Project title:	Exploiting next generation sequencing technologies to understand pathogenicity and resistance in Fusarium oxysporum
Project number:	HAPI Funded Project AHDB Project CP 116
Project leader:	Dr John Clarkson, Warwick Crop Centre School of Life Sciences, University of Warwick, Wellesbourne Campus, Warwick, CV35 9EF
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Location of project:	Warwick Crop Centre & East Malling Research
Industry Representative:	NA
Date project commenced:	31/03/14

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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.

[Name]

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GROWER SUMMARY

Funding

This project has been funded by the Horticulture and Potato Initiative (HAPI). HAPI is part of BBSRC's strategy to drive innovation in bioscience in order to help meet the global food security challenge.

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, 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 soil borne 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 soil borne 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 propose 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

Objectives

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

WP2 Map FOC resistance loci in onion and identify genetic markers using genotype by sequencing (July 2017). Change: KASP genotyping will be used instead of GBS

WP3 Determine genes expression for pathogenic / non-pathogenic Fusarium and susceptible / resistant onion lines using RNA-seq and NanoString (March 2017)

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

Progress

WP1: to determine the basis for FOC pathogenicity using genome sequencing

1.1 DNA extraction was performed on seven F. oxysporum isolates from onion. Three isolates had previously been identified as pathogenic on onion (125, A23 and FUS2), three were non-pathogenic (A28, D2, PG) and one was an intermediate isolate (55). Genomic libraries for each isolates were prepared using Illumina TruSeq protocols.

1.2 Sequencing of all the F. oxysporum isolates was carried out on an Illumina MiSeq (30-40 x coverage) with the exception of D2. Combining the data for the standard pathogenic FUS2 isolate with a previous genome sequence resulted in 60x coverage. It is suggested that this part of WP1 be expanded to perform PacBio sequencing of

the FUS2 genome to improve genome assembly and aid identification of synteny between FOC strains. This would be an important resource for further work on F. oxysporum genetics.

1.3 Draft genome alignment: 'De novo' assemblies of the F. oxysporum genomes were carried out. From these data the size of the F. oxysporum genome for onion isolates is estimated at 52-58Mb and genome assemblies therefore capture >86% of the genome.

1.4 Refine genome alignment and identify effectors and location: Considerable progress has been made in identification of effectors in the Fusarium genomes. Regions homologous to a broad range of effectors and pathogenicity-related genes held in the database PHIbase were identified using BLAST searches including a characteristic RxLR motif. Furthermore, genomic regions showing homology to SIX genes previously identified as being associated with pathogenicity have been identified in the assemblies.

Relevance to Industry: identification of pathogenicity genes specific to FOC in this WP could provide the basis for a diagnostic test to determine the presence of the pathogen in soil or plant samples. This may help assess potential disease risk in the field or before storage of onion bulbs.

WP2: to map FOC resistance loci in onion and identify genetic markers using genotype by sequencing *(not yet started)*

WP3: to 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*: a sterile, agar-based system (ATS medium, square 10 cm dishes) has been developed and optimised for initial growth of onion seedlings at 15C, followed by inoculation with a spore suspension of *F. oxysporum* and incubation at 25C. Colonisation of roots occurred rapidly with plant death 7 days post inoculation.

3.2 RNA extraction from onion roots (different time points) following inoculation with pathogenic *Fusarium* isolate: onion seedlings were inoculated with FOC isolate FUS2 and root / shoot samples taken at 0, 4, 8, 16, 24, 36, 48, 72 and 96 hours after inoculation. RNA was extracted from roots and additional root samples taken and processed for confocal microscopy. Infection of root cells by FUS2 was apparent after 24 hours and by 96 hours, roots were heavily colonised.

3.3 RNA-seq and an initial transcriptome analysis of samples from 3.2 were conducted to determine the proportion of Fusarium RNA present. 36 hours was the earliest time-point where sufficient Fusarium RNA was potentially present (3%) but after 72 hours, approx.10% of the reads mapped to the FUS2 genome which was selected for a further experiment to examine the expression of pathogenicity genes in *F. oxysporum*.

3.4 RNA extraction from onion roots (single time point) following inoculation with pathogenic/non-pathogenic *Fusarium* isolates: A replicated experiment was set up using three *F. oxysporum* isolates; FUS2 (pathogenic), Fo47 (non-pathogenic) and 55 (intermediate pathogenicity) to inoculate two onion lines (resistant and susceptible). Root and shoot samples were taken at 72 hours and RNA extractions are currently being carried out in preparation for RNA-seq.

Relevance to Industry: this WP will further confirm expression of pathogenicity genes specific to FOC and provide information on which ones are highly expressed and therefore potentially more useful as a basis for diagnostics as outlined for WP1.

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

4.1 Generation and testing of F2 onion populations from two Dihaploid (DH) parents segregating for FOC resistance: To initially produce DH plants of FOC resistant onion lines (R1-R5) onion bulbs were vernalised and planted for flower and DH production by gynogenesis (7900 flowers, Jun 14). DH response (embryo counts) was initially assessed at 0.15-1.25% for the five lines. If the plants were big enough, leaf samples were analysed by flow cytometry to determine nucleus size (n or 2n). Any DH (2n) plants were directly transferred to the greenhouse while non-DH (n) plants were first doubled by dipping roots in colchicine. Of the plants that were too small for analysis, all embryos were directly doubled with colchicine before planting. Plants will develop bulbs in 2015 which will then be vernalised and crosses between resistant (R1-R5) and susceptible DH lines will be made in 2016.

4.2 Generation of onion F3 families: Crosses were made between non-DH plants of the FOC resistant lines R1-R5 and a Hazera DH susceptible line using flowers produced in 4.1. Crosses were made by emasculation of all the anthers of the flower head of one parent and free pollination with pollen of the other parent by isolating both flowers in a plastic bag and the use of blow-flies (Jul 14). F1 seed derived from these crosses was drilled (Sep 14) and vernalised (Dec 14) ready for flower production and production of F2 seed later in 2015.

Relevance to Industry: this WP begins the process of developing genetic markers for FOC resistance and introgression of resistance genes into elite onion cultivars.

Knowledge and Technology Transfer

Presentations and Posters

1) Andrew Taylor presented a poster entitled ‘Understanding the genetic control of pathogenicity and resistance for *Fusarium oxysporum* in onion at the ‘New Technologies for Crop Improvement’ workshop in Antalya, Turkey 23-26 Feb 2015.
2) John Clarkson presented a poster entitled ‘Exploiting next generation sequencing technologies to understand pathogenicity and resistance in *Fusarium oxysporum*’ at the BBSRC HAPI meeting in London, UK 25-26 Feb 2015.
3) Andrew Armitage will present a poster entitled ‘Characterisation of lineage specific regions in the onion basal rot pathogen *Fusarium oxysporum* f.sp. *cepae*’ at the Molecular Biology of Plant Pathogens conference at the University of the West of England, UK 8th - 9th April 2015.
4) Andrew Taylor will present a talk entitled ‘Understanding the genetic control of pathogenicity and resistance for *Fusarium oxysporum* in onion’ at the 7th International Symposium of Edible Alliacae in Nigde, Turkey, 21-25 May 2015.
5) John Clarkson is an invited speaker at The Carrot and Onion Conference on 4/5th Nov 2015 and will present a talk entitled ‘Fusarium research on onion’.

Publications

6) A paper entitled ‘Identification of pathogenicity-related genes in *Fusarium oxysporum* f. sp. cepae had been drafted and will be submitted to Molecular Plant Pathology this year.
7) An article was produced for HDC News on Fusarium diseases in onion.