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Continuing Researchers Support Program Final Report

Agricultural Research Foundation Board

Research Objectives

Originally, we had three research objectives for this project. First, to identify beet-derived signaling molecules that could induce the *Pantoea agglomerans* pathovar *betae* (*Pab*) type III secretion system (T3SS). Second, to identify genes from the *Pab* genome that regulate the expression of the T3SS in response to those metabolites. Third, to characterize the infection of plants by *Pab* with *in vivo* pathogenicity assays in live plants.

Deviation From Original Objectives

The overall objectives of this project are thus far unchanged. In the 2022-2023 academic year for which this project was funded by the Agricultural Research Foundation, we have been able to identify that the T3SS of *Pab* is strongly induced in response to several plant derived sugars, and have therefore reached a significant milestone on objective one. It still remains to be seen if there are more beet-derived metabolites to which the *Pab* T3SS is responsive, so there still may be more work to be done as part of that objective.

Methods

Preparation of Arabidopsis and beet exudates. Beets (*Beta vulgaris*) were grown in a 10-14 light-dark cycle on 0.5X Murashige and Skoog (MS) medium solidified with 2.87% plant agar and supplemented with 20 g/L sucrose. To produce exudate, each seedling was soaked in 1 mL of deionized water for 24 hours. The exudate was filter-sterilized and stored at -20°C until further use. Arabidopsis (*Arabidopsis thaliana*) exudate preparation was the same, except seedlings used were one week old.

Gibson assembly of *hrpS*$_{\text{promoter}}$:*gfp* (1 kb) plasmid. *E. coli* carrying the plasmid pPROBE-NT were inoculated into LB broth supplemented with 30 µg/mL kanamycin for plasmid selection, and the inoculated cultures were grown at 28°C for 24 hours. The Qiagen plasmid midi kit (#12123) was used to purify the pPROBE-NT plasmid from the cultured bacteria, and the purified plasmid DNA was digested with the SmaI endonuclease. *Pab* genomic DNA was isolated using the Qiagen QIAprep Spin Miniprep Kit (#27106) from an overnight culture of *Pab*. Oligonucleotides A and B were used to PCR amplify a region approximately 1 kb upstream of *hrpS* from the isolated gDNA. The NEBuilder Hi-Fi DNA Assembly Master mix (NEB #E2621S) was used to ligate the PCR product into the linearized pPROBE-NT, with the product containing the 1 kb *hrpS*$_{\text{promoter}}$ upstream of *gfp*. The assembled *hrpS*$_{\text{promoter}}$:*gfp* pPROBE-NT construct plasmid was mixed with chemically competent DH10β *E. coli* cells and incubated at 42°C for 45 seconds. After heat shock, the cells recovered in LB broth for 1 hour at 28°C, then plated on LB agar with 50 µg/mL rifampicin and 30 µg/mL kanamycin. After 24 hours, transformants were confirmed by colony PCR using oligonucleotides and by Sanger sequencing to confirm the presence of the *hrpS*$_{\text{promoter}}$ insert.

Triparental mating to clone pPROBE-NT *hrpS*$_{\text{promoter}}$:*gfp* into *Pab*. The *Pab* 4188 parental strain, *E. coli* DH10β pPROBE-NT *hrpS*$_{\text{promoter}}$:*gfp* strain, and *E. coli* pRK600 helper strain bacteria were grown on LB agar containing, 50 µg/mL rifampicin, 30 µg/mL kanamycin, and 30 µg/mL chloramphenicol,

respectively. Each bacterial culture was centrifuged, and the resulting bacteria pellet resuspended in 1 mL of sterile deionized water. To transfer the plasmids from *E. coli* to *Pab* via conjugation, 100 µL of the *Pab* parent strain, 20 µL of the DH10β pPROBE-NT $hrpS_{\text{promoter}}$:*gfp* strain, and 20 µL of the pRK600 helper strain were mixed, and 20 µL of the mixture was plated on a nitrocellulose membrane placed on the surface of an LB agar plate supplemented with 50 µg/mL rifampicin and 30 µg/mL kanamycin. After 24 hours at 28°C, the bacteria were resuspended in water and 1x, 10x, and 100x (volume/volume) dilution series were plated again on LB agar containing 50 µg/mL rifampicin and 30 µg/mL kanamycin. Transformants were confirmed by colony PCR using oligonucleotides and positive colonies were stored at -80°C in 25% glycerol stocks.

Microplate reader Absorbance and GFP fluorescence assays. To assay for hrp_{promoter}:*gfp* expression, *Pab* were scraped from the surface of the agar and resuspended into 1 mL of sterilized deionized water. The bacteria were washed three times in 1 mL of water and adjusted to an optical density at 600 nm (OD_{600}) of 1.0. In a 96-well plate, a 10 µL aliquot of 1.0 OD_{600} bacteria was inoculated in a T3SS-inducing minimal medium supplemented with either 25 mM of fructose, glucose, galactose, mannitol, or sucrose or with 30 µL beet exudate (final concentration of 30%). Culture density (Absorbance_{600}) and GFP fluorescence (485 nm excitation, 535 nm emission) of each well was measured every half-hour using a Tecan Spark 10M plate reader. The plate was kept at 25°C and shaken in between reads. To measure the expression of promoter:*gfp* constructs, fluorescence values were divided by Abs_{600} values to account for growth of the bacteria in the media, and Abs_{600}-adjusted fluorescence values from wells inoculated with pPROBE-NT empty vector strains were subtracted from wells inoculated with hrp_{promoter}:*gfp* constructs strains to account for background fluorescence.

Key Findings

Beet exudate contains an array of sugars detected by gas chromatography-mass spectrometry. We prepared beet exudate by soaking beet seedlings in water for 24 hours. We then used gass chromatography-mass spectrometry (GC-MS) to identify metabolites present in the beet exudate. The exudate contained many known plant-derived metabolites, including sugars, organic acids, and amino acids.

***Pab hrpL* and *hrpXY* transcription is strongly induced by a variety of sugars.** To determine if individual sugars can induce *hrpL* expression in *Pab*, we cultured our $hrpL_{\text{promoter}}$:*gfp* and $hrpXY_{\text{promoter}}$:*gfp* reporter strains in various minimal media each supplemented with only one type of sugar. The cultures were maintained in wells of a 96-well assay plate, and a plate reader was used to measure both GFP fluorescence at multiple time points post-inoculation. To assess bacterial growth, the plate reader also measured the Absorbance at λ=600 nm of each culture at each time point. Each of the sugars tested significantly induced *hrpL* and *hrpXY* expression within six hours after inoculation. The timing of maximal expression for each sugar varied across independent replicates of the experiment. Despite this variability, fructose and mannitol consistently led to the highest levels of *hrpL* and *hrpXY* expression. In comparison, galactose and sucrose induced *hrpL* and *hrpXY* at a mid-range level, whereas glucose consistently led to a relatively weak but still statistically significant level of *hrpL* and *hrpXY* induction.

***Pab hrpS* transcription is not strongly induced by sugars.** We also tested if the same sugars induce *hrpS* expression in our *Pab* 0.4 kb $hrpXY_{\text{promoter}}$:*gfp* reporter strain. In contrast to *hrpL* and *hrpXY*, fructose did not strongly induce the expression of *hrpS* based on levels of GFP fluorescence from this reporter strain. A small statistically significant increase in fluorescence was seen but low expression of the promoter was seen overall both when treated only with minimal media and when treated with 50 mM fructose. It is possible that our 0.4 kb *hrpS* promoter construct may be missing one or more of the

important control elements. Therefore, we increased the region of upstream *hrpS* promoter to 1 kb in size; however, we did not observe any significant induction of *hrpS* expression using thus second reporter construct. In fact, a small decrease in GFP fluorescence was observed in response to fructose, glucose, galactose, mannitol, or sucrose. It is possible that even our 1 kb promoter is still missing important control elements because in bacteria they are sometimes within the open reading frame.

***Pab* can use a variety of sugars as growth substrates.** Sugars exuded from beet seedlings induce increased culture density of *Pab* over time when supplemented in a T3SS-inducing minimal medium. Sugar-induced growth was monitored by measuring the Abs_{600} of *Pab* cultured in the same minimal media used for *hrp* expression. Each of the sugars tested significantly increased the growth of *Pab* (pPROBE-NT empty vector) relative to bacteria cultured in a minimal medium without sugar. Fructose and mannitol nearly doubled the peak Abs_{600} reached by the cultures, while galactose or sucrose caused a 1.75-fold increase in the peak Abs_{600}. Glucose caused only a 1.3-fold increase in the peak Abs_{600}.

The magnitude of sugar-induced expression of *hrpXY* and *hrpL* and the magnitude of sugar-induced growth of *Pab* are positively correlated. When the Abs_{600} values for sugars inducing $hrpL_{promoter}$:*gfp* expression are plotted against the GFP fluorescence values obtained in the same well, a strong linear fit is obtained ($R^2 = 0.9117$). The same is true for sugars inducing $hrpXY_{promoter}$:*gfp* expression ($R^2 = 0.9725$). From the strong linear fit, we believe that sugars which are preferred by *Pab* to induce *hrpL* and *hrpXY* expression are also preferred to be used for energy metabolism.

Next Steps

Based on the work I completed for this project, we now that *hrpXY* and *hrpL* are induced in response to sugars found in beet exudate, suggesting that the T3SS of *Pab* is responsive to signals derived from plant hosts. Despite this, we do not yet know how this process is controlled or where exactly in the signaling pathway that these signals come in. For the future of this project, further genetic screening can help to confirm how *Pab* regulates the use of plant-derived signals to regulate expression of its T3SS. We can do a forward genetic screen, where we identify random gene silencing mutations that lead to differences in the sugar-mediated expression of the genes *hrpXY* and *hrpL*. Another option is a reverse genetic screen, where we test the expression of *hrpS* and *hrpL* in response to sugars in a genetic background where *hrpXY* is silenced. This would allow us to further characterize how the *hrp* pathway responds to the presence of plant-derived sugars.

Presentations Given On This Work:

Poster Presentation at Spring Ag & Natural Resources Day

https://drive.google.com/file/d/1HSsmgO36CuMCiNwi-tt547yXdGSIWHxI/view?usp=drive_link

Honors College Thesis Defense

https://media.oregonstate.edu/media/t/1_od5vota0