF	qTEF F2/qTEF R2	GGTCAGGTCGGTGCTGGTTACG/TGGATCTCGGCGAACTTGCAGG	63	3
SIX8	FOL4 SIX8 F1/FOL4 SIX8 R1	ACGTTGAGGGTGGACAGAAC/TCGTGTACCGCTTGTGAGAG	59	3

SIX9	FOL SIX9 F2/FOL SIX9 R2	CTAGCCCAAGGAGTTGCGGT/GCATTGTCCCATACTGAATCC	59	3
SIX14	FOLaSIX14_Exon1F/FOLaSIX14_Exon1R	GCATTTCCACTATGTATTTCTTC/AACCACCACCTGCGTCTAG	58	3
PSE1	FOL4 qPSE1 F1/FOL4 qPSE1 R1	GGCACTTGCTGACTTACTACAG/GCCACATCGGTCTTTTCACACTA	61	3
FOL4 diagnostic	G23490 FOR3/G23490 REV	TGGTTGACAGCCAGATCATAG/GTTGACAAGCCTGCTTTAGCG	60	4

References footnote

[1] Taylor et al., 2016
[2] Lievens et al., 2009
[3] This study
[4] Dr. A. Taylor, unpublished

Table 3 Primer pairs used in the production of SIX8 sgRNAs, the assembly of the SIX8 knockout donor DNA plasmid, and screening of the ⊿SIX8 mutants.

Primer pairs	Sequence 5'-3' (forward/reverse)	Annealing temp. (°C)	Reference
Primers used for sgRNA synthesis			
SIX8 sgRNA1 oligo	TTCTAATACGACTCACTATAGCAGCCACAGAGACGGCTAAGTTTTAGAGCTAGA	37	This study
SIX8 sgRNA2 oligo	TTCTAATACGACTCACTATAGGAAGAGTAAAAGAACGCGTGTTTTAGAGCTAGA	37	This study
sgRNA universal oligo	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	37	This study
Primers used for assembly of donor DNA plasmid			
SIX8 Left flank Fwd/ SIX8 Left flank Rev	ATGATTACGAATTCTTAATTAAGATTTATGTAATATTAAGACCGAGAAGG/AAGATCCCCGGGTACCGAGCTCGATCGTAGGGGTTGCATAGCC	62.8	This study
SIX8 Right flank Fwd/ SIX8 Right flank Rev	TCTCCACTCGACCTGCAGGCATGCAGCCGTCTCTGTGGCTGCTAC/CGTTGTAAAACGACGGCCAGTGCCATTATCCCTATCGGGCCTAATCC	62.8	This study
CRISPR Genotyping oligos			
11772/SIX8 flanks rev	ACATCCTTTCGTACCGCATC/CAGCATCCATATCCACGCCATA	55	This study
1605/8251	GATGTAGGAGGGCGTGGATA/CGTCTGCTGCTCCATACAAG	57	This study
10322/751	ATAGTTGGGCAGAACGCAGG/CCTTCAGCGGATGATCGACTG	57	This study
745/SIX8 flanks rev	GCATGTTTCTTCCTTGAACTCTC/CAGCATCCATATCCACGCCATA	61	This study

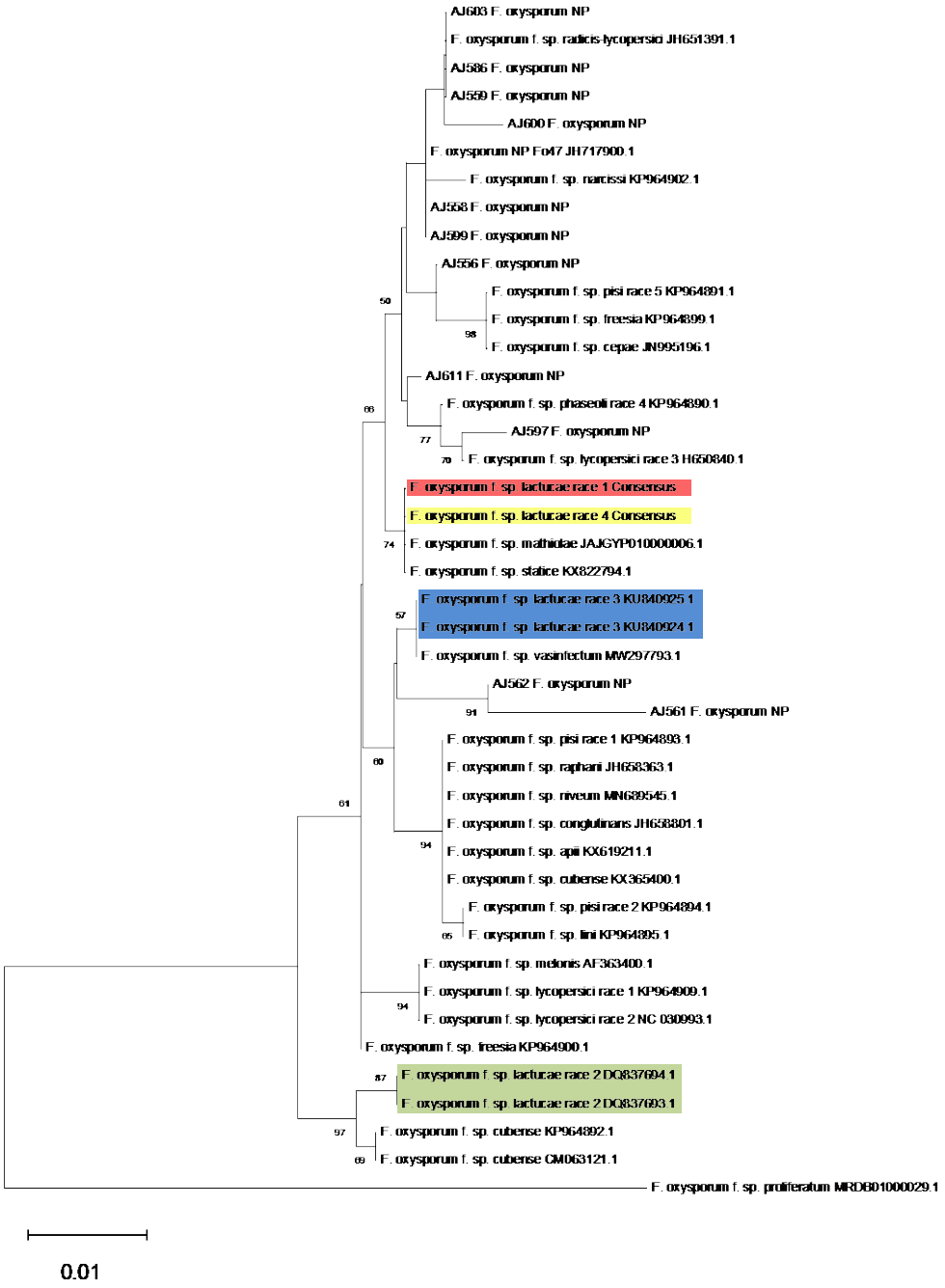

Figure 6 TEF phylogenetic tree for FOL isolates and other F. oxysporum f. spp.. Evolutionary history was inferred using the Minimum Evolution method. FOL1, FOL2, FOL3, and FOL4 isolates are denoted by the colours red, green, blue, and yellow respectively. "NP" following the isolate ID denotes FOL non-pathogenic isolates. The tree is rooted with a TEF sequence from F. proliferatum. Numbers represent bootstrap values from 1000 replicates. Scale bar indicates 0.01 substitutions per site.

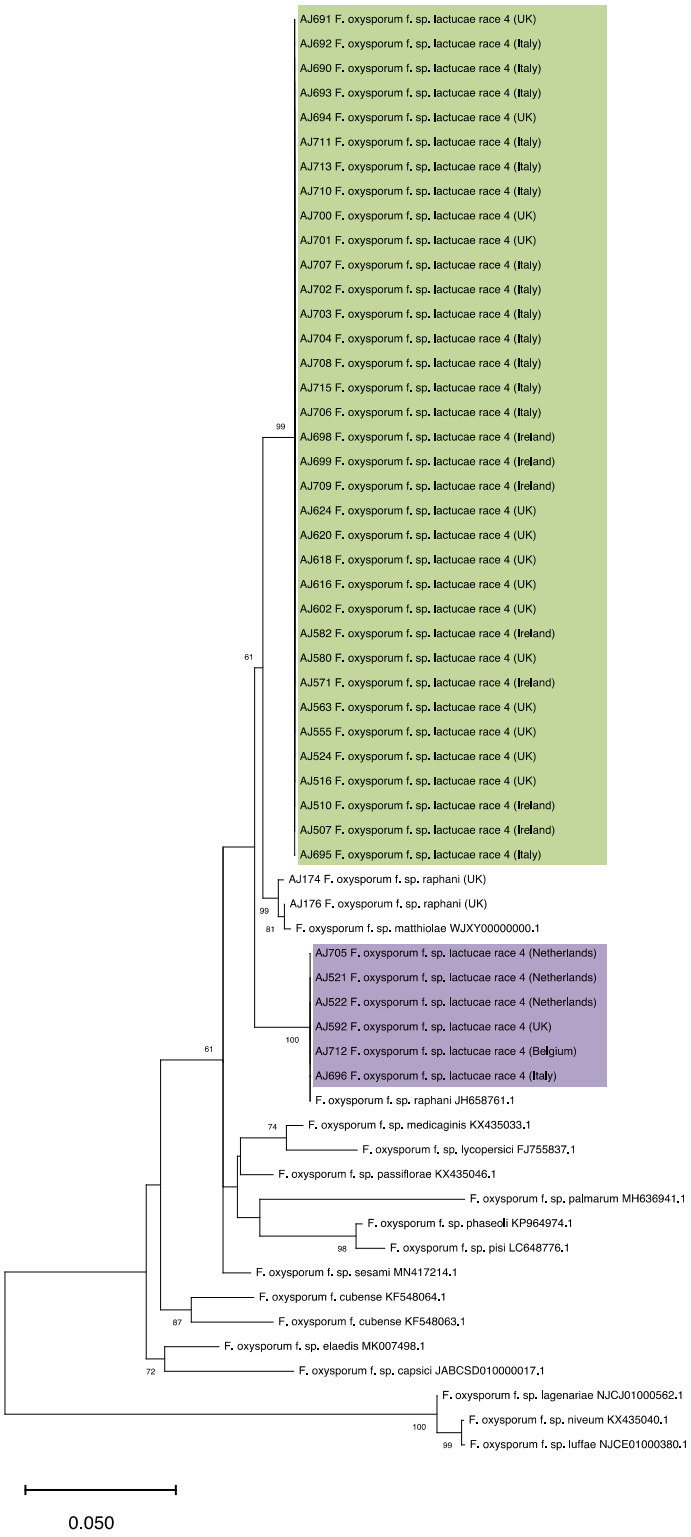

Figure 7 SIX8 phylogenetic tree for FOL4 isolates and other F. oxysporum f. spp.. Evolutionary history was inferred using the Minimum Evolution method. Two separate FOL4 SIX 8 clades are highlighted in different colours. Numbers represent bootstrap values from 1000 replicates. Scale bar indicates 0.05 substitutions per site.

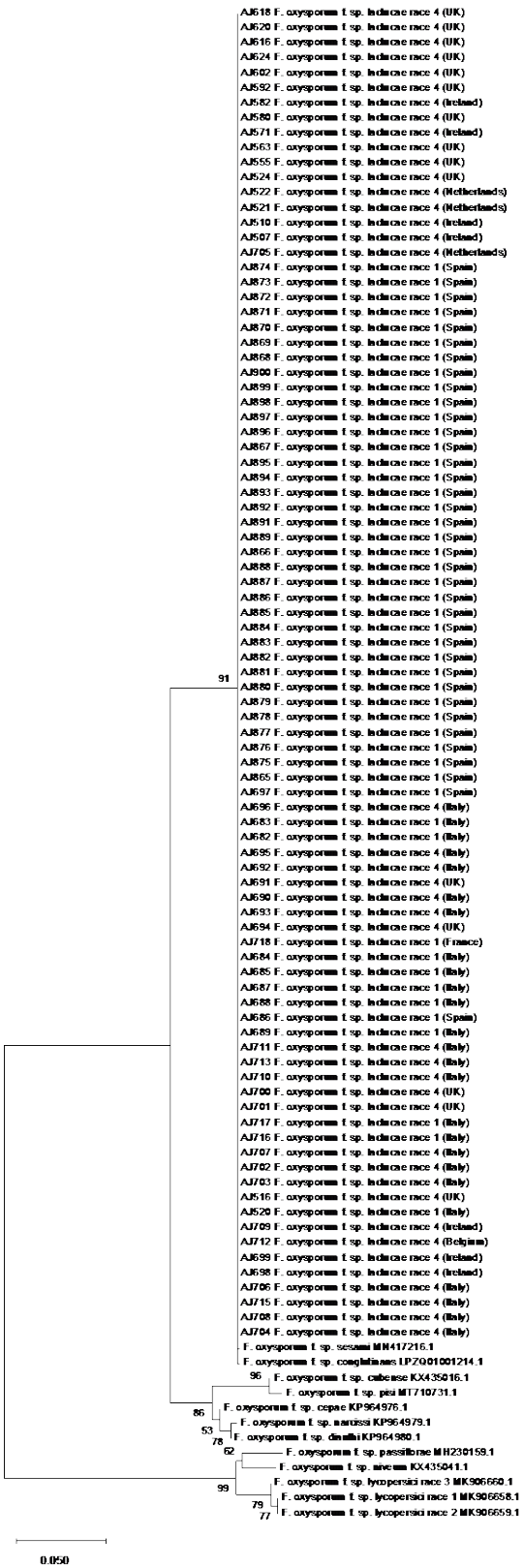

Figure 8 SIX9 phylogenetic tree for FOL1 and FOL4 isolates and other F. oxysporum f. spp.. Evolutionary history was inferred using the Minimum Evolution method. Numbers represent bootstrap values from 1000 replicates. Scale bar indicates 0.05 substitutions per site.

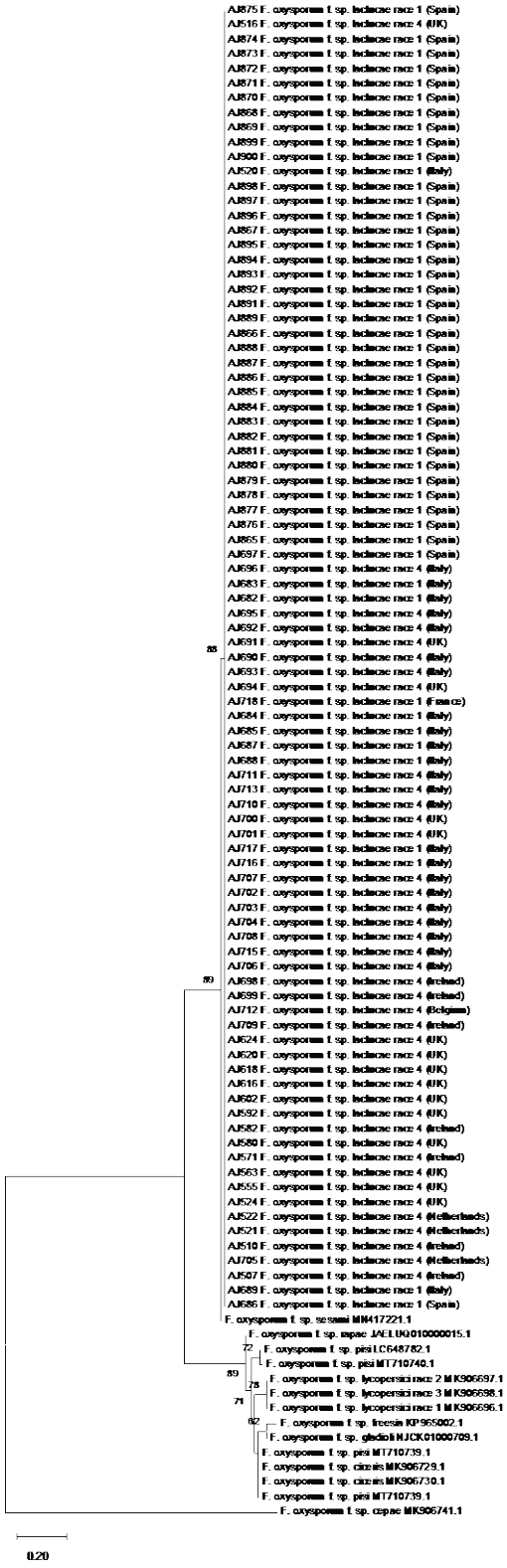

Figure 9 SIX14 phylogenetic tree for FOL1 and FOL4 isolates and other F. oxysporum f. spp.. Evolutionary history was inferred using the Minimum Evolution method. Numbers represent bootstrap values from 1000 replicates. Scale bar indicates 0.20 substitutions per site.

4.2. Objective 2: Identify putative FOL4 effector genes expressed during lettuce infection through RNAseq and confirm their roles in virulence

In the infection time course experiment where three lettuce cultivars Temira, Steamboat and Webbs Wonderful were inoculated with FOL4 AJ516, the relative expression of *SIX8*, *SIX9* and *SIX14* was examined between 0 and 120 hpi in all lettuce lines (Figure 10).
For all *SIX* genes tested, there were overall significant differences in log relative expression (ANOVA: $p < 0.001$ for *SIX8*; $p < 0.001$ for *SIX9*; $p < 0.001$ for *SIX14*) over the time courses in all lettuce varieties. Initial detection of *SIX* gene expression varied depending on the gene and the lettuce variety. *SIX9* was detected earlier than the other two *SIX* genes (6 hpi in Temira and Webbs Wonderful; 12 hpi in Steamboat).

SIX9 expression increased at every timepoint in both Temira and Steamboat. However, in Webbs Wonderful *SIX9* expression increased from 12 hpi until 96 hpi at which it then decreased at 120 hpi. Although, comparing expression values with 5% LSD there was no statistically significant difference in expression of *SIX9* between the 96 and 120 hpi timepoints in all lettuce varieties. There was however a statistically significant difference in *SIX9* expression between 72 and 96 hpi in Temira but not in the other two varieties.

SIX8 expression was first detected at 12 hpi in Webbs Wonderful and later for Temira and Steamboat (24 hpi). *SIX8* expression increased overtime in all lettuce varieties up to 96 hpi at which point it decreased in Webbs Wonderful and increased for the other two varieties at 120 hpi. Again, comparing relative expression with 5% LSD there was no statistically significant difference in *SIX8* expression between the 96 and 120 hpi timepoints in all lettuce varieties. A statistically significant difference was seen between the 72 and 96 hpi timepoints in Webbs Wonderful but not in the other lettuce varieties.

SIX14 was also first detected at 12hpi in both Webbs Wonderful and Steamboat but later in Temira (24 hpi). *SIX14* expression, like *SIX8*, increased overtime in all lettuce varieties up to 96 hpi at which point it decreased in Webbs Wonderful and increased for the other two varieties at 120 hpi. There was no statistically significant difference in expression of *SIX14* at 96 and 120 hpi in all lettuce varieties upon comparing against 5% LSD, although, similar to *SIX9*, there was a statistically significant difference in *SIX14* expression between the 72 and 96 hpi timepoints in Temira.

The amount of expression of the *SIX8* and *SIX9* genes varied between lettuce varieties (ANOVA: $p < 0.04$). Comparing relative expression to 5% LSD between lettuce varieties at the 96 hpi timepoint for the *SIX8* and *SIX9* genes showed statistically significant differences between both Steamboat and Webbs Wonderful compared to Temira but no significant difference between each other. The same comparison done for *SIX14* showed no significant difference in *SIX14* expression between all lettuce varieties. Additionally, all SIX gene qPCR reactions also included negative controls (roots inoculated with SDW + tween and harvested at 96 hpi) but no *SIX* gene expression was seen in any of these samples (data not shown).

In addition to using qPCR to examine relative *SIX* gene expression, an RNAseq approach was also utilised to further investigate expression of other putative pathogenicity genes expressed during infection. To ensure early expression genes would not be missed, the 96h timepoint was selected for the RNAseq analysis. RNAseq was carried out on resistant (Webbs Wonderful), and susceptible (Steamboat) lettuce tissue infected with FOL4 isolate AJ516 after 96 hpi, as well as on FOL4 AJ516 mycelium grown on PDA and harvested at the same timepoint.

Overall similarity of sample replicates was assessed by calculating the distance matrix between samples and plotting as a heatmap. Distances between replicates within the treatments indicated replicates were highly similar. FOL4 expression profiles between replicates of the different lettuce lines were also highly similar. Moreover, there was substantial differences seen between the FOL4 infected lettuce root samples when compared to FOL4 mycelium plate samples (Figure 11).

Additionally, a principal component analysis (PCA) was carried out to further examine sample distances. The analysis showed that PC1 accounted for 90% of the variance and indicated there were large differences between FOL4 infected lettuce root samples and the FOL4 mycelium plate samples across this PC. However, there was no real difference between the different lettuce lines root samples across PC1 (Figure 12). PC2 accounted for 4% of the variance. Both resistant and susceptible root samples clustered closely with the exception of 1 replicate outlier (from 96 hpi susceptible treatment) indicating hardly any differences between replicates and between these two treatments. FOL4 mycelium plate replicates showed larger variance across PC2 (Figure 12). Due to their high similarity, the expression data for the resistant and susceptible lettuce lines were pooled and compared to the expression data *in vitro* for all subsequent differentially expressed gene analyses.

The differentially expressed gene analysis identified a range of highly expressed putative effectors including *SIX8*, *SIX9* and *SIX14*. In addition, eight genes were identified as homologues of effectors previously identified in *Fusarium oxysporum f. sp. apii*, while three were homologues of effectors in other f.spp (not including *SIX8*, *SIX9* and *SIX14*). 24 genes had no clear identification and the remainder appeared to be various cell wall degrading enzymes or transcription factors (Table 5).

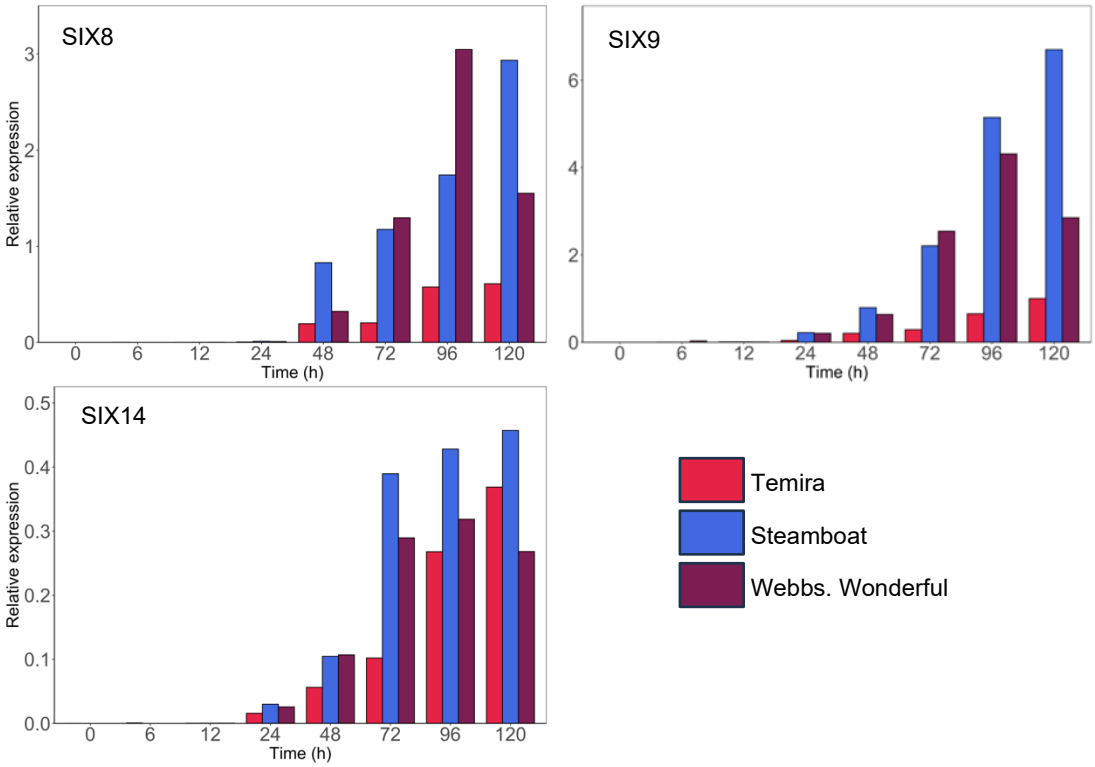

Figure 10 Quantitative expression of Secreted In Xylem (SIX) genes for RNA extracted from lettuce roots of three different lettuce varieties infected with Fusarium oxysporum f. sp. lactucae race4 (FOL4) isolate AJ516 as determined by reverse transcription qPCR. Expression was calculated relative to translation elongation factor 1a (TEF), and log transformed for ANOVA. Values plotted represent means of relative gene expression at eight time points (0 – 120 h) post inoculation.

Table 1 Log transformed ANOVA means of the expression of Secreted In Xylem (SIX) genes relative to the translation elongation factor 1a (TEF) gene for RNA extracted from lettuce roots infected with Fusarium oxysporum f. sp. lactucae race 4 (FOL4) isolate AJ516 between 0 – 120 hpi. Significant differences between time points can be calculated using the 5% LSD[1], and between lettuce varieties for the same time point (in this case 96 hours) using the 5% LSD[2]. NA denotes missing or undetectable levels of expression.

Time (h)	**Relative expression of SIX gene to *TEF* (transformed means)**								
	8			**9**			**14**		
	Temira	Steamboat	Webbs.	Temira	Steamboat	Webbs.	Temira	Steamboat	Webbs.
0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
6	*NA*	*NA*	*NA*	-2.48	*NA*	-1.91	*NA*	*NA*	*NA*
12	*NA*	*NA*	-2.86	-2.03	-2.04	-2.15	*NA*	-3.43	-3.42
24	-2.45	-1.95	-2.02	-1.4	-0.66	-0.69	-1.82	-1.53	-1.59
48	-0.75	-0.52	-0.51	-0.71	-0.21	-0.22	-1.26	-0.99	-1
72	-0.69	0.06	0.1	-0.54	0.33	0.41	-0.99	-0.41	-0.54
96	-0.29	0.23	0.4	-0.2	0.61	0.58	-0.59	-0.38	-0.5
120	-0.22	0.47	0.18	-0.01	0.81	0.41	-0.46	-0.34	-0.57
LSD[1]	0.43	0.70	0.30	0.25	0.43	0.30	0.26	0.17	0.22
LSD[2]		0.49			0.54			0.25	

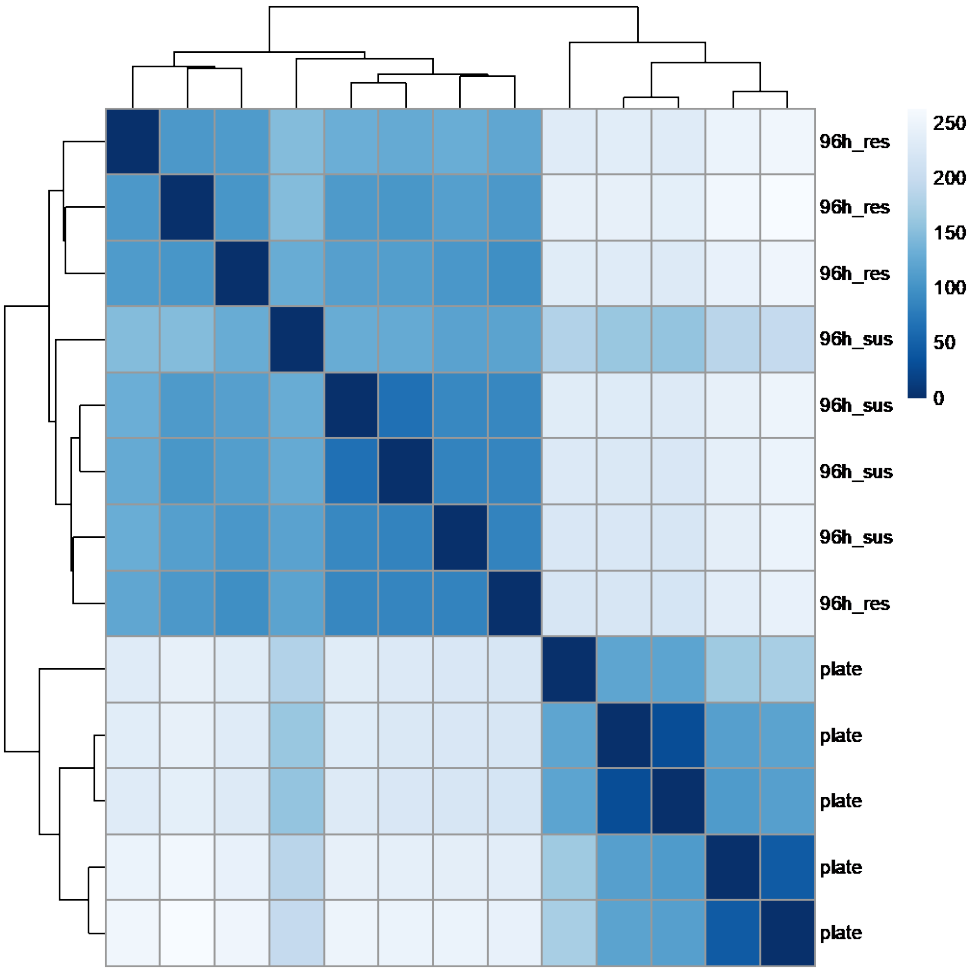

Figure 11 Sample distances heatmap of normalised RNAseq reads from infected lettuce roots of resistant and susceptible lettuce varieties (96 hpi); and agar-grown mycelium samples of F. oxysporum f. sp. lactucae (FOL) isolate AJ516. All reads aligned to the AJ516 genome.

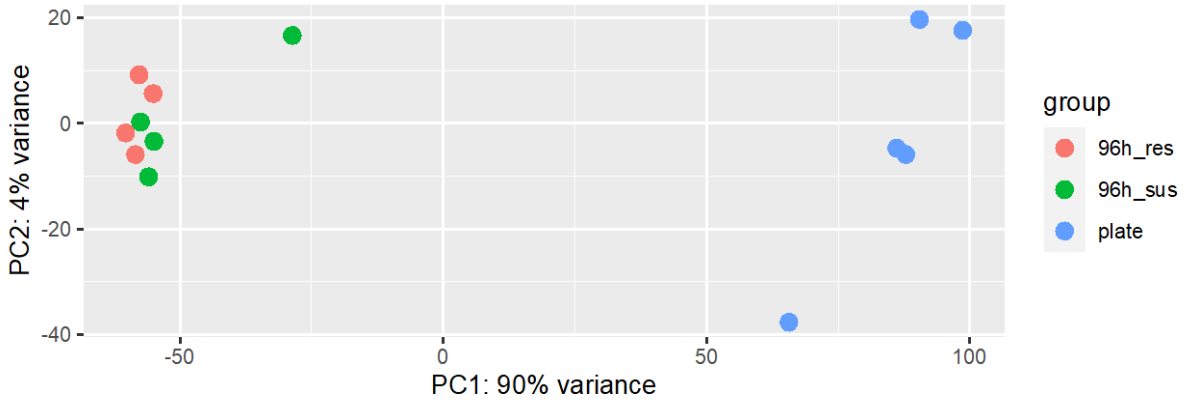

Figure 12 Principal component analysis (PCA) of normalised RNAseq reads from infected lettuce roots of resistant and susceptible lettuce varieties (96 hpi); and agar-grown mycelium samples of F. oxysporum f. sp. lactucae (FOL) isolate AJ516. All reads aligned to the AJ516 genome.

Table 2 Table outlining the most promising putative effector candidates for future knockout studies. For each gene its transcript count, differential expression p value, BLAST hit, and presence/absence on the FOL4 putative pathogenicity chromosome (+ denotes presence and – denotes absence) are shown.

Gene ID	Transcript count	pvalue	Presence on pathogenicity contig	BLAST hits
g8918	30790.4	2.38E-70	+	>MT364389.1 Fusarium oxysporum f. sp. apii isolate FoaR4-274.AC effector protein gene, complete cds
g8911	30763.8	2.10E-70	+	>MT364389.1 Fusarium oxysporum f. sp. apii isolate FoaR4-274.AC effector protein gene, complete cds
g21124	29601.1	3.99E-62	+	>EXM12923.1 hypothetical protein FOTG_18604 [Fusarium oxysporum f. sp. vasinfectum 25433]
g24453	29595.0	3.47E-62	+	>EXM12923.1 hypothetical protein FOTG_18604 [Fusarium oxysporum f. sp. vasinfectum 25433]
g24452	29580.1	3.73E-62	+	>EXM12923.1 hypothetical protein FOTG_18604 [Fusarium oxysporum f. sp. vasinfectum 25433]
g21125	29580.1	3.43E-62	+	>EXM12923.1 hypothetical protein FOTG_18604 [Fusarium oxysporum f. sp. vasinfectum 25433]
g8912	19892.1	2.63E-141	+	>QOE88867.1 effector protein [Fusarium oxysporum f. sp. apii]
g8917	18989.1	1.34E-141	+	>QOE88867.1 effector protein [Fusarium oxysporum f. sp. apii]
g12530	14500.4	1.01E-60	+	>AEN94579.1 SIX9a [Fusarium oxysporum]
g9012	14485.5	1.74E-60	+	>AEN94579.1 SIX9a [Fusarium oxysporum]
g12532	6199.9	1.68E-83	+	>RYC78141.1 hypothetical protein BFJ63_vAg18986 [Fusarium oxysporum f. sp. narcissi]
g9017	6191.9	1.75E-83	+	>RYC78141.1 hypothetical protein BFJ63_vAg18986 [Fusarium oxysporum f. sp. narcissi]
g20675	3518.8	4.12E-70	+	>KAG7408591.1 Polygalacturonase [Fusarium oxysporum f. sp. rapae]
g12454	3460.3	3.85E-65	+	>KAG7408591.1 Polygalacturonase [Fusarium oxysporum f. sp. rapae]
g9993	3436.5	2.60E-65	+	>KAG7408591.1 Polygalacturonase [Fusarium oxysporum f. sp. rapae]
g10013	2463.3	9.42E-85	+	>TVY73907.1 hypothetical protein Focb16_v006228 [Fusarium oxysporum f. sp. cubense]
g9602	2013.2	1.27E-15	+	>EWZ77789.1 hypothetical protein FOWG_17837 [Fusarium oxysporum f. sp. lycopersici MN25]
g9601	1091.9	1.29E-22	+	>HQ260604.1 Fusarium oxysporum SIX8 gene, complete cds
g9989	962.8	1.25E-100	+	>XM_028640592.1 Verticillium nonalfalfae uncharacterized protein (D7B24_006462), partial mRNA
g18830	860.7	9.81E-09	-	>PCD22022.1 hypothetical protein AU210_015824 [Fusarium oxysporum f. sp. radicis-cucumerinum]
g12862	465.3	5.43E-37	+	>QOH31766.1 secreted in xylem 14 [Fusarium nirenbergiae]
g9460	379.6	6.98E-44	+	>KAI8406429.1 hypothetical protein FOFC_13899 [Fusarium oxysporum]
g20708	337.4	1.87E-55	+	>AKC54395.1 exopolygalacturonase [Fusarium oxysporum f. sp. cubense]
g12538	335.5	9.49E-56	+	>AKC54395.1 exopolygalacturonase [Fusarium oxysporum f. sp. cubense]
g9023	335.2	4.55E-56	+	>AKC54395.1 exopolygalacturonase [Fusarium oxysporum f. sp. cubense]

Gene ID	Transcript count	pvalue	Presence on pathogenicity contig	BLAST hits
g20057	327.8	1.19E-65	-	>EXM12421.1 murein transglycosylase [Fusarium oxysporum f. sp. vasinfectum 25433]
g9977	303.9	9.86E-61	+	>EXK76576.1 hypothetical protein FOQG_18687 [Fusarium oxysporum f. sp. raphani 54005]
g9484	210.8	1.23E-23	+	>QQY97452.1 secreted in the xylem 15, partial [Fusarium oxysporum f. sp. physali]
g18764	92.0	5.58E-15	-	>KAF4420389.1 Rapid ALkalinization Factor, partial [Fusarium acutatum]
g18849	61.6	2.15E-14	-	>KAH7194414.1 rapid alkalinization factor-domain-containing protein [Fusarium oxysporum]
g18788	53.5	3.41E-17	-	>KAH7186595.1 hypothetical protein DER44DRAFT_904557 [Fusarium oxysporum]
g23935	32.7	5.40E-22	+	>KAH6985451.1 Alpha/Beta hydrolase protein, partial [Ilyonectria destructans]
g8937	30.1	2.24E-11	+	>EXM12638.1 catalase-peroxidase 2 [Fusarium oxysporum f. sp. vasinfectum 25433]
g12559	29.4	7.60E-41	+	>KAG7402788.1 Polygalacturonase [Fusarium oxysporum f. sp. raphani]
g9617	29.4	1.19E-40	+	>KAG7402788.1 Polygalacturonase [Fusarium oxysporum f. sp. raphani]
g4778	22.2	2.60E-05	-	>KAH7481096.1 hypothetical protein FOMA001_g8688 [Fusarium oxysporum f. sp. matthiolae]
g23863	18.0	1.48E-23	+	>QOE88858.1 effector protein [Fusarium oxysporum f. sp. apii]
g9612	16.5	8.64E-20	+	>QOE88858.1 effector protein [Fusarium oxysporum f. sp. apii]
g9669	9.1	1.93E-07	+	>KAJ0129718.1 putative transcriptional regulatory protein [Fusarium oxysporum f. sp. albedinis]
g20748	3.8	5.73E-15	+	>APP91304.1 SIX15 [Fusarium oxysporum f. sp. lycopersici]
g10014	0.7	2.85E-05	+	>XM_031194900.1 Fusarium oxysporum NRRL 32931 uncharacterized protein (FOYG_16648), mRNA
g9475	1109.6	6.76E-14	+	>MT364396.1 Fusarium oxysporum f. sp. apii isolate FoaR4-274.AC effector protein gene, partial cds
g19656	444.7	1.62E-130	-	>XM_046241596.1 Ilyonectria robusta endo-1,3-beta-glucanase (BGZ61DRAFT_365307), partial mRNA
g9964	437.8	1.48E-128	+	>XM_046241596.1 Ilyonectria robusta endo-1,3-beta-glucanase (BGZ61DRAFT_365307), partial mRNA
g23733	283.2	1.42E-85	+	>MT364401.1 Fusarium oxysporum f. sp. apii isolate FoaR4-274.AC metalloproteinase gene, complete cds
g8965	189.0	1.88E-13	+	>EWY79970.1 hypothetical protein FOYG_16911 [Fusarium oxysporum FOSC 3-a]
g12492	188.7	2.19E-13	+	>EWY79970.1 hypothetical protein FOYG_16911 [Fusarium oxysporum FOSC 3-a]
g8955	166.8	3.10E-60	+	>EXA29084.1 hypothetical protein FOVG_19372 [Fusarium oxysporum f. sp. pisi HDV247]
g12478	166.5	3.31E-60	+	>EXA29084.1 hypothetical protein FOVG_19372 [Fusarium oxysporum f. sp. pisi HDV247]
NA	137.9	9.94E-29	+	>XM_031195880.1 Fusarium oxysporum NRRL 32931 uncharacterized protein (FOYG_17556), mRNA
NA	100.8	0.01844516	+	>XM_031195183.1 Fusarium oxysporum NRRL 32931 uncharacterized protein (FOYG_16911), mRNA
NA	100.8	0.01956395	+	>XM_031195183.1 Fusarium oxysporum NRRL 32931 uncharacterized protein (FOYG_16911), mRNA

Gene ID	Transcript count	pvalue	Presence on pathogenicity contig	BLAST hits
g19655	37.6	8.52E-54	-	>JX204293.1 Fusarium oxysporum f. sp. fragariae transposon Fot3
g9956	34.8	2.04E-51	+	>JX204293.1 Fusarium oxysporum f. sp. fragariae transposon Fot3
g9429	29.2	3.11E-46	+	>XM_018397452.1 Fusarium oxysporum f. sp. lycopersici 4287 hypothetical protein mRNA
g12560	28.4	4.35E-30	+	>XM_044867847.1 Hirsutella rhossiliensis transposase (HRG_09376), partial mRNA
g8930	11.9	2.04E-32	+	>XM_018396232.1 Fusarium oxysporum f. sp. lycopersici 4287 hypothetical protein mRNA
g23845	2595.8	3.25E-67	+	>MT364410.1 Fusarium oxysporum f. sp. apii isolate FoaR4-274.AC effector protein

4.3. Objective 3: CRISPR Cas9 mediated knockouts of FOL4 putative effectors

The ability of Cas9/sgRNA RNPs to cleave *SIX8* at specific target site was tested *in vitro*. A ~1.6kb fragment containing the *SIX8* coding sequence was mixed with a complimentary sgRNA and Cas9 protein. The two designed NLS-Cas9-*SIX8*sgRNA complexes were able to cleave the *SIX8* target sequence with ~100% efficiency yielding fragments of ~1kb and ~0.6kb (Figure 13B).

Donor DNA transformed into *SIX8* mutants contained a *hph* gene fused with *GFP*, as well as ~500bp of homologous sequences flanking either side of the *hph* cassette with homology to flanking regions either side of the *SIX8* cleavage site (Figure 13A). Therefore, *SIX8* knock out mutants should grow on selective hygromycin media as well as exhibit GFP fluorescence. Putative transformants were screened for the presence of the donor DNA inserted within the *SIX8* locus, of 12 selected putative transformants all tested positive for *hph* PCR amplification (Figure 13C) and exhibited GFP fluorescence (Figure 14), and 7 contained the *hph* cassette inserted in locus shown by the absence of *SIX8* amplification and presence of *hph* cassette amplicon of ~6kb (Figure 13D). Seven *SIX8* mutants were tested in a pathogenicity test against a susceptible lettuce line (cv. Steamboat). Steamboat exhibited significantly less root browning when infected with *SIX8* mutants compared to when infected with wild type FOL4 (Figure 15A). ANOVA on browning scores revealed that there were statistically significant differences between the treatments at all the different timepoints ($p < 0.0001$). Tukey HSD test was used to interpret data at the 28 dpi timepoint.There was a statistically significant difference between root browning caused by the *SIX8* mutants compared to wild type FOL4 AJ516 ($p < 0.05$) (Figure 15B). Moreover, there was also a statistically significant difference between root browning caused by the *SIX8* mutants when compared to uninoculated control plants ($p <$

0.0001). There appeared to be no statistically significant difference between average browning scores of the mutants when compared to each other (data not shown).

Reduction in pathogenicity of the *SIX8* knockout mutants due to potential reduced fitness caused by deleterious effects as a by-product of transformation was assessed by comparing isolate growth rates on PDA. ANOVA revealed that there were significant differences between the growth rates of isolates on PDA ($p<0.003$). However, when the growth rate of the *SIX8* knockout mutants was compared with the wildtype FOL4 using Tukey HSD it was found that there was no statistically significant difference in their growth after 5 days (Figure 16A). There were significant differences of growth rate between other mutants when compared to ΔSIX8 2-4 ($p<0.005$). However, when mutants were grown on PDA and in the presence of hygromycin, varying levels of growth were recorded after 5 days (ANOVA $p < 0.0001$). After analysis using Tukey HSD mutants separated out into three growth groups with ΔSIX8 2-1 exhibiting the most growth and being significantly faster growing than the rest. ΔSIX8 1-1, ΔSIX8 2-14, ΔSIX8 2-4, and ΔSIX8 2-7 did not statistically differ from each other except from when ΔSIX8 2-14 was compared with ΔSIX8 2-7. Additionally, these isolates exhibited significantly faster growth than the remaining two mutants ΔSIX8 1-2 and ΔSIX8 2-18. ΔSIX8 1-2 and ΔSIX8 2-18 were the slowest growing and did not statistically differ from each other (Figure 16B).

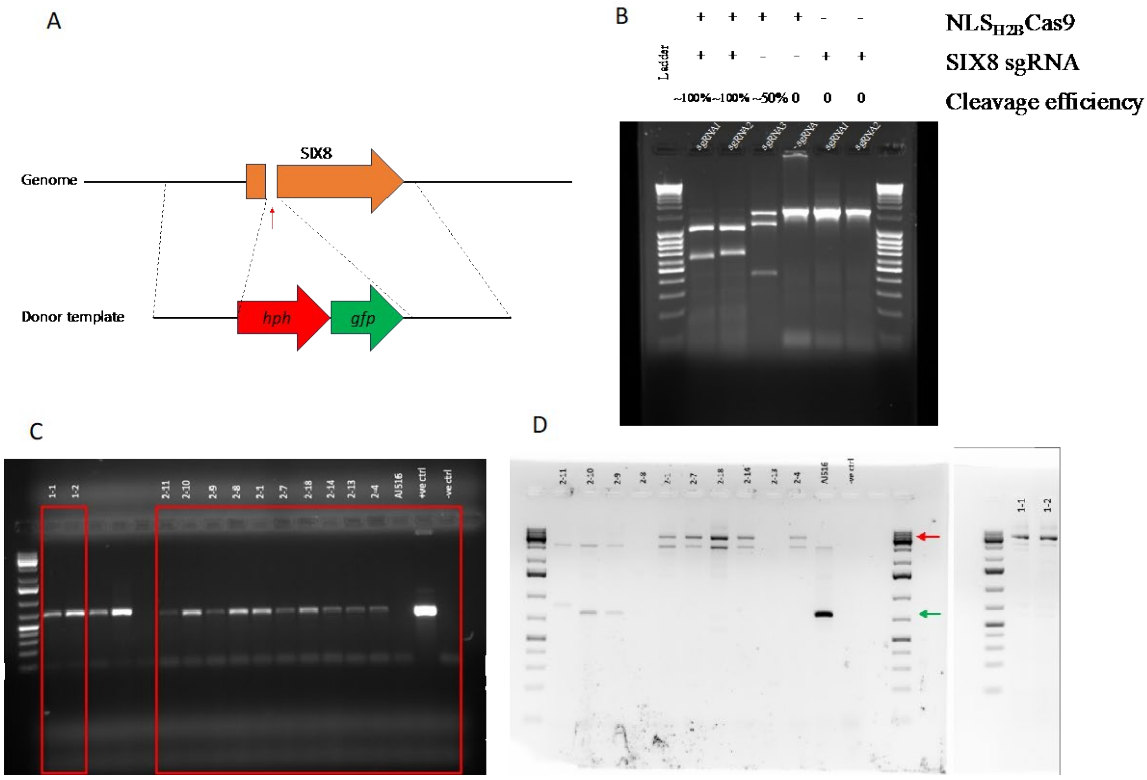

Figure 13 (A) SIX8 homologous directed repair knockout infographic depicting insertion site of hph and gfp genes. (B) The cleavage efficiency of sgRNA for SIX8 in vitro after incubation at 37C for one hour. (C) Gel image of hph PCR amplification of putative SIX8 mutants. (D) Gel images of SIX8 amplification of putative SIX8 mutants; green arrow indicates approximate size of SIX8; and red arrow indicates approximate size of donor DNA insert.

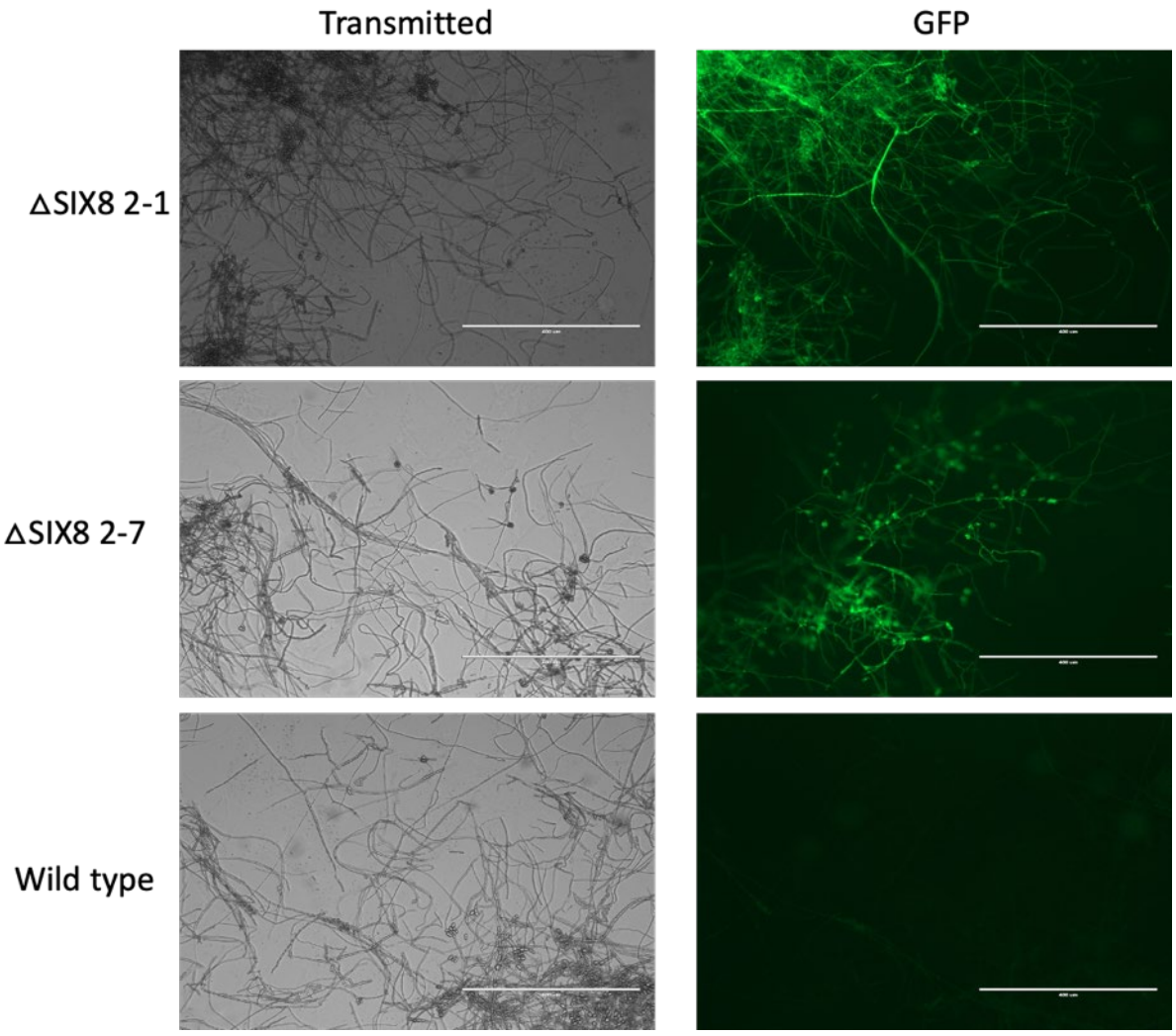

Figure 14 Shows transmitted and GFP fluorescent images of two putative SIX8 mutants compared to the FOL4 AJ516 wild type isolate.

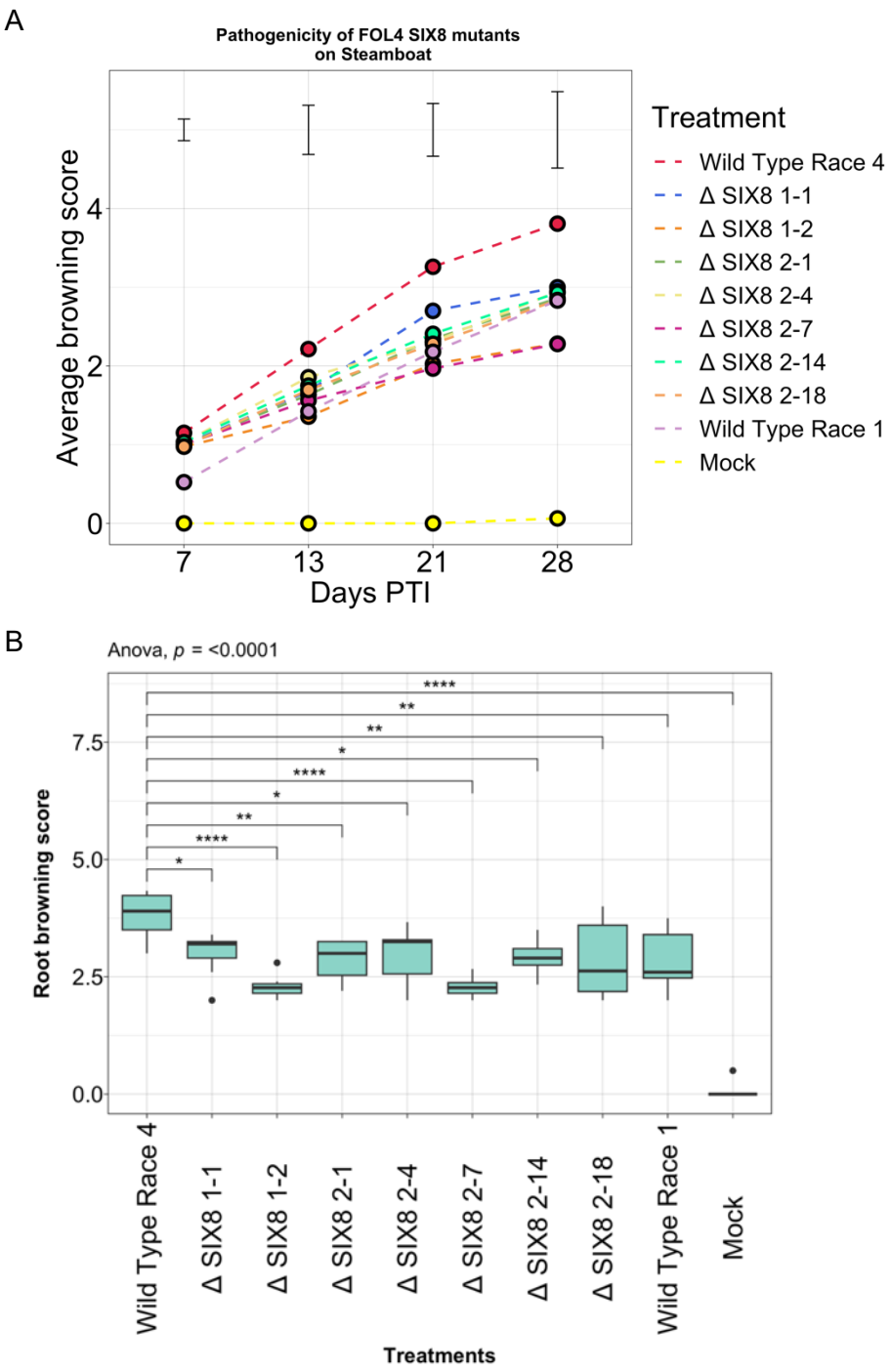

*Figure 15 (A) Average root browning scores in lettuce line cv. Steamboat infected with FOL4 over 28 days. Error bars indicate the least significant difference (LSD) at 5% level. A score of 0 denotes a healthy plant with no browning, scores of 1,2,3, and 4 denote % browning ranges of 1-25%, 26-50%, 51-75%, >75% respectively. A score of 5 denotes whole plant death. (B) Box and whisker plots illustrating the final root browning score of susceptible line cv. Steamboat at 28 days PTI across the ten treatments. ANOVA p value indicated in the top left corner. Brackets indicate Tukey HSD comparisons of treatments against wildtype FOL4. * indicates statistical significance (p <0.05).*

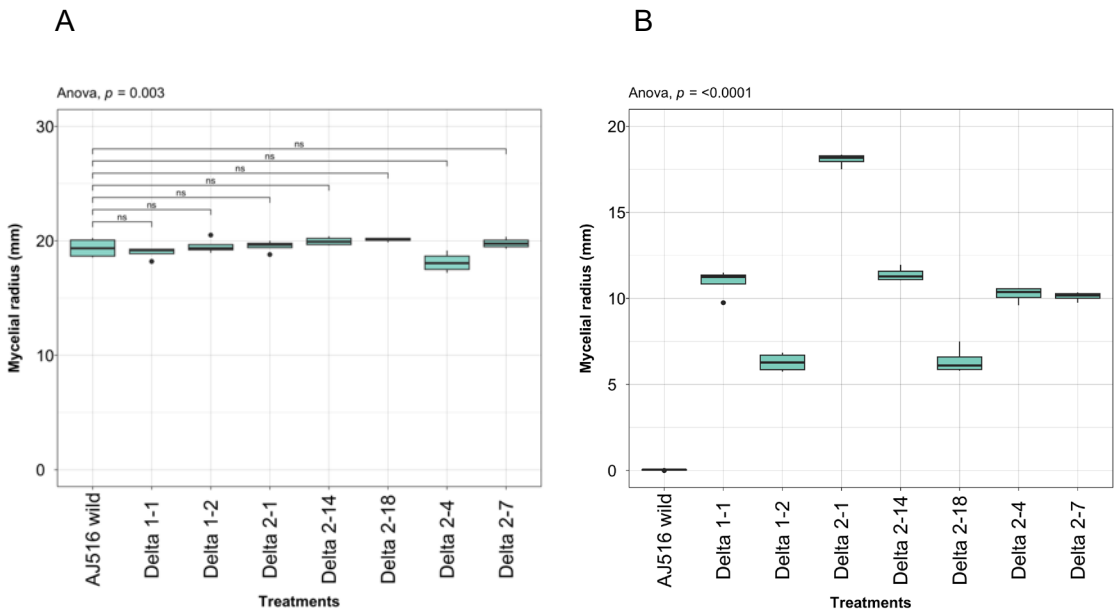

Figure 16 A) Mycelial radii measurements of isolate growth rate on standard PDA plates after 5 days. Brackets indicate Tukey HSD comparisons of treatments against wildtype FOL4. "ns" indicates no statistical significance (p >0.05). B) Mycelial radii measurements of isolate growth rate after 5 days on PDA plates appended with hygromycin. ANOVA p value indicated in the top left corner.

4.4. Objective 4: Confirm resistant and susceptible lettuce phenotypes of selected lettuce lines and examine segregation of a new lettuce mapping population

4.4.1. Confirming resistance / susceptibility of lettuce lines by screening against FOL4

Screening of 15 lettuce lines in a FOL4 infested polytunnel was carried out to confirm their predicted phenotypes. Eleven of the lines were predicted to be resistant to FOL4 and the remainder predicted to be susceptible. A standard susceptible variety cv. Amica was included as a positive control for disease symptoms (Table 6). ANOVA on wilt scores revealed that there were statistically significant differences between the lettuce lines at all the different timepoints ($p < 0.0001$). Average wilt scores at 37 days post inoculation (dpi) indicated that all lines significantly differed from the standard susceptible control (LSD = 0.268, 5% level) (Figure 17). The lines Iceberg, Batavia Blonde, Bloody Warrior, Bibb, Webbs Wonderful, Smaragd, Floricos 83, Cobham Green, Kavir, RZ423-2049 and E01B.11914 exhibited the lowest wilt scores and did not statistically significantly differ from each other based on 5% LSD (Figure 17). Of these lines all had predicted resistant phenotypes except for Kavir and Cobham

Green (Table 6). The lines that exhibited the highest wilt scores were Sabauda, Steamboat, and L sativa however the latter significantly differed from the other two based on 5% LSD showing slightly lower wilt scores. Of these lines, two were predicted susceptible varieties (Steamboat and Sabauda) and one was predicted to be resistant (L sativa) (Table 6). When comparing average wilt scores of Steamboat and Sabauda from 27 dpi to 37 dpi there is no significant difference between wilt scores at these two time points (ANOVA $p > 0.05$). In contrast average wilt scores of the standard susceptible at these two time points are significantly different (ANOVA $p < 0.0001$). This could indicate that the rate of disease progression is lower for these two susceptible lines compared to Amica.

A Kruskal Wallis H test on vascular browning scores 37 dpi indicated that there was an overall significant difference in browning scores between lettuce varieties ($p< 0.0001$). Further analysis using Dunn's multiple comparison test indicated that all the predicted resistant lines had significantly lower average vascular browning scores when compared to the susceptible control ($p< 0.05$) (Figure 18). Moreover, the predicted susceptible lines Cobham green and Kavir had largely significantly lower average vascular browning scores when compared to the susceptible control ($p< 0.0001$; Figure 18). The remaining two susceptible varieties (Steamboat and Sabauda) exhibited no significant difference in average vascular browning score when compared to the susceptible control (Figure 18).

4.4.2. Mapping population screen

Enza produced F2 seed which was used to raise 654 individual plants which were transplanted into the FOL4 infested polytunnel. Figure 19 shows the number of individuals that exhibited different vascular browning scores. Individuals that scored a vascular browning score >1 were considered susceptible. Individuals with a vascular browning score of below 1 were said to be resistant. These criteria produced a segregation ratio of 2.3:1.

Table 6 Summary table of resistant and susceptible parental lines used in FOL4 inoculated poly tunnel trial, along with their phenotypes as observed in previous glasshouse screening tests.

Cultivar	**Source**	**Predicted phenotype**
Iceberg	Warwick crop centre	Resistant
Batavia Blonde de Paris	Warwick crop centre	Resistant
Bloody Warrior	Warwick crop centre	Resistant
Bibb	Warwick crop centre	Resistant
Webbs Wonderful	Warwick crop centre	Resistant
Smarged	Warwick crop centre	Resistant
Floricos 83	Warwick crop centre	Resistant
L sativa (no name)	Warwick crop centre	Resistant
Cobham Green	Warwick crop centre	Susceptible
Kavir	Enza Zaden	Susceptible
Steamboat	Enza Zaden	Susceptible
Sabauda	Enza Zaden	Susceptible
RZ 42-109	Early commercial release Rijk Zwaan	Resistant
RZ 42-2049	Early commercial release Rijk Zwaan	Resistant
E01B.11914	Early commercial release Enza Zaden	Resistant
Amica	Warwick crop centre	Susceptible

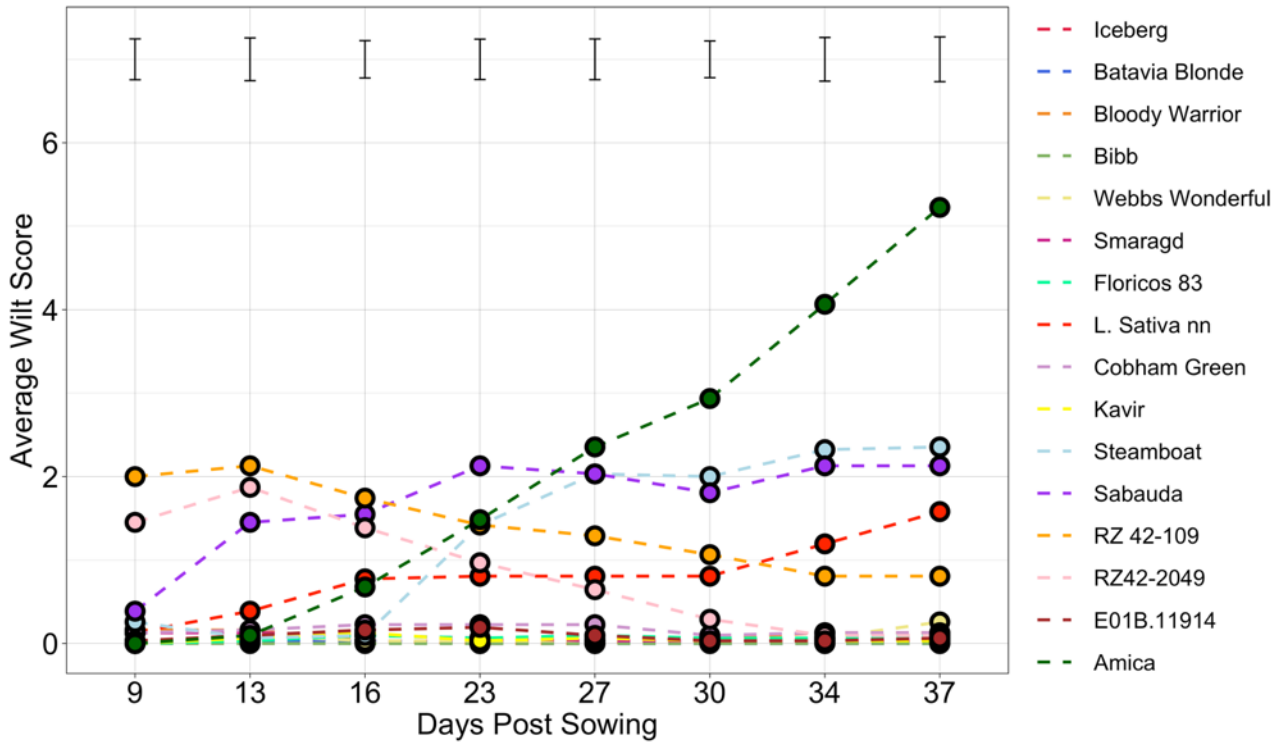

Figure 17 Average wilt scores in resistant and susceptible lettuce lines infected with FOL4 over 37 days. Error bars indicate the least significant difference (LSD, 5% level) at each timepoint. A wilt score of 1 denotes wilting of 1-2 leaves, wilt scores of 2,3,4,5,6 and 7 denote % wilting ranges of <10%, 10-25%, 25-50%, 50-75%, 75-99%, and 100% respectively.

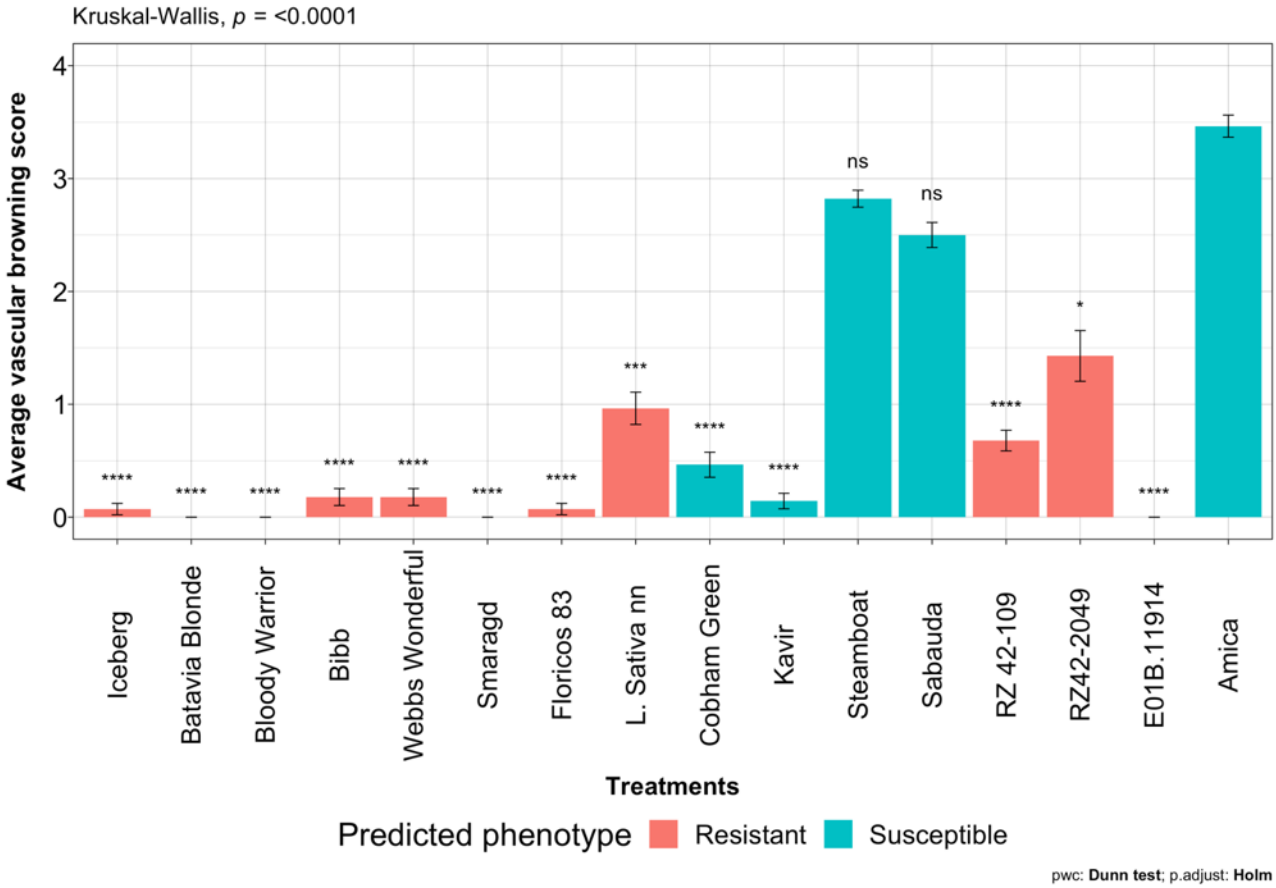

Figure 18 Average vascular browning scores in resistant and susceptible lettuce lines 37 days post infection with FOL4. Error bars indicate standard error of the mean. Significance of each treatment compared to the standard susceptible cv Amica is indicated above each bar ($p < 0.05$). Vascular browning scores 0,1,2,3 and 4 denote the categories of no symptoms, mild vascular browning, vascular browning, severe vascular browning, and plant death respectively. Red bars indicate lines predicted to be resistant and blue bars indicate lines predicted to be susceptible.

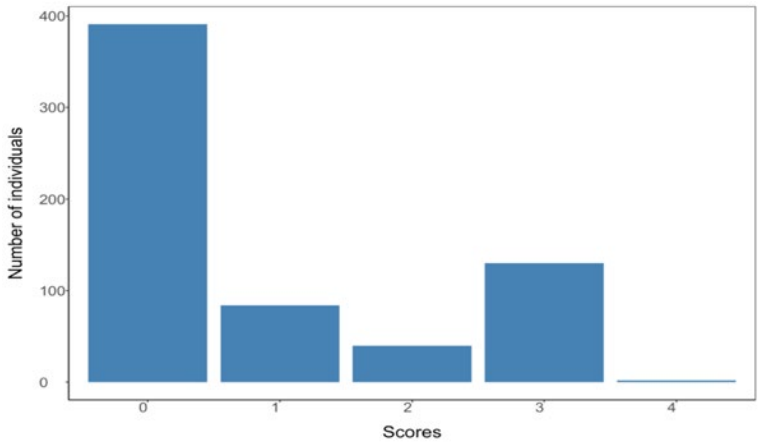

Figure 19 Number of mapping population individuals exhibiting respective vascular browning scores. Vascular browning scores 0,1,2,3 and 4 denote the categories of no symptoms, mild vascular browning, moderate vascular browning, severe vascular browning, and plant death respectively.

4.5. Objective 5: Investigate the extent of root colonisation of resistant and susceptible lettuce lines by FOL4

Nine lettuce cultivars of varying resistance and susceptibility to FOL4 based on previous screens (shown in Table 7) were investigated to determine the extent that FOL4 can colonise the tap root. Leaf wilt scores were taken during the course of infection. ANOVA analysis on the wilt scores at each individual timepoint indicate statistically significant differences between the treatments at all timepoints ($p < 0.05$). Susceptible varieties exhibited higher wilt symptoms more rapidly therefore corresponding to earlier harvests and vascular browning assessments (section 3.4.2) (Figure 20). Visual inspections of vascular browning were carried out with browning ranked on a scale from 0 to 3. An ANOVA revealed overall significant differences in browning score between lettuce lines ($p < 0.0001$) at their respective harvest times (section 3.4.2). Moreover, the vascular browning scores for all the resistant lines were not statistically different from the uninoculated susceptible control based on a Tukey HSD test. Conversely, the intermediately resistant cultivar exhibited significantly more vascular browning compared to the uninoculated susceptible control and the resistant lines ($p <0.01$). However, this line was not statistically significant compared to the susceptible line Steamboat. Lastly, all of the susceptible lines were significantly more diseased than the control ($p < 0.0001$) and exhibited the highest vascular browning scores (Figure 21).

FOL4 could be isolated from different locations inside the taproot. PCR detection of FOL4 was used to confirm identity of FOL4 like morphologies after isolation (section 3.5.2). Three FOL4 like morphologies (purple, white, and orange pigment types) were isolated from the lettuce root tissues and exemplar samples from different lettuce lines and from the different locations were selected to screen against FOL4 specific PCR primers. The results indicate that two of the three morphology types (purple and white) were found to be PCR positive for FOL4. Therefore, the purple and white pigment types were considered as positive FOL4 identifications whilst the orange pigment type was considered as negative (Table 8). Recovery of FOL4 was then recorded across the lettuce lines and across the three root locations.

A Fishers Exact test revealed that there were overall significant differences between lettuce treatments in all three root locations ($p < 0.0001$). The results indicated that the pathogen could be isolated from the bottom and middle locations in all lettuce lines. Although resistant lines (with the exception of Bloody Warrior) showed lower percentage recovery rates from these locations (Figure 22B). Isolation of FOL4 from the top of the taproot was observed in all susceptible lines (with the exception of Sabauda) and in the intermediate line. Interestingly

FOL4 also colonised the top location in resistant lines Bloody Warrior and L sativa but was not isolated from the top location in Webbs Wonderful or Bibb (Figure 22B). The extent of recovery of FOL4 in resistant line Webbs Wonderful was not significantly different to that of the susceptible lines in the middle and bottom locations ($p< 0.05$). The standard susceptible variety Amica exhibited the highest percentage recovery in all locations and exceeded 89% in all locations. Treatments were compared to the standard susceptible using pairwise Fishers tests. This revealed that in the bottom location percentage recovery only significantly differed in resistant lines Webbs Wonderful, Bibb, and L sativa ($p < 0.05$) when compared to Amica. In the middle section all lines except for Bloody Warrior and Banchu red fire significantly differed from Amica ($p < 0.05$). Finally, in the top section all lines significantly differed from Amica ($p < 0.05$) and only Amica significantly differed from the uninoculated control ($p < 0.001$).

Combining isolations from all root sections and comparing pooled counts using Fishers Exact test indicated that there was an overall significant difference between FOL4 recovery in the different locations ($p < 0.0001$). Interpreting this using pairwise Fishers Exact tests it was found that there was no significant difference between FOL4 recovery in the bottom and middle locations. However, there were large significant differences between FOL4 recovery when the bottom and middle locations were compared to the top location ($p < 0.0001$). When the locations were compared within lettuce treatments using Fishers Exact test it was found that for Amica there was no significant difference in FOL4 recovery between the three locations. However, in the other two susceptible lines as well as in the intermediate resistant line there was a significant difference in FOL4 recovery between the three locations ($p < 0.05$). For all resistant lines (except for Bloody Warrior) there was no significant difference in FOL4 recovery in the different locations.

Table 7 Summary table of resistant, intermediate, and susceptible lines used in glass house trial investigating root colonisation by FOL4.

Cultivar	Source	Phenotype
Webbs Wonderful	Warwick crop centre	Resistant
Bloody warrior	Warwick crop centre	Resistant
Bibb	Warwick crop centre	Resistant
L sativa (no name)	Warwick crop centre	Resistant
Banchu red fire	Warwick crop centre	Intermediate
Sabauda	Enza Zaden	Susceptible
Steamboat	Enza Zaden	Susceptible
Amica	Warwick crop centre	Susceptible

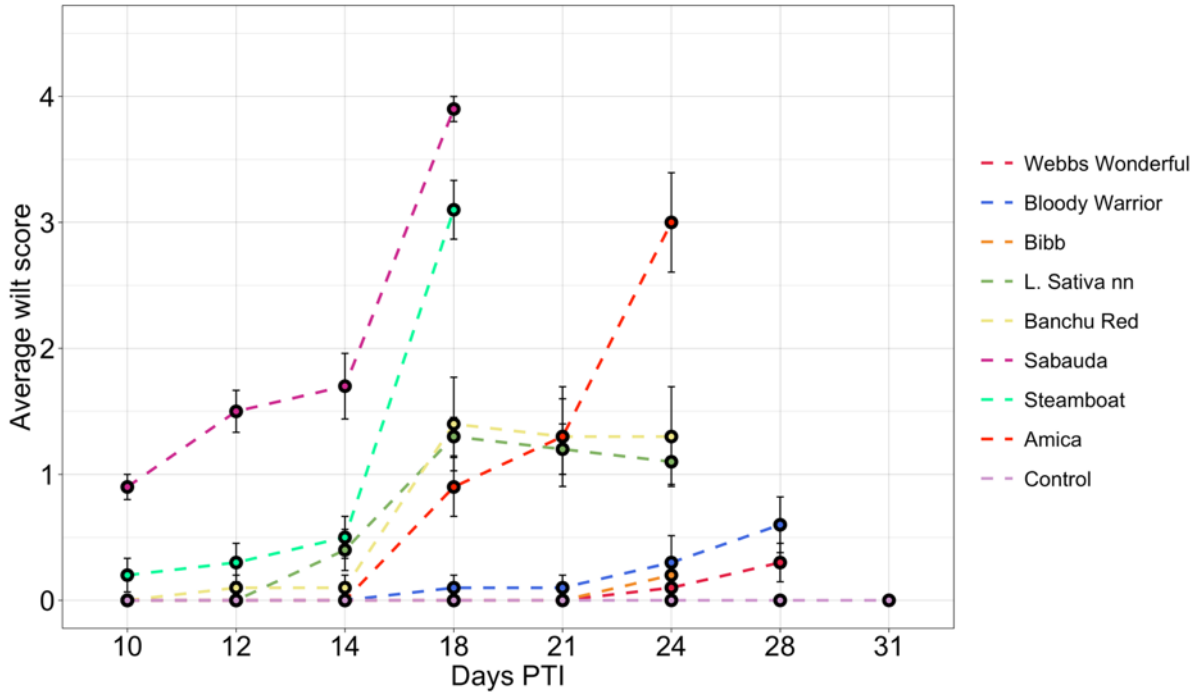

Figure 20 Average wilt scores in resistant, intermediate resistant, and susceptible lettuce lines infected with FOL4 over 31 days. Error bars indicate the standard error of the mean. A wilt score of 1 denotes wilting of 1-2 leaves, wilt scores of 2,3,4,5,6 and 7 denote % wilting ranges of <10%, 10-25%, 25-50%, 50-75%, 75-99%, and 100% respectively.

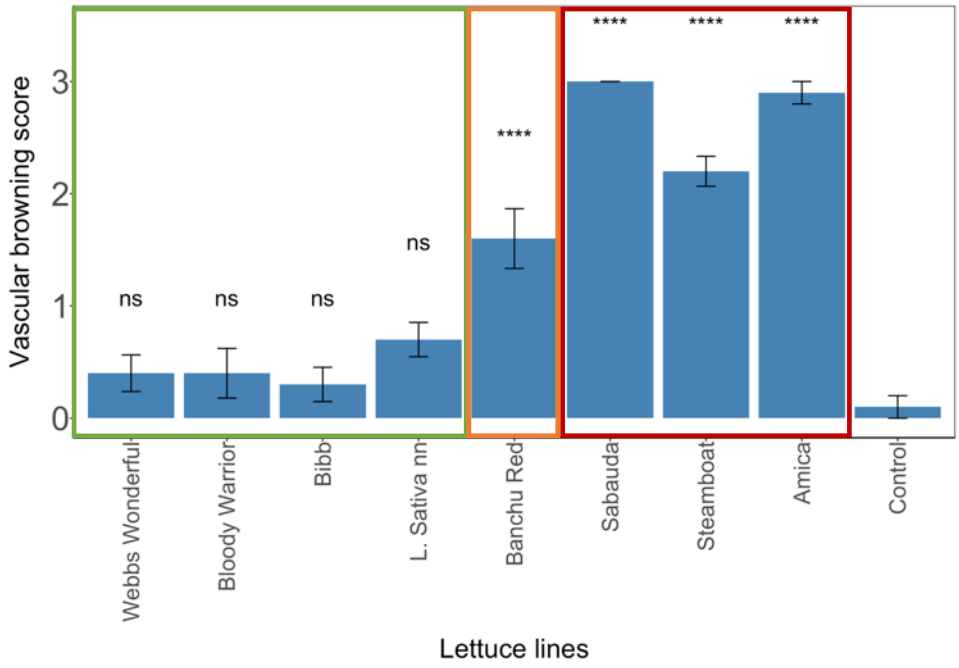

Figure 21 Average vascular browning scores in resistant (denoted within the green box), intermediate resistant (denoted within the orange box), susceptible lettuce lines (denoted within the red box) and an uninoculated control; recorded either when the majority of individuals of a line reached a wilt score of 3-4 or if latter wilt scores were not achieved 3-5 weeks post infection with FOL4. Error bars indicate standard error of the mean. Vascular browning scores 0,1,2,3 and 4 denote the categories of no symptoms, mild vascular browning, vascular browning, severe vascular browning, and plant death respectively. Significance of each treatment compared to the control is indicated above each bar ($p < 0.05$).

Table 8 FOL4 diagnostic PCR results from mycelium taken from exemplar PDA plates of the different morphology types in the different root locations of the different lettuce lines tested. "+" indicates a positive PCR result whilst "-"indicates a negative PCR result.

Line	Root location	Morphology type	FOL4 Diagnostic PCR
Webbs Wonderful	1	FP	+
Bloody Warrior	1	FP	+
Bloody Warrior	1	FW	+
Bibb	1	FW	+
Banchu Red Fire	1	FP	+
Banchu Red Fire	1	FW	+
Sabauda	1	FP	+
Sabauda	1	FW	+
Steamboat	1	FP	+
Amica	1	FP	+
Amica	1	FW	+
Webbs Wonderful	2	FP	+
Bloody Warrior	2	FP	+
Bloody Warrior	2	FW	+
L sativa (no name)	2	FW	+
Banchu Red Fire	2	FP	+
Banchu Red Fire	2	FW	+
Sabauda	2	FP	+
Steamboat	2	FO	-
Amica	2	FP	+
Amica	2	FW	+
Bloody Warrior	3	FW	+
L sativa (no name)	3	FW	+
Banchu Red Fire	3	FW	+
Steamboat	3	FO	-
Amica	3	FP	+
Amica	3	FW	+

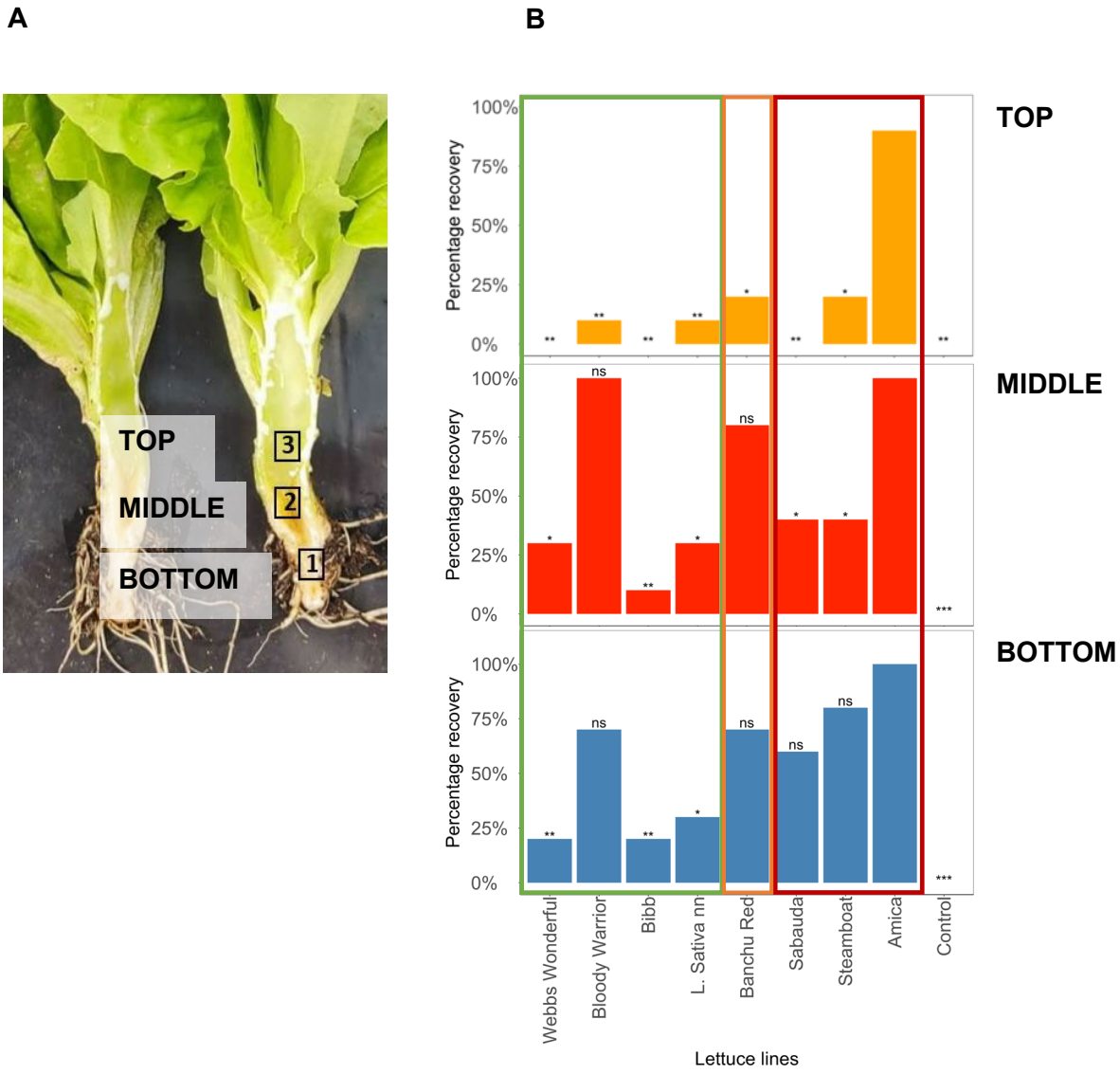

Figure 22 A) Locations on lettuce tap root used for FOL4 isolations. B) Percentage recovery of FOL4 from lettuce root pieces from different isolation locations for resistant (denoted within the green box), intermediate resistant (denoted within the orange box) and susceptible (denoted within the red box) lettuce lines. Significance of each treatment compared to the standard susceptible (cv. Amica) is indicated above each bar ($p < 0.05$).

5. Conclusions / Discussion

5.1. Objective 1: Characterise FOL isolates from different European locations.

Results indicated that FOL1 and FOL4 shared identical *TEF* sequences therefore indicating that *TEF* sequencing can't distinguish between FOL1 and FOL4 isolates. It was also found that FOL1 and FOL4 *TEF* sequences differed from FOL2 and FOL3 *TEF* sequences. Moreover, FOL2 and FOL3 sequences (acquired from NCBI) differed from each other. This could indicate that FOL1 and FOL4 have a monophyletic origin as opposed to FOL2 and FOL3 which likely have differing origins from each other and FOL1/FOL4 (Figure 6).

Investigations into FOL1 and FOL4 *SIX* gene complements found that FOL1 isolates contained *SIX9* and *SIX14* whilst FOL4 isolates contained *SIX8*, *SIX9* and *SIX14*. These results were found to be consistent amongst all FOL1 and FOL4 isolates tested. Moreover, these results correctly correlated with results from FOL1 and FOL4 diagnostic tests therefore allowing for confirmation of race type based on SIX gene presence / absence (Table 1).

Variation in SIX gene sequences was also investigated and no variation was seen in *SIX9* and *SIX14* sequences between and within the two FOL races (Figure 8 and Figure 9). However, variation was found within FOL4 *SIX8* sequences. Here it was found that there were two sequence types which loosely correlated with isolate origin (Figure 7).

5.2. Objective 2: Identify putative FOL4 effector genes expressed during lettuce infection through RNAseq and confirm their roles in virulence

Results indicated that there were significant differences in relative expression over time in all lettuce varieties ($p < 0.001$ for all genes) with the overall highest expression of the genes seeming to occur between 96 to 120 hpi. There was no significant difference between the 96 and 120 hpi timepoints for all treatments, which could indicate a plateau effect or slight reduction in expression past 96 hpi (Figure 10 and Table 4). This led to the selection of the 96 hour timepoint for further RNAseq analysis.

Based on heatmap and PCA analysis there were large differences in FOL4 expression *in planta* when compared with *in vitro* grown FOL4. However hardly any differences in FOL4 expression between the two different lettuce varieties (Figure 11 and Figure 12).

The differentially expressed gene analysis identified a range of highly expressed putative effectors (Table 5). *SIX8*'s role in pathogenicity was investigated further but the remainder may also have important roles. Interestingly some *lactucae* specific uncharacterised putative effectors were uncovered from the analysis, which could be involved in FOL specificity on lettuce.

5.3. Objective 3: CRISPR Cas9 mediated knockouts of FOL4 putative effectors

Results indicate that there was a significant difference between root browning caused by the *SIX8* knockout mutants compared to wild type FOL4 AJ516 after 28dpi ($p < 0.05$). A significant difference was also observed between root browning caused by the *SIX8* knockout mutants when compared to uninoculated control plants ($p < 0.0001$). This indicates that the loss of SIX8 reduced pathogenicity but did not abolish it, indicating that SIX8 plays a significant role in FOL4 pathogenicity on lettuce. Future *SIX8* complementation tests aiming to restore full pathogenicity of mutants should robustly indicate *SIX8*'s role in virulence.

Growth assays were carried out to ensure loss of pathogenicity wasn't due to loss of fitness from transformation. These results indicated that the *SIX8* knockout mutants did not grow significantly different from the wild type (Figure 16A) thus eliminating reduced fitness from the reason for reduced pathogenicity. It was also recorded that *SIX8* mutants grew differentially in the presence of the selection antibiotic hygromycin. This could be due to multiple insertions of the selection cassette which could increase total expression of the hygromycin resistance gene enabling faster growth.

5.4. Objective 4: Confirm resistant and susceptible lettuce phenotypes of selected lettuce lines and examine segregation of a new lettuce mapping population

Disease assessments indicated that lettuce lines Steamboat and Sabauda were very susceptible to FOL4 exhibiting high vascular browning and wilt scores, although not as susceptible as the standard susceptible cv. Amica (Figure 17 and Figure 18). Conversely, the susceptible lines Cobham Green and Kavir did not differ greatly from some of the resistant lines (Figure 17 and Figure 18). Of the resistant lines the majority displayed low levels of vascular browning. However, resistant lines L sativa, RZ 42-109 and RZ 42-2049 showed low levels of disease indicating mild susceptibility to FOL4 (Figure 17 and Figure 18). These results have therefore identified some clear phenotypic differences between selected resistant and susceptible lines which could therefore be used as parents of mapping populations for future genetic analyses. This could potentially lead to identification of novel lettuce resistance loci/genes which could culminate in the breeding of new FOL4 resistant lettuce cultivars.

5.5. Objective 5: Investigate the extent of root colonisation of resistant and susceptible lettuce lines by FOL4

Nine cultivars exhibiting differing levels of resistance to FOL4 based on phenotypic wilt and vascular browning were assessed to investigate the extent of root colonisation by FOL4. Results showed that susceptible lines showed higher wilt and vascular browning scores, therefore leading to earlier harvests and vascular browning assessments (section 3.4.2; Figure 20). A similar trend was seen in the vascular browning scores, which showed that the vascular browning of the resistant cultivars didn't statistically differ from the uninoculated control. Conversely, the susceptible lines exhibited the highest vascular browning scores, and all significantly differed from the control ($p < 0.0001$) (Figure 21). The intermediate resistant line (Banchu red fire) significantly differed from the control, and only differed from two of the three susceptible lines. Therefore, confirming phenotypes of the different lines moving forward.

Three FOL like morphologies were seen to grow out of infected root isolations. PCR diagnostic tests revealed that two of the three morphologies (purple and white) tested positive for FOL4 when exemplar samples were screened (Table 8). In the bottom location all lines except for three of the resistant lines (Webbs wonderful, Bibb, and L sativa) ($p <0.05$) did not significantly differ in their percentage recovery of FOL4 when compared to the standard susceptible Amica (Figure 22). In the middle section, only Bloody Warrior (resistant) and Banchu red fire (intermediate resistant) did not significantly differ from Amica. Finally, in the top section all lines significantly differed to Amica ($p < 0.05$), and only Amica significantly differed from the uninoculated control ($p < 0.001$). The results indicate that the pathogen could be isolated from the bottom and middle locations in all lettuce lines. Therefore, showing FOL4 is able to colonise different parts of the taproots of resistant lines without causing symptoms. Moreover, the majority of the resistant lines (with the exception of Bloody Warrior) exhibited significantly lower percentage recovery in all locations compared to the standard susceptible. The two non-standard susceptible lines also differed compared to the standard susceptible in that they showed significantly lower recovery in the middle and top locations, indicating reduced colonisation of FOL4 on these lines. The intermediate resistant line (Banchu Red Fire) and the resistant line Bloody Warrior showed similar recovery profiles across all the locations. Interestingly they showed similar levels of recovery compared to the standard susceptible in the bottom and middle locations but significantly lower recovery in the top location.

Interpreting comparisons between locations within lettuce treatments it was seen that there was no significant difference in FOL4 recovery between the three locations in Amica, indicating that FOL4

can easily and rapidly colonise all parts of the roots in Amica. However, in contrast in the other two susceptible lines as well as in the intermediate resistant line there was a significant difference in FOL4 recovery between the three locations, indicating limitation of FOL4 colonisation to the upper parts of the taproot in these lines, therefore differing levels of colonisation was seen within susceptible lines exhibiting similar phenotypic scores. Finally, there was no significant difference in FOL4 recovery in the different locations in the resistant lines (except for Bloody Warrior), potentially indicating a different mechanism of resistance or tolerance in Bloody Warrior. Bloody Warrior therefore still maintained phenotypic resistance with low wilting and vascular browning scores whilst still showing comparable levels of FOL4 recovery to susceptible lines. Overall, it appears that FOL4 behaves endophytically in all resistant lines and pathogenically in the intermediate and susceptible lines.

These findings indicate use of resistant material in the field may not reduce inoculum levels of FOL4 as the pathogen is still able to colonise and grow on resistant material. Good hygiene practices to prevent pathogen spread and incidence is therefore still of importance for FOL4 control. It has been shown that single gene resistance and harsh avirulence responses can lead to increased selection pressure for a pathogen to break resistance (Pagán & García-Arenal, 2018). Therefore, future investigation of resistance mechanisms leading to tolerant or intermediate resistant phenotypes seen in Bloody Warrior and L sativa may be of interest.

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