Investigating Effector Virulence Components in Plant Pathogens

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Introduction

Fungal and oomycete pathogens utilize effectors, small secreted proteins, to overcome host defense responses. To screen and characterize pathogen effectors, we established a working assay based on agroinfiltration to examine phenotypic results. We investigate *Peronospora belbahrii*, an oomycete pathogen that causes basil downy mildew, an agriculturally significant disease. Since *P. belbahrii* cannot be transformed, heterologous expression is one of the only ways that we can examine the effectors as critically important virulence factors. Our lab previously identified a set of canonical RXLR family effectors hypothesized to be important to *P. belbahrii* infection of basil (Figure 1). This summer I focused on characterizing one of these effectors, REL1, to assess whether it can suppress a programmed cell death response (hypersensitive response or HR). REL1 and REL1-RXLR were chosen as effectors of choice from an RNA-seq data from my lab, and significant because they were highly upregulated during infection. We hypothesize that REL1 will interact with the plant immune system to facilitate infection.

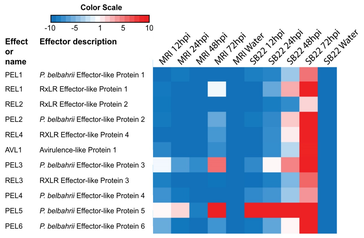


Figure 1. Heatmap of *P. belbahrii* candidate cytoplasmic effectors

Candidate *P. belbahrii* cytoplasmic secreted effectors with increasing expression over time in SB22, a susceptible breeding line of sweet basil. MRI is a downy mildew resistant cultivar of sweet basil and shows limited expression of the effectors in comparison. Color changing to red indicates increasing transcript abundance (represented by relative fold change) compared to the *P. belbahrii* sporangia control

Background

Heterologous expression is a common way to screen RXLR effectors from downy mildew pathogens because their lifestyle is obligate, and suppression of HR helps to keep the plants alive for pathogen infection. Heterologous expression using *Agrobacterium tumefaciens* mediated-transient transformation (Figure 2) is performed by infiltrating *A. tumefaciens* into intact plant leaves. What makes this bacteria special is how it can insert its own *Transfer-DNA* (T-DNA) into the plant cells. This T-DNA also encodes for genes, known as virulence factors, allowing for the transfer to occur. When the T-DNA is inserted, these genes are activated to relocate the T-DNA into the nucleus. Which then inserts into the genome and the genes will be transcribed and expressed by the plant cell, resulting in potentially phenotypic changes[1].

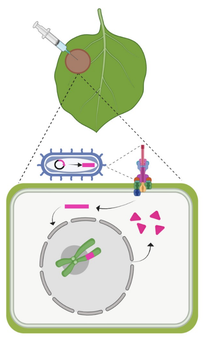


Figure 2. Heterologous expression of effectors using agroinfiltration in *Nicotiana* spp. – unpublished data

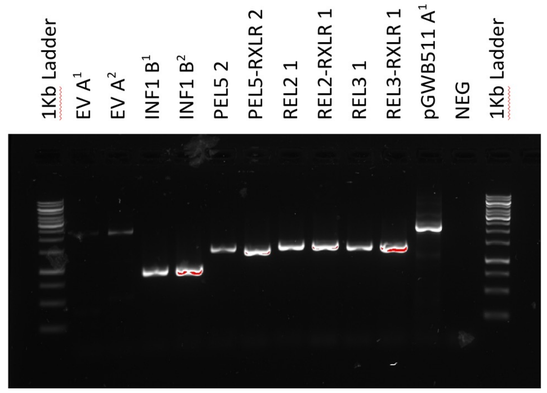


Figure 3. Colony PCR selection of successful expression vector clones.
Agrobacterium colonies selected and propagated after successful gateway cloning and transformation.

Material and Methods

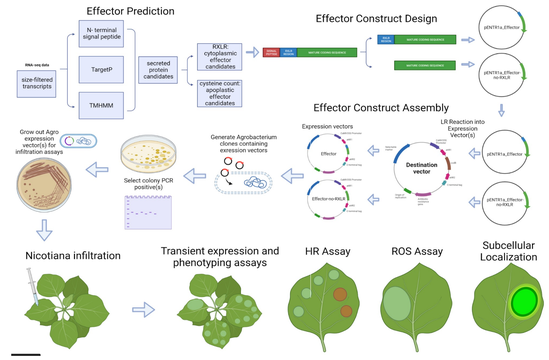
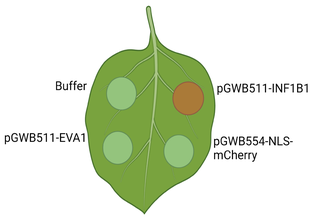


Figure 4. Outline of *Peronospora belbahrii* effector analysis procedures

Effectors predicted to be essential for pathogenicity which results in two types, cytoplasmic and apoplastic. Once the cytoplasmic ones are chosen, a construct is designed, one containing the RXLR motif, and one without it[3]. Expression vectors are created using Gateway cloning, and then transformed into *E. coli* and then Agrobacterium. Colony PCR is used to select successful clones, which are then propagated for agroinfiltration into *Nicotiana* species. The RXLR sequence is downstream of signal peptide but upstream of effector.

A. Agroinfiltration screening and optimization design



B. Effector Agroinfiltration screening design

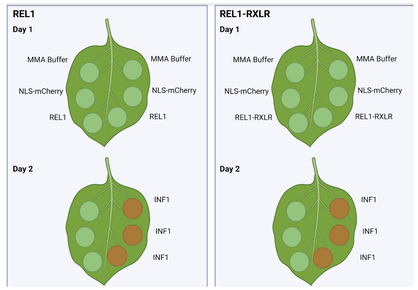


Figure 5. Illustration of infiltration pattern on leaves and effector screening of REL1 & REL1-RXLR constructs

5A. *N. benthamiana* leaves were infiltrated in the 4 sites shown on the left. Buffer was a standard MMA Buffer, pGWB511-EVA1 is an empty vector containing the backbone, acting as a negative control. The pGWB511-INF1B1 is a well-known HR inducer from the oomycete pathogen *Phytophthora infestans*, serving as a positive control. pGWB554-NLS-mCherry is used to visualize fluorescence underneath a confocal microscope.

5B. *N. benthamiana* leaves were infiltrated in 6 sites, each side mirroring the other to provide a control for the INF1 HR inducer infiltration on one side. The expected outcome is the buffer and NLS will show a strong HR response due to the INF1, and the RXLR effectors may demonstrate HR inhibition.

Results

Phenotype Caused By Different Cultures (HR Scoring)

Figure 6. Leaf Images of Trial E and Scores

Six leaves taken from the same plant show optimal results. All six of these leaves have no HR response on 3 of the 4 cultures (Buffer, EVA1, and NLS) as predicted. While the HR-inducer, INF1, shows significant cell death with scores of 4. We examine the variation of results within and across individual plants and found that leaves from the same plant often performed similarly (data not shown).

Phenotype Caused by Different Cultures (Quantification)

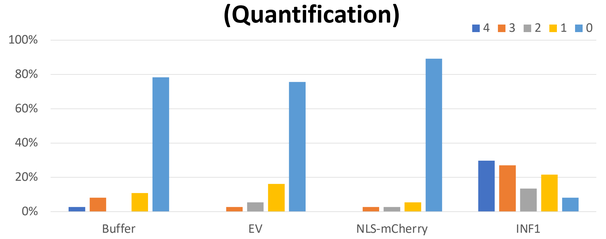


Figure 7. Graph of percentage of HR score on all 4 different conditions

Sample Size of 36 individual leaves and spots. Buffer is an MMA buffer acting as a negative control alongside the empty vector (EV), and NLS-mCherry. Since INF1 is a HR inducer, the spots should be experiencing cell death.

Buffer, EV, and NLS-mCherry all experience most spots showcasing no HR response of any kind, which aligns with what is expected. INF1 has roughly 60% of spots with HR response scores of either 3 or 4, indicating the HR inducer is working as intended. On the other hand, Buffer, EV and NLS-mCherry had close to 90% of scores around 0-1 where there was no HR at all.

Confocal Microscopy

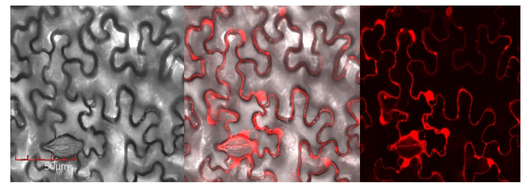


Figure 8. Confocal microscopy of Nuclear Localization Signal (NLS) constructs shows successful heterologous expression as early as 36 hours post-infiltration (hpi)

Confocal microscopy images were taken from leaves 36 hpi to 72 hpi with NLS-mCherry constructs to ensure showed fluorescence. The *Nuclear Localization Signal (NLS)* is included as a control to localize a protein (in this case mCherry) to the nucleus. NLS can be used to help visualize cytoplasmic vs. apoplastic effectors, however, here it is used to confirm Agroinfiltration results. This control can be used prior to visible HR induction from the INF1 construct.

HR Percentage Scores by Different Cultures (Effector + INF1 different day)

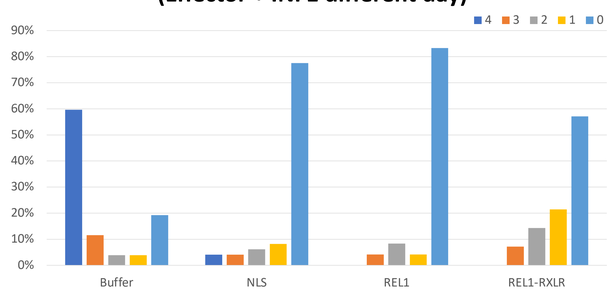


Figure 9. Graph of percentage of score on all four cultures

Sample size of 52 spots for Buffer, 49 for NLS, 24 for REL1 & 28 for REL1-RXLR. INF1 was added 1 day after initial infiltration of Buffer, NLS and effectors. The effectiveness of *INF1* was measured in each culture, in which, there should be inhibition of HR response caused by *REL1* & *REL1-RXLR*. The buffer and NLS were also measured, in which there should be no inhibition of the HR response.

These two effectors were measured to understand if the effectors will cause any difference in how strong HR response inducer like *INF1* will be able to overtake it. However, when data of NLS was taken, roughly 80% of spots were very resilient to the necrosis effect, which was not predicted.

HR Percentage Scores by Different Cultures (Effector + INF1 same day)

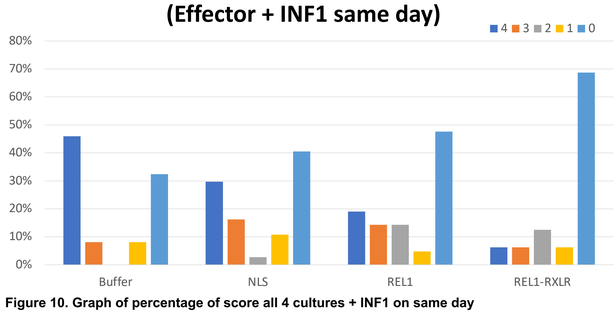


Figure 10. Graph of percentage of score all 4 cultures + INF1 on same day

Sample size of 37 for buffer and NLS, 21 for REL1 and 16 for REL1-RXLR. INF1 was added on the same day as the cultures by mixing cultures at OD1 together. The effectiveness of *INF1* was measured in each culture, in which, there should be inhibition of HR response caused by *REL1* & *REL1-RXLR*. The buffer and NLS were also measured, in which there should be no inhibition of the HR response.

When INF1 is added within the same day, the NLS showed much more congruent results as predicted. However, while *REL1* has a majority inhibiting the HR response, there is also a large percentage of cell death this trial. On the contrary, *REL1-RXLR* has a higher percentage of inhibition than the prior trial.

HR Response Leaves (Effector + INF1 same day)

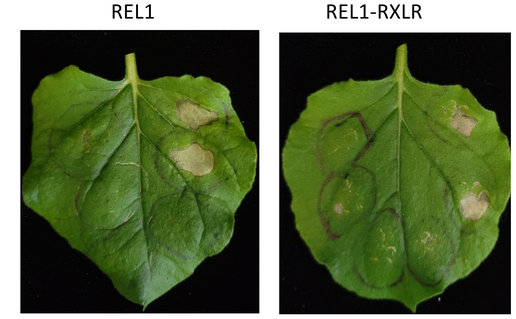


Figure 11. Leaf Images of Effector + INF1 same day

2 leaves were taken from different plants, one containing the *REL1* effector and the other containing the *REL1-RXLR* effector. These images show the optimal results. On both leaves the buffer and NLS show heavy HR response, whereas both the different effectors showcase inhibition of the cell death.

Conclusion and Future directions

What I did:

This summer, the main objective was to examine whether *P.Belbahrii* effector REL1 will interact with host immune system using a HR based infiltration assay to collect phenotypic results. I was able to learn how to engineer and transform a plasmid, observe and collect plant phenotypes, and operate the confocal microscope to look at fluorescent tagged proteins. I also was able to run whole weeklong experiments for growing out agrobacterium in different cultures and prepare them for agroinfiltration.

What I learned:

These many trials of Agroinfiltration taught me how to work well independently and how to think more like a scientist. There were many obstacles and optimizations that were added every week. In my opinion, this summer has taught me what research really takes. I had thought every experiment would go smoothly, and I would get to experience many different techniques during these three months. However, reality was research took a lot of time and effort, it wasn't until I took a step back from that thinking that I was able to get much better at the work I was doing, and really see things through

Future directions:

- learn more about pathogen effectors,
- apply protocols for more effectors and species
- explore different possibilities to characterize effectors
- develop improved protocols for assessing their activities.

References and Acknowledgments