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Title: Analysis of protein-DNA interactions using FLIM-FRET microscopy

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Background

Plant-pathogen interactions feature a complex signaling exchange between host and pathogen. The host attempts to perceive the presence of the pathogen and mount an effective defense response, while the pathogen tries to avoid detection by the host and perturb defense strategies to cause infection. Many plant pathogens have large repertoires of secreted proteins, termed effectors, which translocate into host tissues and cells and manipulate plant processes to aid the infection process. Effectors are thought to modify their host by binding plant proteins or DNA, altering protein activity or gene expression. While pathogen genome sequencing efforts have identified many pathogen effectors, the activities of most have yet to be elucidated.

Phytophthora capsici is a broad host range pathogen with hundreds of RXLR and Crinkler (CRN) effectors found within its genome. Previous efforts have attempted to identify the activity of some of these effectors using yeast-2-hybrid (Y2H) screens to determine their plant interacting protein partners. One such screen using the CRN effector 12_997, found that this effector binds plant TCP transcription factors. We have several lines of evidence suggesting that CRN12_997 and SlTCP14 (tomato) interact in the nucleus and that this interaction is important for virulence. We also have some evidence suggesting that CRN12_997 affects the ability of TCP to bind DNA, according to chromatin fractionation assays. However, further evidence of a CRN12_997-TCP interaction and the impact of CRN12_997 on the DNA binding properties of TCP is required.

In our effort to understand effector function within the home lab, we have also conducted Y2H screens with the *P. capsici* effector CRN83_152. This effector was found to interact with a plant sumo ligase and endonuclease in yeast. CRN83_152 causes cell death when overexpressed *in planta*, localises to the nucleus and causes the re-localisation of chromatin. While effector and its targets localise to the plant nucleus, we have struggled to confirm the direct interaction between these proteins *in planta*.

Within the home lab we are also interested in the ability of effectors to bind host DNA. Using a DNA binding prediction pipeline developed within the lab we have identified a number of effectors with predicted DNA binding activity. Chromatin fractionation assays have allowed us to narrow down our set of predicted DNA binding effectors. However, direct effector-DNA binding must be confirmed.

Objectives/ Purpose of the visit

The STSM was divided into 3 main objectives outlined below. All of these objectives were based on FLIM (fluorescence lifetime imaging microscopy) and FRET (fluorescence resonance energy transfer) for examining protein-protein and protein-DNA interactions. This method has been successfully used at the LIPM in Toulouse and was not available at the home institute.

1) **CRN12_997-TCP14 interaction**
   a) Confirm the interaction between CRN12_997 and TCP transcription factors *in planta.*
   b) Determine whether CRN12_997 has an impact on the DNA binding properties of TCP transcription factors.

2) **CRN83_152-endonuclease and sumo ligase interaction**
   a) Determine whether CRN83_152 interacts with endonuclease and sumo ligase *in planta*.

3) **Predicted DNA binding effectors**
   a) Screen a small selection of predicted DNA binding effectors to determine whether they bind DNA *in planta*.

Description of the work

Interaction experiments were completed using a FLIM system with a streak camera enabling rapid acquisition of data. For protein-protein interaction experiments donor proteins were tagged with GFP and acceptor proteins tagged with RFP. For protein-DNA interaction experiments donor proteins were tagged with GFP while DNA was stained with sytox fluorescent stain.

<u>Protein combinations</u>

We tested the ability of RFP tagged CRN12_997 to bind the tomato transcription factors TCP14-1 and TCP14-2, which were both GFP tagged. To test the impact

of CRN12_997 on the DNA binding properties of TCP, CRN12_997 was tagged with HA, while TCP were tagged with GFP.

For CRN83_152 interaction experiments we used GFP tagged effector and RFP tagged endonuclease and sumo ligase.

Predicted DNA binding effectors (RXLRs 04145, 471, 180 and 16294) were GFP tagged, while DNA was stained with sytox.

Infiltrations were done using 3-4 week old *N. benthamiana* plants and imaging or fixing/sytox treatment was done 48 hours post-infiltration.

Main results

1) **CRN12_997 interacts with TCP transcription factors**
   I have been able to confirm the interaction of CRN12_997 with TCP14-1 and TCP14-2, supporting previous observations using CO-IP experiments in the home lab. I have also found that the TCP transcription factors do not interact with free RFP in the nucleus (negative control) while CRN12_997 does not interact with free GFP (negative control). In addition, I was able to show that TCP transcription factors bind DNA and preliminary results suggest that the binding of CRN12_997 with TCP14-1 causes this transcription factor to dissociate with DNA. Experiments are currently on-going in the host lab to complete this data set.

2) **CRN83_152 interacts with endonuclease and sumo ligase**
   FLIM-FRET experiments have confirmed the interaction between CRN83_152 and endonuclease and sumo ligase. To test whether the interaction with endonuclease and sumo ligase was specific to this effector we used another nuclear effector RXLR04145, which like CRN83_152, triggers cell death when overexpressed at high levels in planta. No interaction was observed between RXLR04145 and endonuclease and sumo ligase. In addition, no interaction was observed between CRN83_152 and free RFP (a negative control for the non-specific binding of CRN83_152).

3) **Predicted DNA binding effectors**
   The examination of the DNA binding properties of effectors proved to be challenging. These experiments required plant material to be fixed with paraformaldehyde and subsequently DNA was stained with sytox. This fixing and staining procedure resulted in damage to plant tissue and accurate measurement

of FRET was not possible. Preliminary data suggests that one of the effectors tested may be interacting with DNA, but these experiments must be repeated to generate a robust data set.

Future collaboration with the host institution

The experiments conducted in Toulouse have been extremely informative and have generated valuable data, demonstrating the strength of FLIM-FRET for studying effector activity *in planta*. I have established an excellent working relationship with the host lab and we have agreed to continue working together to complete our data sets for publication. Given the large effector repertoire of *P. capsici*, there will be other effectors to examine in the future in collaboration with Susana Rivas at LIPM.

Projected publications/articles related to or resulting from the STSM

Results from this scientific mission will contribute directly towards at least 2 publications in the near future. The CRN-TCP interaction data will be incorporated into a manuscript which is currently under revision for resubmission to Plant Physiology. CRN83-152 interaction data confirms our Y2H results and a manuscript examining the activity of this effector and its host targets will be prepared for submission later this year. Follow-up experiments in collaboration with Susana Rivas examining the activity of DNA binding effectors, will contribute to multiple projects within the home lab and will likely provide valuable data for future publications.