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Introduction

Plants and plant pathogens live in a constant molecular arm's race. Secreted virulence proteins, named effectors, are used to manipulate the host to promote successful colonisation. By contrast, plants have cell surface and intracellular immune receptors to mount a defence against pathogen attack. The most common of which, NLRs (nucleotide-binding, leucine-rich repeat receptors) are able to detect and elicit an immune response upon detection of pathogen effectors. The effector repertoire of pathogens is highly adaptive, with mutations and deletions allowing pathogens to evade immune recognition, while NLRs also adapt to new effector variants. This area of study is important as plant pathogens cause widespread disease in certain staple crops (e.g., wheat, rice, maize, potato) resulting in significant yield loss and if left unchecked serious scarcity in food supply chains. With a growing global population to feed, it is crucial to understand plant disease so as to better engineer resistance and avoid food shortages. During this project we studied an effector from the fungal plant pathogen *Magnaporthe oryzae* that can infect a wide range of cereal crops. Intriguingly, this effector is recognised by a novel configuration of geneticially paired immune receptors in the host.

Aims of the project

The project aims to understand the specific effector-receptor dynamics of one of *M. oryzae* virulence proteins. To do so the project will 1) Investigate whether the effector is recognised by the paired immune receptors by observing levels of plant cell death in the model plant *Nicotiana benthamiana*. A product of successful plant immune receptor response is cell death, which prevents the spread of infection of the biotrophic pathogens that require living tissue for their life cycle. 2) Model and then experimentally determine the effector protein structure. For the latter, we express and purify the effector protein from *E. coli*, crystallise the protein, and obtain *X*-ray diffraction data.

Description of work

For the cell death assays, *Agrobacterium tumefaciens* was transformed with DNA constructs to enable gene delivery into plant cells (*via* agroinfiltration) where the effector and immune receptors would then be expressed. Following agroinfiltration, *N. benthamiana* leaves were left for 5 days prior to imaging cell death areas with UV light and scoring using a discrete scoring index (Maqpool *et* al., 2015). For the protein structure studies, AlphaFold2 was used to predict the structure of the effector, but it is important to confirm any model using experimental data that can be obtained with *X*-ray crystallography. To express the effector, *E. coli* 'SHuffle' cells were transformed with the appropriate plasmid. The protein was produced in *E. coli* culture and then purified using affinity and gel chromatography. The integrity of the purified effector was confirmed with mass spectrometry. Purified, concentrated protein sample was then subjected to crystallisation experiments, set-up using a Douglas Instruments Oryx8 robot. Crystallisation experiments were monitored over the period of 1 week to assess crystal growth.

Assessment of results and outcome

Protein structure: the effector was successfully purified and confirmed as the protein of interest (figure 1A). The protein readily crystallised under multiple conditions over the course of 24 hours (figure 1B). The crystals formed were harvested and shipped to Diamond Light Source synchrotron and observed to diffract X-rays to a resolution of 1.2 Angstroms. However, current attempts to "solve" the structure by molecular replacement methods, using the AlphaFold2 model, were unsuccessful. Interestingly, the effector structure model shares some similarity with proteins of known structure (figure 1C), but confirmation of this requires further study.

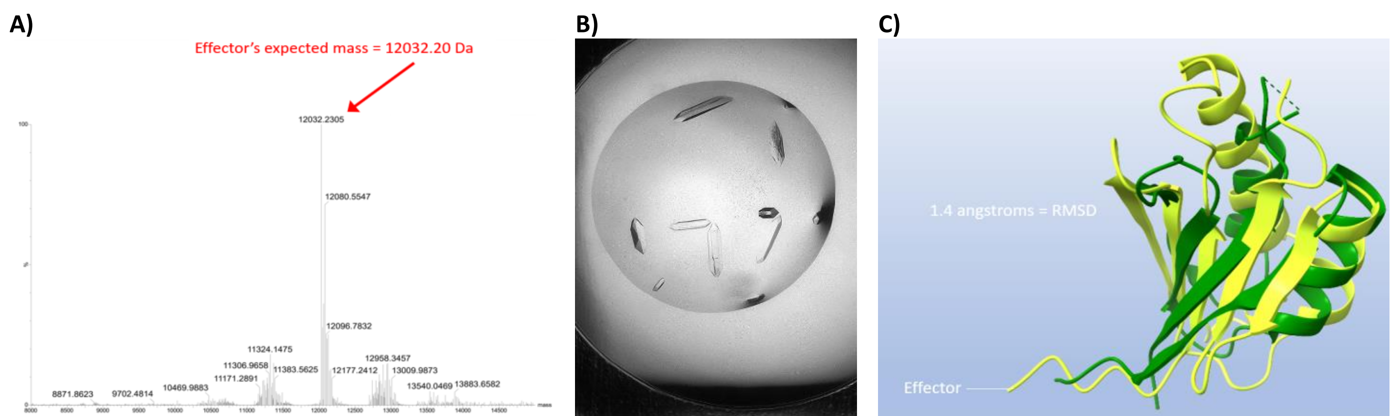

Figure 1. The effector was purified to homogeneity and crystallised readily. **A)** Mass spectrometry of the purified sample shows a high peak at the effector's expected mass. **B)** Effector crystals from an optimisation plate following successful early crystallisation tests. **C)** Alphafold2 prediction of the effector aligned with another protein.

In planta cell death studies: only when paired with both immune receptors, did the effector elicit a significant level of cell death as compared to a positive control for cell death (figure 2) whose mechanism of recognition has been prepreviously established to also require these paired immune receptors.

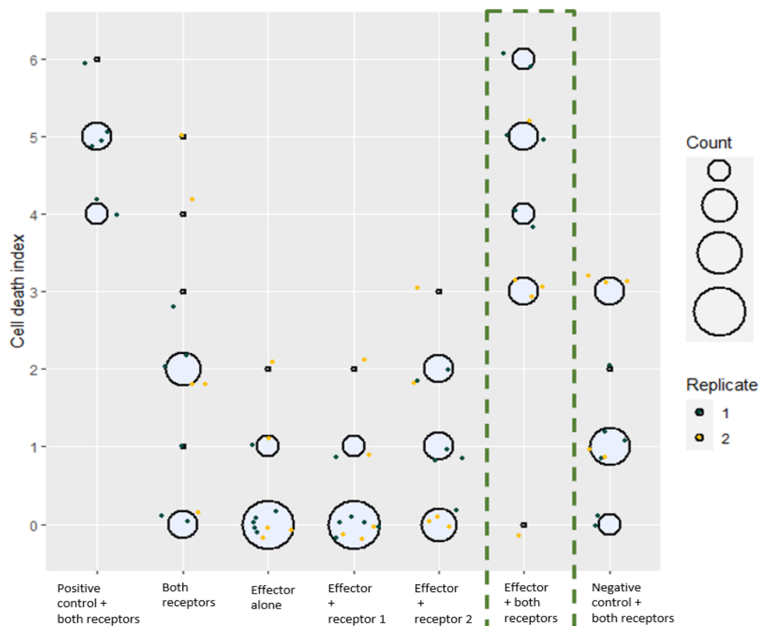

Figure 2. The studied effector (highlighted group) produced similar cell death levels (first condition group) only when expressed together with both immune receptors.

Future directions in which the project could be taken

A significant next step will be to focus on solving the effector structure through experimental phasing, for example using crystal soaks with heavy atoms. Once the effector's structure is determined, comparisons can be made to other effector families to see whether this effector adopts shared fold or is novel. It is hoped that this information, through the use of additional in planta cell death assays, can inform understanding of the mechanism of recognition. Ultimately, such information may help engineer new disease resistance in crops.

Departures from original proposal

While not strictly a departure, I was lucky enough to be present and involved in remote *X*-ray data collection from the protein crystals I obtained.

Value of studentship to student and supervisor

Student: I have learned a range of lab/machine/bioinformatic techniques (agrobacteria preparation + leaf agroinfiltration, protein crystallisation, spectrophotometry, Western blotting, protein purification through affinity and gel chromatography, DNA extraction, modelling predictive protein structure with AlphaFold2, plasmid assembly in Benchling, etc) through first-hand experience, and through observing experts in the laboratory. Additionally, I got to refine my pipetting skills and revisit techniques introduced at university such as Golden Gate cloning as well as programming with R during data analysis. What I found most invigorating was the ability to perform the complete cycle of research. That is; from preparing my lab space, data collection/analysis and lastly presentation; as opposed to the "pre-digested", truncated steps I had been used to – this studentship gave me a tangible insight into the life of a wet lab scientist and inspiration to seek a plant biology module in my third year studying BSc Molecular Biology & Genetics.

Supervisor: The studentship with Enzo was a valuable experience for me as the direct day-to-day supervisor in the laboratory. As an early career scientist myself, this was my first time directly mentoring a student. From this experience I was able to develop my leadership and organisational skills to train Enzo to eventually become an independent and productive summer student in a short space of time to advance the project along very well. There are few short-term project students who manage to produce diffracting protein crystals in such a short time!

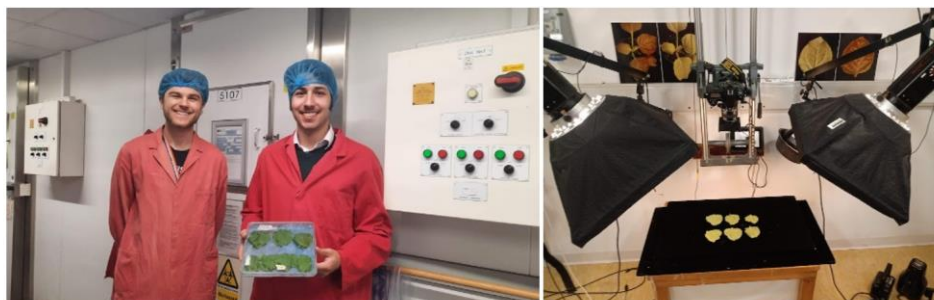

Figure 3. Josh Bennett and Enzo with in planta cell death assays ready to be imaged.

Reference: A Maqbool, H Saitoh, M Franceschetti, CEM Stevenson, A Uemura, H Kanzaki, S Kamoun, R Terauchi, MJ Banfield (2015) 'Structural basis of pathogen recognition by an integrated HMA domain in a plant NLR immune receptor', *eLife* 4:e08709.