Project title:	Exploiting next generation sequencing technologies to understand pathogenicity and resistance in *Fusarium oxysporum* (HAPI)
Project number:	CP 116
Project leader:	John Clarkson, Warwick Crop Centre
Report:	Annual report, August 2016
Previous report:	Annual report, July 2015
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Location of project:	Warwick Crop Centre, School of Life Sciences, University of Warwick, Wellesbourne Campus, Warwick, CV35 9EF
Industry Representative:	N/A
Date project commenced:	31/03/14
Date project completed (or expected completion date):	31/03/17

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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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CONTENTS

GROWER SUMMARY

Headline

This Grower Summary was extracted from the BBSRC/HAPI Report produced by Dr John Clarkson. The content has been slightly abridged for this purpose. A copy of the full report is available from AHDB Horticulture on request.

Background

Onion is an important horticultural crop which is cultivated by every agricultural nation in the world and is also the second most valuable vegetable crop in the world behind tomato. Onions are a staple crop in many countries and deliver a range of health benefits including anti-carcinogenic, antithrombotic and antibiotic effects. Despite the value of the crop, research into breeding and genetic improvement is limited as few resources such as onion diversity sets, segregating populations, pure breeding lines and genomic information are available.

Diseases are one of the major constraints to onion production and one of the most important is caused by the soilborne plant pathogen *Fusarium oxysporum*. This fungus is diverse and has many different sub-species (formae speciales, f.spp.) which attack various crop plants. In onion, *F. oxysporum* f.sp. *cepae* (FOC) can infect plants at any stage causing a 'damping-off' symptom on seedlings and a basal rot on more mature plants and bulbs. This results in severe pre- and/or post-harvest losses and has been estimated to cost farmers in the UK in the region of £11M per annum. As FOC is a soilborne pathogen which produces long-lived spores that survive for many years, control approaches are difficult and have previously relied on the use of soil sterilisation / pasteurisation, drenches with fungicides or seed treatments. This approach has largely been unsuccessful, has undesirable environmental effects and is threatened by legislation governing restrictions in pesticide use. In the absence of effective control measures for Fusarium basal rot, identifying resistance in onion is extremely desirable but so far has been relatively unsuccessful. However, using a highly pathogenic FOC isolate in a rapid screening test we developed using onion seedlings, we have identified onion lines with much higher levels of basal rot resistance than current commercial cultivars.

In a collaborative project between the University of Warwick, East Malling Research and the international vegetable breeding company Nickerson-Zwaan, we aim to provide information, tools and resources which will lead to more effective and sustainable control of Fusarium basal rot, primarily through the development of FOC-resistant onion lines. This will benefit a wide range of stakeholders including breeders, growers and other researchers. The main outcomes of the project will be 1) the identification of FOC pathogenicity/effector genes which could be used as markers to distinguish this pathogen from other *F. oxysporum* f. spp. or non-

pathogenic isolates, 2) the identification of FOC resistance loci and associated genetic markers in onion for use in future breeding programmes, 3) the production of new onion populations segregating for FOC resistance and pre-breeding onion lines to enable the development of basal rot resistant onion cultivars for the industry. Growers and the industry will clearly benefit from this research as the deployment of resistant cultivars will give them a more sustainable and attractive option for basal rot control in onion in the future. In addition, DNA markers associated with genes controlling FOC pathogenicity should also provide a platform for developing diagnostic and quantitative tests for the pathogen in soil, onion seed, sets and bulbs which will help farmers make decisions about disease risk and develop management options. Overall this means that the public will benefit from better quality onions grown with reduced pesticide inputs.

Summary

Project Objectives

WP1 Determine the basis for FOC pathogenicity using genome sequencing (Sep 2015)

WP2 Map FOC resistance loci in onion and identify genetic markers (July 2017)

WP3 Determine gene expression for pathogenic / non-pathogenic Fusarium isolates (March 2017)

WP4 Produce pre-breeding resistant onion lines and additional onion mapping populations (March 2017)

WP1: Determine the basis for FOC pathogenicity using genome sequencing

All the milestones for this WP1 were completed at the end of January 2016, but further sequencing, refinement of genome assembly and analysis is continuing with the remaining allocated resources.

1.1 Extract DNA from different Fusarium isolates; 1.2 Genome sequencing of Fusarium isolates

DNA extraction and genome sequencing (Illumina) was carried out for an additional four *Fusarium oxysporum* isolates from onion this year bringing the total number to eleven isolates comprising pathogenic, non-pathogenic and intermediate types. An additional eight Fusarium isolates were also genome sequenced, comprising five *F. oxysporum* pathogens from pea where pathogenicity is being confirmed in a related PhD project, one from daffodil, confirmed to be pathogenic and isolates of *Fusarium avenaceum* (pathogenic on pea) and *F. proliferatum* (isolated from onion). DNA extraction and library preparation for these isolates

was performed using PCR-free Illumina TruSeq protocols. Sequencing of all the *F. oxysporum* isolates was carried out on an Illumina MiSeq.

1.3 Draft genome alignment; 1.4 Refine genome alignment and identify effectors and location

Genomes of *F. oxysporum* isolates from onion were assembled from scratch and continued development of bioinformatic pipelines has allowed improved assemblies of all Fusarium genomes. Assemblies were 48-51 Mb in size, and were considered to have a good representation of the core *F. oxysporum* genome. Important progress has also been made in gene prediction as a result of RNAseq data generated as part of WP3 being used to aid training of programs (Braker1) to predict genes in assembled genomes. As noted in last year's report, regions homologous to a broad range of effectors and pathogenicity-related genes held in the database PHIbase have been identified including genomic regions showing homology to the seven SIX genes (SIX3, SIX5, SIX7, SIX9, SIX10, SIX12 and SIX14) previously identified as being associated with pathogenicity on onion through PCR tests (Taylor *et al*., 2015). This year, comparative genomics analyses have also been carried out for three *F. oxysporum* pathogenic and three non-pathogenic isolates on onion. Orthology analysis (Fig. 1) between predicted genes has identified 202 orthogroups (gene families) only found in pathogenic isolates which likely represent genes from lineage-specific regions of FOC. Furthermore, functional annotation has allowed identification of two additional transcription factors, which may be involved in regulation of effectors. This list of effector candidates now provides targets for functional characterisation using gene knockouts.

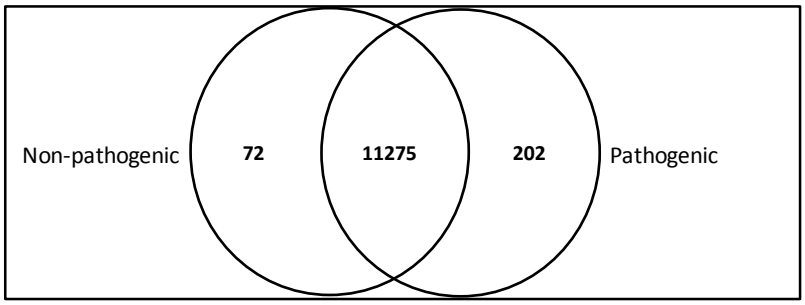

Figure 1. Results of orthology analysis showing number of gene families exclusive or common to pathogenic and non-pathogenic isolates for *F. oxysporum*

Relevance to Industry: further identification of specific genes associated with *F. oxysporum* pathogenicity on onion is providing more targets for a potential diagnostic test for FOC. This will help determine the presence of the pathogen in seed, soil, plant or bulb samples and hence assess potential disease risk in the field or before storage of onion bulbs. Furthermore, identifying specific effectors in FOC may also allow identification of corresponding resistance genes in onion.

WP2: Map FOC resistance loci in onion and identify genetic markers

2.1 extract DNA from 192 segregating onion F2 individuals; 2.2 KASP genotyping of 192 onion individuals; 2.3 screen 192 onion individuals for FOC resistance

A number of onion lines were selected for quantifying resistance to FOC in a mature plant bulb test using the method described by Taylor *et al*., (2013). These comprised two FOC resistant half sib onion lines (R1, 152 individuals; R5, 173 individuals), two further half sib lines from the same parental line (HSR3, 200 individuals; HSR5, 62 individuals), parental lines for each source of resistance (PR3, 119 individuals; PR5, 120 individuals) as well as a susceptible commercial control line (cv. Sturon) to give a total of 946 individuals assessed for FOC resistance. Briefly, onion seedlings from each line were transplanted into compost inoculated with FOC isolate FUS2 (1 x 105 cfu/g) in a heated glasshouse (25°C day, 18°C night) and plant mortality scored on a weekly basis. After nine weeks, bulbs were allowed to dry out for two weeks and basal rot symptoms then scored on a scale of 0-3 (where 3 is severe rot). Data were converted to a resistance score ranging from 1 (very susceptible, plant dies in week 2) to 11 (completely resistant, healthy onion bulb). For resistant line R1, 32% of the plants had the maximum resistance score of 11 and an additional 14% had a score of 6-10 compared to the susceptible control where 53% of plants had a resistance score of 3 and the maximum score of any individual plant was 5 (Fig. 2). Line R5 also showed a good level of resistance, with 21% of plants having a score of 11 and an additional 18% having a resistance score of 6-10. As expected, both parent lines also showed some segregation for resistance. The additional half sib lines HSR3 and HSR5 demonstrated low and high resistance to FOC (3% of plants with a score of 11 and 40% of plants with a score of 11 respectively). Leaf samples were taken from all plants three weeks after transplanting, and then lyophilised before DNA was extracted from all 152 R1 plants and 34 plants from R5 (individuals with a range of resistance scores). Genotyping was then carried out using the 450 KASP markers identified previously as being polymorphic using the service at Hazera Seeds. As the parent lines of both R1 and R5 were hybrid F1 onion varieties, the observed segregation of FOC resistance in these half sib F2 populations was expected and the

corresponding genotyping data will now allow us to carry out a QTL analysis for FOC resistance. Although the genotyping data has only just been generated, initial analysis indicates that a number of markers are significantly linked to FOC resistance in R1. This suggests that it should be possible to identify QTL for FOC resistance once a genetic map has been generated. The project will continue to focus on R1 as this was originally the best source of FOC resistance for a long day onion line, but clearly the R5 line also shows promise and may contain a different source of resistance that can be exploited in the future.

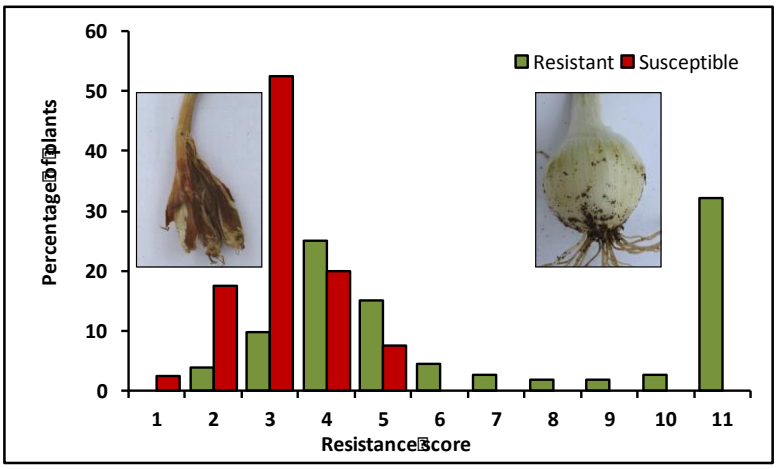

Figure 2. FOC resistance score for line R3 (green bars, resistant) and a susceptible control line (red bars).

Relevance to Industry: good levels of resistance to FOC have been confirmed in two onion lines and genotyping and subsequent preliminary analysis of one of these has already indicated potential genetic markers associated with this important trait. Once confirmed, these markers will enable the more rapid selection of resistant onion lines without the need for screening, hence speeding up the breeding process to produce an elite onion variety with good resistance to FOC.

WP3: Determine differential gene expression in interactions between pathogenic / non-pathogenic Fusarium isolates and susceptible / resistant onion lines using RNA-seq and nanostring

3.1 Optimise onion inoculation procedures and conditions for Fusarium (completed year 1)

3.2 RNA extraction from onion roots (different time points) following inoculation with pathogenic Fusarium isolate (completed year 1)

3.3 RNA-seq and initial transcriptome analysis of samples from 3.2 (completed year 1)

3.4 RNA extraction from onion roots (single timepoint) following inoculation with pathogenic/non-pathogenic Fusarium isolates

A replicated experiment was set up using a sterile, agar-based system to grow and inoculate onions with FOC as described in the previous report. Three *F. oxysporum* isolates were used to inoculate a susceptible onion line and comprised FUS2 (pathogenic), Fo47 (non-pathogenic) and 55 (intermediate pathogenicity). Root and shoot samples were taken 72 h post-inoculation, followed by RNA extraction. Inoculation of a FOC resistant onion line however was not carried out as initial experiments indicated that in the agar system, the resistant line was overcome and became infected with FOC at a similar rate to the susceptible control. Further attempts to promote a resistant phenotype by manipulating FOC inoculum levels and temperature were unsuccessful. Part of the reason for this is that the current FOC resistant lines available still segregate for resistance with only approx. 30% of individuals showing high levels of resistance (see WP2). This means that of the 12 onion individuals used in each agar plate, only four on average will be resistant. Until we have a FOC resistant line with a higher level of resistance (WP4) it will be difficult to develop a system whereby we can compare gene expression in resistant and susceptible onion lines. Overall however, the main emphasis of this WP was always to examine gene expression differences between pathogenic and non-pathogenic *F. oxysporum* isolates rather than and make comparisons between resistant and susceptible onion lines. Nonetheless, alternative inoculation systems suitable for transcriptome analysis will continue to be tested where possible, in order to reproducibly obtain the FOC resistant onion phenotype.

3.5 RNA-seq of 3.4, transcriptome analysis and nanostring probe development

Transcriptome sequencing of the root samples from 3.4 was carried out using Illumina Hi-Seq with Illumina TruSeq library preparation. Paired-end RNA reads were aligned to the available FOC FUS2 genome (WP1) and alignment rates ranged between 3 and 7% over the three isolates Fo47, FUS2 and 55. Differential gene expression analysis was then performed which clearly showed large differences in gene expression between the pathogenic isolate FUS2 and non-pathogenic isolate Fo47 with 782 and 1143 genes significantly differentially up- or down-regulated respectively. Visualisation of these revealed a subset of genes highly upregulated in FUS2 (Fig. 3) and these included SIX genes and other putative effectors also identified in the genome analysis (WP1). These genes therefore represent potential targets for further expression analysis using nanostring or RNA-seq.

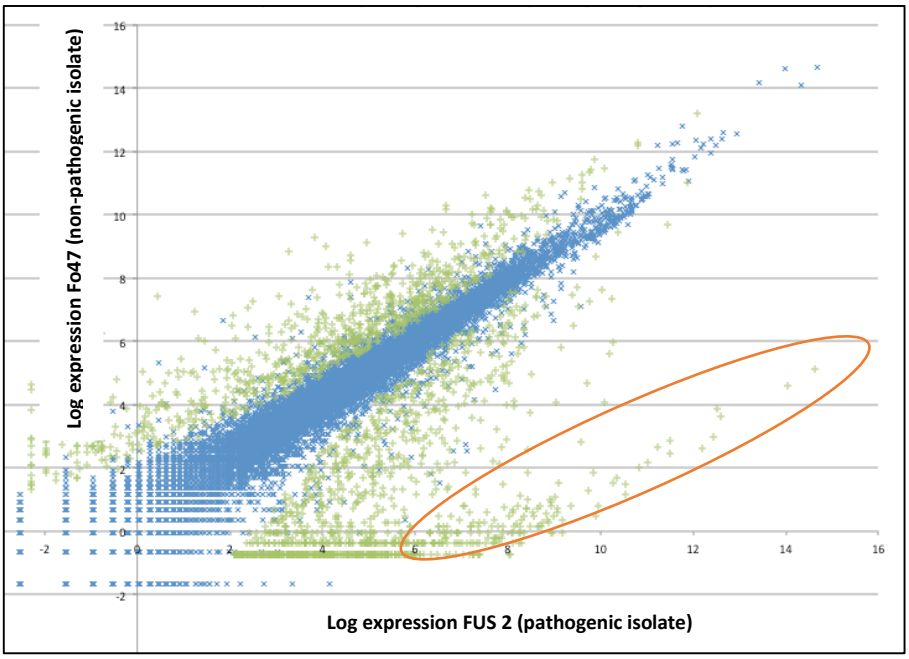

Figure 3. Differentially expressed genes between pathogenic (FUS2) and non-pathogenic (Fo47) isolates of *F. oxysporum* on onion. Selected area indicates genes highly upregulated in FUS2.

Relevance to Industry: this WP further confirms expression of pathogenicity genes specific to FOC and provides information on expression levels. Again this provides a set of targets for diagnostics as outlined for WP1.

WP4: Produce pre-breeding resistant onion lines and additional onion mapping populations

4.1 Generation and testing of F2 onion populations from two DH parents segregating for FOC resistance (4.1.1-4.1.5)

As indicated in the previous report, DH plants of FOC resistant onion lines (R1-R5) have been produced and crosses with susceptible DH lines will be made in 2016 (4.1.5). This will facilitate further genetic analysis and refinement of QTLs for FOC resistance.

4.2 Generation of onion F3 families (4.2.1-4.2.3)

As indicated in the previous report, crosses were made between non-DH plants of the FOC resistant lines R1-R5 and a Hazera DH susceptible line with the F2 seed produced in 2015 (4.2.2). Approx. 400 different seed lots from different combinations of crosses including selfs of the individual R1-R5 parent plants used to make these crosses (S2) have now been generated. As reported last year, F3 lines will not be available until later in 2016 meaning that phenotyping for FOC resistance using the mature plant bulb test will not be possible within the timeframe of the project. Therefore, we will now phenotype and genotype (as the budget allows) a number of F2 lines derived from the crosses of R1, R2 x susceptible DH line H4 which will be segregating for FOC resistance using the bulb test in 2016 (milestone change). This will allow further refinement of the QTL analysis being carried out in WP2. To make sure we select the F2 families derived from the widest possible crosses for this, a seedling test is currently underway using S2 seed derived from the parent plants to confirm both strong FOC resistance in the R1, R2 parents and susceptibility in the H4 parent plants.

Relevance to Industry: considerable progress has been made in creating new onion populations segregating for FOC resistance which will allow more detailed genetic analysis to be undertaken to refine QTLs and confirm the markers associated with FOC resistance (WP2). This will potentially provide more detailed information of the nature of FOC resistance and speed up the breeding process to produce an elite onion variety with good resistance to FOC.

