IDENTIFICATION AND CHARACTERIZATION OF CANDIDATE EFFECTORS IN GRAPE PHYLLOXERA

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By

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Abstract

Insects are a continual biotic stress on agriculture driving the development of preventative and curative treatments for crops. Grape phylloxera, *Daktulosphaira vitifoliae,* is a parenchyma feeding insect that produces galls on grape (*Vitis)*, likely utilizing specialized proteins in its saliva, called effectors, to manipulate the plant immune system and development. Many other organisms employ effectors to overcome the host plant immune response in a similar manner, targeting the same signaling hubs [1]. The highly conserved nature of the plant immune system suggests that pest effectors performing comparable functions may be similar across species, making discoveries in effectors of a model insect, advantageous for research in other pests. An essential component of plant defenses are the complex calcium ion signaling pathways with calcium ion binding proteins characterized by the presence of EF-hand motifs. This motif was discovered in 124 predicted grape phylloxera effectors, suggesting a role in influencing calcium dependent plant cell responses. A comparative transcriptomics approach with different phylloxera populations and host species in combination with in vitro assays can identify the insect genes and plant-insect protein interaction that facilitate successful colonization and host specificity. Three EF-hand containing, predicted effector genes were chosen for functional validation assays as part of an overall gene expression analysis of *D. vitifoliae*. Yeast-two-hybrid protein interaction assays proved to be a challenging method for visualizing plant-insect interactions, but has potential for use in identifying insect effector functions in the future. A differential gene expression analysis suggested a strong host effect on predicted effector expression profiles. These experiments will improve knowledge on the genetic factors that result in compatibility or incompatibility for phylloxera on *Vitis* and lead to more effective pest management strategies. Results can be applied to experimentation in other herbivorous insects that use similar strategies for colonization as the plant immune system is highly conserved.

Acknowledgments

I would like to acknowledge everyone in the Nabity lab for supporting me through this project and beyond; and give special thanks to Marakee Tilahun who assisted in this project during her time as an REU student.

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Table of Contents

INTRODUCTION

Successful responses to biotic or abiotic stresses can be the determining factor in an organism's survival. Of the various challenges to overcome, interactions with other living organisms may be some of the most complex. Plants face a unique hurdle lacking specialized immunity cells like animals, relying on innate immunity of individual cells and a complicated signaling network to create an appropriate response in altered gene expression [2].

Every stress has a unique molecular marker that interacts with plant cell recognition receptors allowing the plant to perceive and react to the stimulus (Figure 1). Mechanical stress by wounding creates damage-associated molecular patterns (DAMPs) while biotic stresses are recognized by pathogen-associated molecular patterns (PAMPs). These can be further broken down into microbe-associated molecular patterns (MAMPs) and herbivore-associated molecular patterns (HAMPs) [3]. Recognition of these patterns in PAMP-triggered immunity (PTI) induces

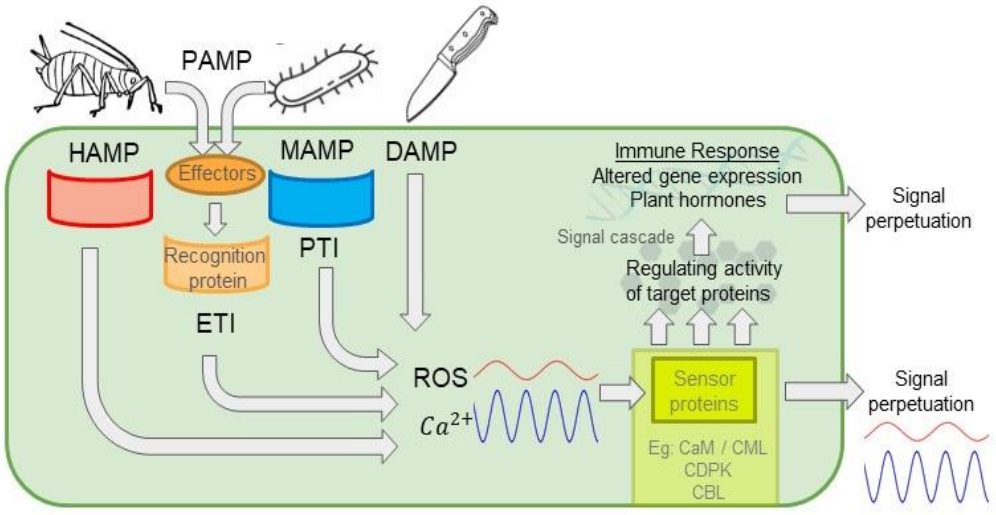

Figure 1: Simplified diagram demonstrating how the plant immune system works. PAMP=pathogen associated molecular pattern, HAMP=herbivore associated molecular pattern, MAMP=microbe associate molecular patterns, DAMP=damage associated molecular patterns, ETI=effector triggered immunity, PTI=pathogen triggered immunity, ROS=reactive oxygen species.

a transient flux of calcium ions and reactive oxygen species (ROS). The calcium signature, defined by a unique pattern of concentration changes, including frequency, duration, amplitude and localization, is specific to the particular stimulus. This signature is interpreted by calcium-binding sensor proteins that can interact with other proteins in a signaling and response cascade [3,4].

Calcium has an integral role as a universal messenger in diverse signaling pathways of eukaryotes and is especially important in the plant immune system [3-5]. Ca^{2+} serves as one of the first signaling elements in coordinating plant stress response, particularly in regards to pathogens or mutualistic organisms [3]. Ca^{2+} influxes to the cytosol, using internal and external sources, through complex interactions between ion channels, exchangers, pumps, receptor proteins, and crosstalk with other signaling pathways. The specificity and successful generation of the Ca^{2+} signature is integral for the plant to respond appropriately to pathogens. These Ca^{2+} signatures are recognized by calcium binding proteins and are translated into a cellular response; regulating the activity of various target proteins, enzymes and genes [4]. Calcium-binding proteins such as calmodulin (CaM) function as a primary Ca^{2+} signature sensor that interacts with CaM-binding Transcription Activators to regulate gene expression as well as channel proteins and Ca^{2+} -ATPases to regulate the Ca^{2+} transport system itself to halt or perpetuate the signal. Plants uniquely contain genes for divergent forms of CaM, called calmodulin-like (CML) proteins that are involved in the suppression of post-transcriptional gene silencing. Mutations in CaM and CML function and expression have been found to greatly affect the plant immune response [4]. Most Ca2+ binding proteins share a conserved motif, a helix-loop-helix structure called the EF-hand [3,4,6]. The EF-hand motifs function in pairs as a single domain and can undergo a multitude of unique conformational changes allowing it to interact with a variety of

systems [6]. Primary Ca^{2+} sensors like CaM and CML only contain EF-hand domains, but other proteins like Ca^{2+}-dependent protein kinases calcineurin B-like proteins contain other functional domains, acting as calcium sensors and directly affecting processes in the plant immune system [3,4].

Many organisms have developed specialized proteins called effectors that are secreted into the plant to manipulate the host plant immune system, metabolism, growth, or nutrient transport [7]. Plants can evolve effector recognition proteins in response to combat the pest as explained by the zig zag model of plant immunity [2]. When the effectors are recognized, they induce a similar signaling cascade; however, while typical PAMP-triggered immunity involves transient cytosolic calcium fluctuations that return to normal levels within minutes, effector-triggered immunity (ETI) elicits a prolonged cytosolic calcium increase that can last for hours. The effector-triggered immunity is also often associated with a hypersensitive response resulting in apoptosis, localized cell death [3,4]. This supports that Ca^{2+} is indispensable in the plant immune response and could itself be a target of effectors.

Studies have previously found insects that utilize calcium-binding proteins to overcome the plant responses to herbivory. Vetch aphid (*Megoura viciae*) saliva has been discovered to contain calcium-binding components that act as Ca^{2+} scavengers, effectively reducing the availability of Ca^{2+} that would normally trigger a phloem occlusion response [8]. An EF-hand containing effector protein was identified in the brown planthopper (*Nilaparvata lugens*) that decreases cytosolic calcium and modulates H_2O_2 levels to decrease plant defenses [9]. Planthopper has a history of overcoming rice resistance and developing new virulent populations, with effector evolution as a possible driver.

Grape phylloxera (*Daktulosphaira vitifoliae*) is a parenchyma feeding insect native to eastern North America that has become a worldwide pest of grapevine with the appearance of new biotypes. Grape phylloxera feed and produce galls, abnormal growths of plant tissue, on leaves and roots of grape induced by feeding activity and effector inoculation [10,11]. The root feeding types have been the primary focus in viticulture as it makes the plants vulnerable to secondary infections. Resistant rootstocks are the preferred management strategy against phylloxera, yet the rootstocks fail in some locations with the arrival of new virulent phylloxera populations, seen to develop in as little as 10 years in South Africa, costing growers billions of dollars [11]. Grapes are currently the fifth most important consumable crop in the US with the US ranking third in global grape production (FAO, United Nations 2013). Identification of effectors that compromise the grape immune system and understanding the mechanisms that allow for successful phylloxera feeding may prove integral in developing more effective vineyard protection strategies. Although it is a pest of great economic importance, genetic understanding of different biotypes and resistant determinants in *Vitis* is limited.

Effectors from pathogens across kingdoms have been shown to converge on the same plant signaling hubs to induce similar responses in the host [1]. The conserved nature of the plant immune system suggests that effector families targeting it are likely to be shared across pest species. The role of effectors in manipulating plant defense and development have been characterized for many pathogens, but little has been discovered on insect effector induced plant immune responses and gall formation, which can be explored in grape phylloxera.

A preliminary experiment that examined how geographically isolated populations of grape phylloxera differentially feed on the same host plant revealed a difference in survival on different *Vitis* hosts. A hypersensitive plant response was observed when the Arizona population

attempted to colonize incompatible hosts, indicative of ETI., whereas the Illinois populations survived to induce the typical gall phenotype. Preliminary analysis of transcriptome data between the populations on their native hosts revealed a difference in expression of predicted effector genes. The Phylloxera Genome working group identified over 2740 genes that could act as effectors, 124 of which contain EF-hand motifs. A comparative transcriptomics approach examining how different phylloxera populations respond to native and novel host species in combination with in vitro assays on these EF-hand containing effectors may identify the insect genes and plant-insect protein interaction that facilitate successful colonization, host specificity, and effector evolution.

RESULTS & DISCUSSION

Characterization of Chosen Genes

Three EF-hand containing predicted effector genes were chosen for a functional analysis of their interactions with proteins in the plant based on their original gene expression patterns. DV3023045.2 (predicted effector 2155-4000), hereby referred to as ARZ for simplicity, was expressed very highly in the Arizona population (113 cpm) but lowly expressed in the Illinois population (27 cpm). This difference may underlie the idea that Arizona elicited a hypersensitive response through effector triggered immunity with this gene. ARZ has a coding sequence 318 base pairs long that translates into a predicted protein of 105 amino acids. A signal peptide was predicted in the N-terminus of the amino acid sequence with a cleavage site between amino acids positions 21 and 22. DV3009582.2 (predicted effector 425-4600), or ILL, was highly expressed in the Illinois population (54 cpm) but was absent in the Arizona population. This suggests the alternative hypothesis that the Arizona population could be missing an integral calcium-binding effector to suppress the plant immune system. ILL has a coding sequence 555 bp long and a

predicted protein 184 amino acids long. A signal peptide was predicted in the N-terminus between amino acids of positions 18 and 19. DV3007389.2 (predicted effector 213-30231), hereby referred to as ALL, was expressed in both populations, 77 cpm in Arizona and 81 cpm in Illinois. This predicted effector may be conserved across populations because it is fundamental in the ability of phylloxera to colonize grape. The gene's coding sequence is 675 bp long and the predicted protein is comprised of 224 amino acids. A signal peptide at the N-terminus was predicted between amino acids at positions 18 and 20. Coding region sequences for each gene are provided in Appendix A.

Cloning

To identify what plant proteins these predicted effectors interact with in the plant host, the three chosen genes were prepared for yeast two-hybrid screening. Specific primers were designed for each gene to amplify it from a DNA background of either Wisconsin or Arizona cDNA (appx. B). At the time, exisiting cDNA was available for a population from Wisconsin that is genetically similar to Illinois, and reduced the amount of time and cost associated with cDNA preparation. Agarose gel electrophoresis confirmed specific amplification of the target genes (Figure 2). The genes were then successfully cloned into pGEM-T Easy vectors and transformed into *E. coli* (Figure 3). Subcloning was carried out into pGBKT7 vectors, which are better suited for eventual protein expression in yeast, with DNA isolated from the transformed colonies and new primers designed to trim the signal peptides from our chosen genes and restriction sites (appx. C). The signal peptide is removed to more accurately replicate the protein in protein interaction assays as it would exist in the organism. The subcloning transformations were not successful. While PCR amplifications of our target genes supported the presence of our target genes (Figure 4), they also indicated some sort of error in the cloning of ILL. Sanger

sequencing results were inconclusive for ALL and ILL samples but indicated that ARZ was not cloned into the vector before transformation. This was likely due to human error and is not a representation of the utility of yeast two-hybrids in identifying plant-effector protein interactions as they have been used for effector validation in microorganisms and insects before [13,14] and are being successfully completed by other researchers in our lab (unpublished data). Due to time constraints, efforts for correcting the functional assays preparation steps were paused. Yeast two-hybrid experiments were not completed in this project but will be attempted in the future.

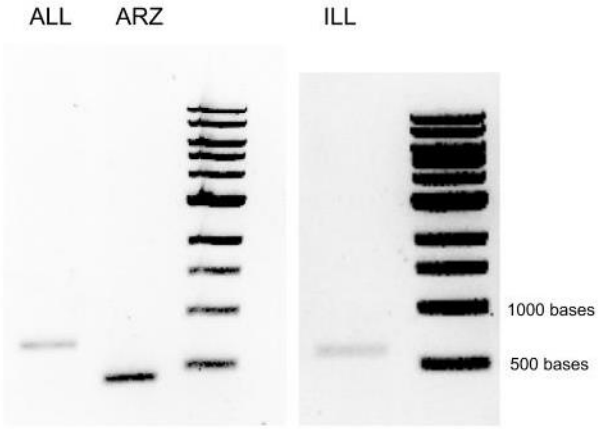

Figure 2: Agarose gel of initial PCR of target genes showing amplicons of the expected sizes, supporting correct amplification.

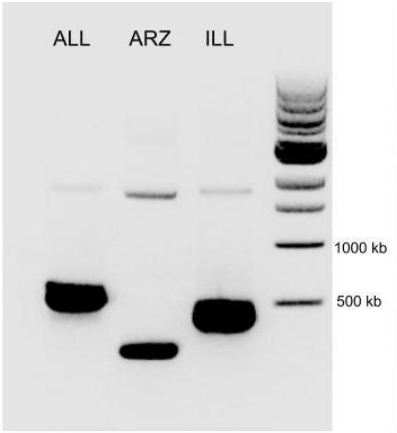

Figure 3: Agarose gel of PCR products in transformed colony DNA using target gene specific primers, indicating the presence of our target genes.

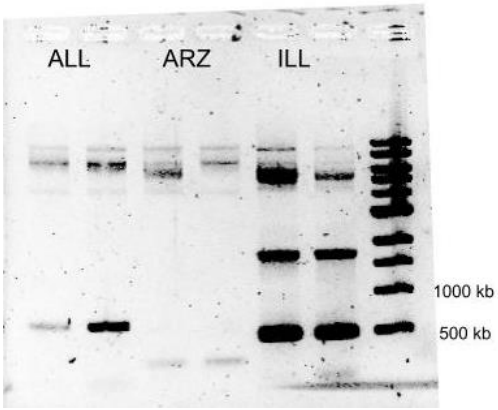

Figure 2: Agarose gel of PCR products from subcloning colony purified DNA and primer set 2. An unexpected band at 2000 bp in ILL indicates an error in the cloning or transformation process.

Population Expressions

Differential gene expression analysis of phylloxera samples from different populations feeding on different hosts revealed that predicted effector gene expression clustered based on host species (Figure 5). This suggests a strong host effect on the effector profile of phylloxera and may provide a signature of local adaptation. Gene expression profiles of phylloxera feeding on *V. riparia*, a species widely distributed across central North America, are distinct from those feeding on *V. arizonica*, a species native to the southwestern United States. Gene expression of phylloxera feeding on *V. x* Frontenac, a cultivated variety with European grape and *V. riparia* lineage, is more loosely clustered, likely due to the shared ancestry between the host species.

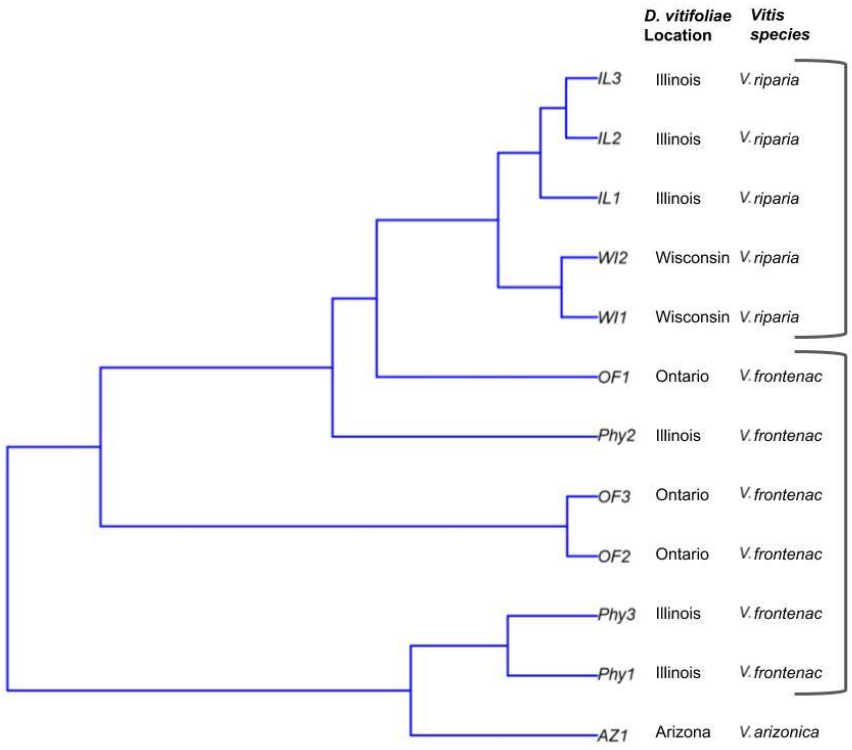

Figure 3: Dendrogram for samples of feeding stage phylloxera representing different populations and the species of grape they were feeding on. The algorithm clusters them based on similarity in gene expression profiles of the predicted effectors.

Differential Gene Expression (DGE) Analysis

An RNAseq analysis between each distinct treatment type (indicated by the sample acronyms) revealed 1106 differentially expressed predicted effector genes with 10-fold change (FC) and a 0.1 false discovery rate (FDR) (Figure 6).

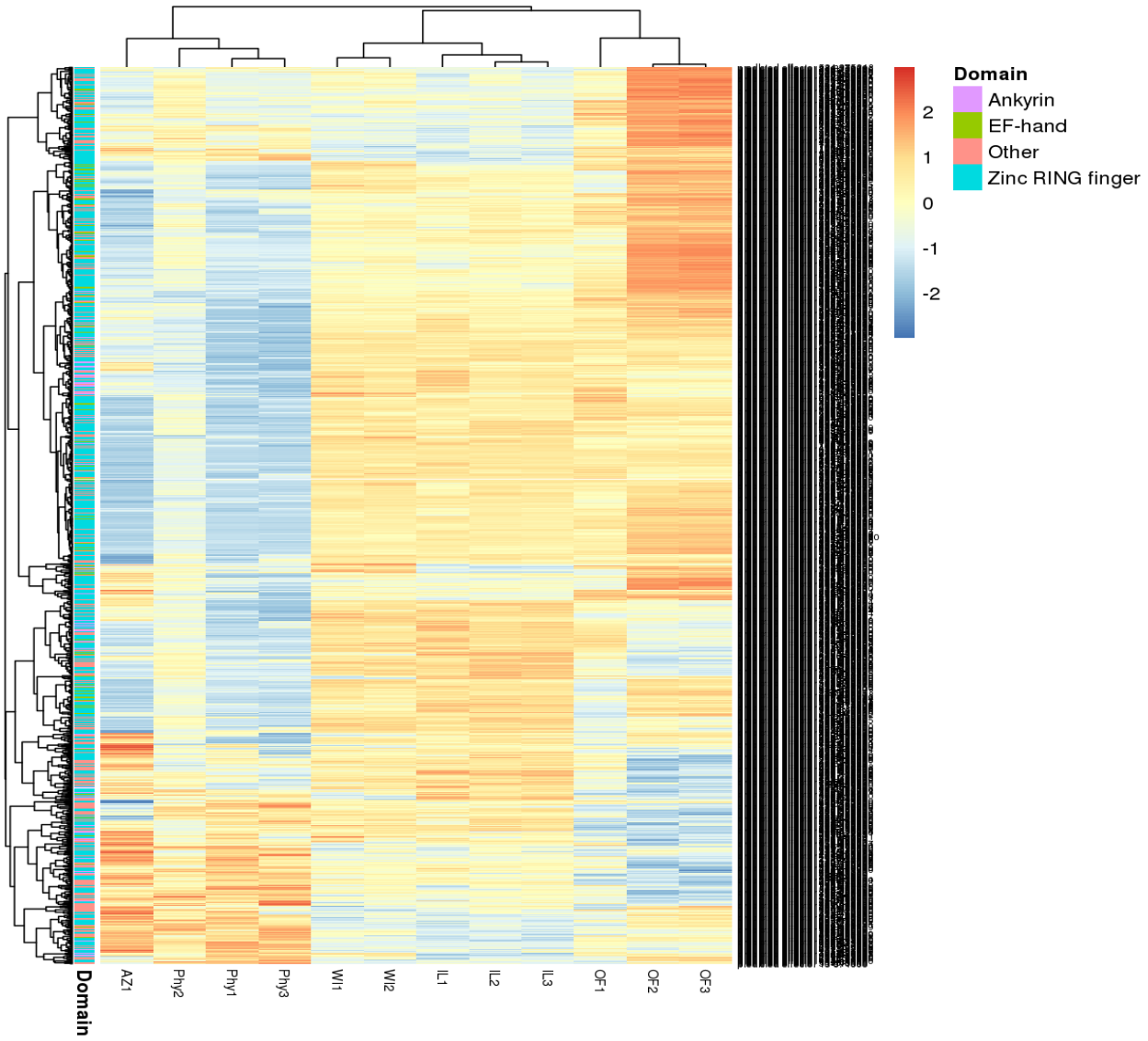

Figure 4: **Population+Host Treatment Heatmap, 10 FC, 0.1 FDR.**
Heatmap of feeding stage phylloxera analyzed for gene expression similarities or difference between the different treatments of population and host species (IL, WI, OF, Phy, and AZ). Annotated are the most common predicted protein motifs.

The gene names are not legible in the corresponding heatmap due to the high volume of results, exemplifying the need for further analysis to narrow down true effector genes. The comparison between IL and Phy samples reveal a large number of differentially expressed genes (DEG) (Table 1). This is of special interest as IL and Phy samples are of the same phylloxera population, only differing in the species of host they are feeding on. This supports that there is a strong host effect on the predict effector gene expression in phylloxera.

Comparisons	Up	Down
IL-WI	10	21
IL-AZ	217	84
IL-Phy	449	75
IL-Ont	108	166
WI-AZ	201	65
WI-Phy	427	51
WI-Ont	78	63
AZ-Phy	49	9
AZ-Ont	136	253
Phy-Ont	118	504

Table 1: **Population+Host Treatment DGE Summary**. Number of up or down regulated genes between each comparison. Highlighted is the comparison between Il and Phy samples that show a great number of differentially expressed genes even though that are of the same population

To investigate this host effect further, a DGE analysis was conducted on these same samples using only host species as the treatment, making three treatment groups to compare between: *V. riparia*-feeding (R), *V. x* Frontenac-feeding (F), and *V. arizonica*-feeding (AZ) . There were 106 total predicted effector genes that exhibited distinct differences in expression between host species (Figure 7). Predicted effector 965-61049 stands out as it showed higher expression in the AZ sample than any other sample and also codes for an EF-hand motif. This gene is also constitutively expressed in phylloxera of feeding stages on *V. arizonica* and not eggs, making it more likely to be a true effector (unpublished data). This would support the hypothesis that the Arizona phylloxera population may have one or more effectors that triggers a hypersensitive response in incompatible hosts. While this gene may be necessary in colonizing

V. arizonica, it may be working against the insect as it attempts to colonize other grape species. However, due to the lack of replication for this sample, there is a possibility the difference we see is not significant.

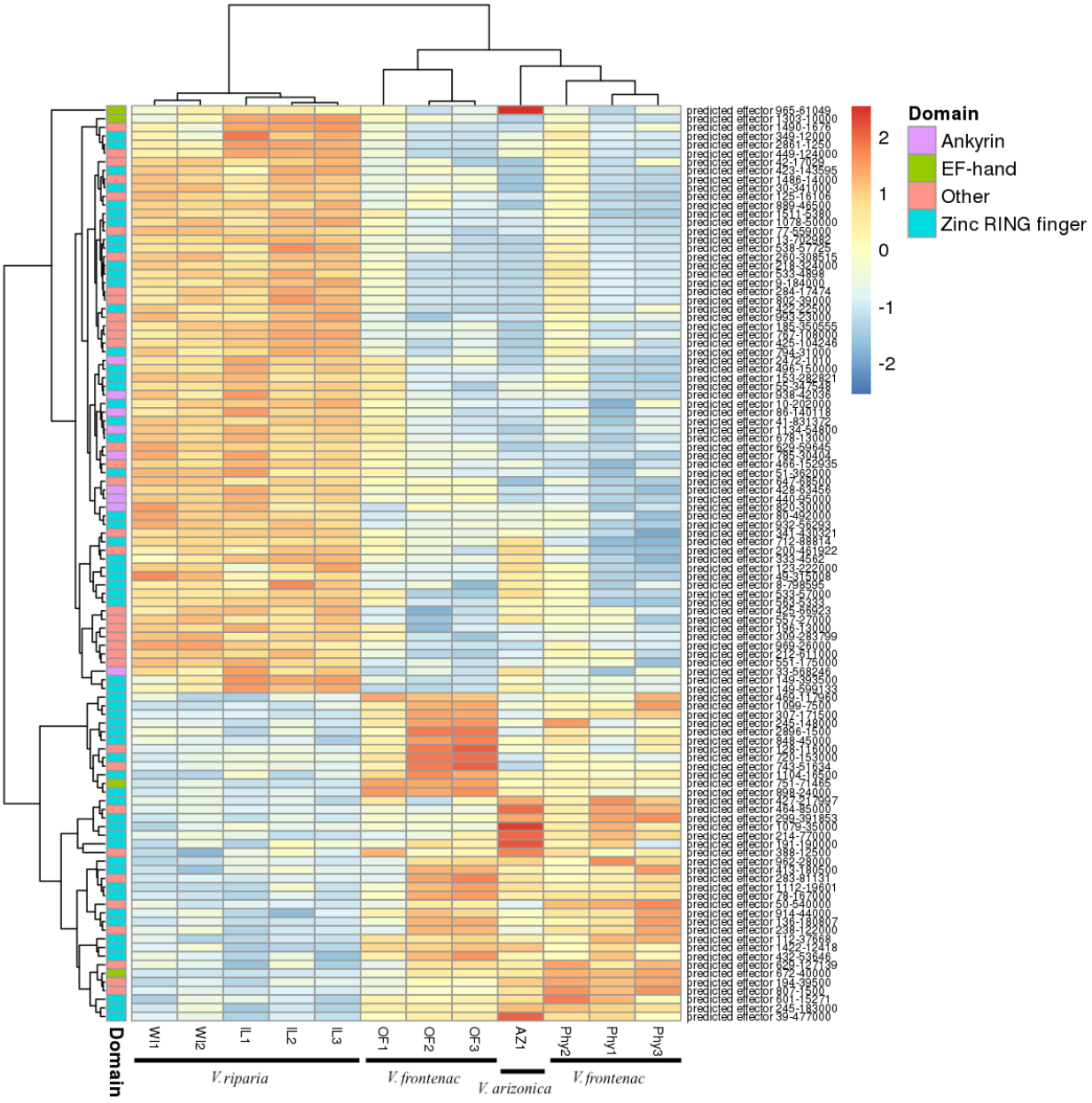

Figure 5: **Host Treatment Heatmap, 10 FC, 0.1 FDR**.

Heatmap of samples IL, WI, OF, Phy, and AZ analyzed for DEGs using host species as the treatment group.

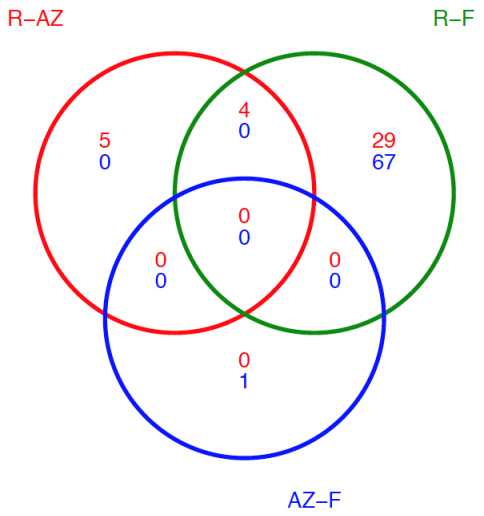

Figure 6: **DEG Host Venn Diagram.**
Diagram displaying the number of up or differentially expressed genes between each host species: *V. riparia* (R), *V. x* Frontenac (F), and *V. arzonica* (AZ) using the same samples. Red indicates the number of genes that are up-regulated genes in the second group. Blue indicates the number of genes that are down-regulated in the second group. For instance, the comparison between AZ and F revealed 1 gene that is down-regulated in F compared to AZ.

There are 100 differentially expressed genes discovered between *V. riparia*-feeding, and *V. x* Frontenac-feeding groups, higher than any other host comparison (Figure 8). Of these, 4 genes were downregulated in the *V. riparia*-feeding group compared to feeding on either other host. *Vitis riparia*-feeding and *V. x* Frontenac-feeding phylloxera varied the most in gene expression, even though *V. x* Frontenac has *V. riparian* lineage. This suggests that the traits inherited from the novel European hosts in *V. x* Frontenac's ancestry influence the gene expression of phylloxera tested. Predicted effector 965-61049 is represented in Figure 8 as the only significant DEG in the AZ-F comparison, showing down-regulation in *V. x* Frontenac-feeding samples compared to *V. arizonica*-feeding samples. Genes that are not up or down-regulated between comparisons are equally expressed among populations and may be conserved as integral effectors in overcoming the plant immune response and establishing galls; or they are not true effectors and do not function to manipulate the plant, but function as secretory proteins

in insect homeostasis. Further data analysis and functional assays in the future will elaborate on this.

CONCLUSION

A comparative transcriptomics approach was used to investigate population and host specific predicted effector gene expression in grape phylloxera. The analysis revealed a strong host species effect on the effector profiles of phylloxera. Most notable was the comparison between *V. riparia*-feeding and *V. x* Frontenac-feeding Illinois population phylloxera, that had 524 differentially expressed genes even though they were collected from the same location. The data created during this project can be used towards creating an effector co-expression network, and in combination with plant response data, can better predict the function of unknown effectors. These bioinformatics-based analyses helps to narrow down possible true effectors in grape phylloxera to be examined in future experiments. While yeast two-hybrid assays were not completed in this project, they will be attempted in the future for genes of interest, like predicted effector 965-61049. These effector studies will lead us closer to understanding insect-plant interactions, and the genes in conferring compatibility or incompatibility on hosts. This information can be built upon to breed resistant plants that can better recognize conserved effector proteins and develop better pest management strategies that target effector functionality.

METHODS

Plants and Insects

Populations of *Daktulosphaira vitifoliae* were collected from several wild grape species across North America: *V. riparia* in Urbana, IL (40.078943,-88209237), *V. riparia* in La Crescent, MN (43.885364,-91.338169), *V. arizonica* in Arizona (34.538409, -111.687592), and

V. riparia in Ontario, Canada (43.47589,-79.89012). Individuals from the Illinois population were colonized on V. x Frontenac in a separate experiment (denoted Phy1-3). Insects were collected across multiple vines at each location to maximize genetic diversity and pooled into samples comprised of 20-50 actively feeding insects.

RNA Extractions, Sequencing, and Analysis

Daktulosphaira vitifoliae RNA was extracted using QIAGEN's RNeasy Mini Kit and following the included protocol. Samples were collected from galls of infested *Vitis* leaves. Using small paint brushes, insects were brushed out of galls onto a petri dish where they could be sorted by life stages; egg, nymph (of any instar), and mature adult, being sure to only include those that were alive. Nymphs from the Arizona population had a low survival rate in the greenhouse, so eggs were collected and temporarily stored in culture tubes until they hatched into nymphs to be collected. RNA was extracted following the QIAGEN RNeasy Mini Kit protocol. RNA extractions were quality checked with a 2100 Bioanalyzer and sequenced with HiSeq 4000 at the University of California at Davis DNA tech core. Alignment to the reference genome (hosted at http://bipaa.genouest.org/is/) [18] was completed using Hisat2 with a k value of 1. RNAseq analysis was conducted with a modified workflow of SystemPipeR utilizing edgeR for a differential gene expression analysis [17]. Transcript counts were compared only for effector genes annotated in the genome using host and population location as contrasts.

Plasmid Construction

Daktulosphaira vitifoliae Arizona population cDNA was synthesized following the SuperScript III First-Strand Synthesis Systems for RT-PCR protocol for oligo$(dT)_{20}$ from Invitrogen. The Minnesota population cDNA was already available in the lab. Candidate effector

genes DV3007389.2 (predicted effector 213-30231) and DV3009582.2 (predicted effector 425-4600) of Wisconsin cDNA and DV3023045.2 (predicted effector 2155-4000) of Arizona cDNA coding sequences were amplified using PCR settings 1 for DV3007389.2 and DV3023045.2 and PCR settings 2 (appx. D) for DV3009582.2 using primers indicated in Appendix B. Amplicons were purified using the QIAquick PCR Purification Kit and cloned into the pGEM-T Easy Vector following the pGEM-T Easy Vector Systems protocol by Promega. This was transformed into Lucigen 5 alpha DUO competent cells following its respective protocol using LB/Amp/IPTG/X-gal selective media. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit by Qiagen. Transformation was confirmed by Sanger sequencing at UCR's Gencore

Subcloning with pGBKT7 Vector

Primers were designed with SfiI and BamHI restriction sites based on consensus sequences, cutting off signal peptide portion as determined by SignalP 4.1 and Phobius [15,16]. PCR was conducted using primer set 2 and PCR settings 1 (appx. D) for all three gene clones then purified using the QIAquick PCR Purification Kit. Restriction enzyme digestion was then conducted in a 50ul reaction using 5ul NEB CutSmart 10x Buffer, 1ug of purified PCR product DNA, and 10U of each restriction enzyme. Digestion was also run for the pGBKT7 vector at twice the reaction volume. This was incubated at 37℃ for 1 hour. The digests were purified using the QIAquick PCR Purification Kit. Ligation was conducted at an approximate 1:3 plasmid to insert ratio. In a 20ul reaction; 2ul of 10x Ligase Buffer, 200ng of digested pGBKT7, 50ng of digested gene insert, and 1ul of T4 DNA Ligase was combined and incubated at room temperature for 2 hours. Transformation into Lucigen 5-alpha DUO competent cells followed the accompanying procedure using Kanamycin selective media. DNA was purified using the

QIAprep Spin Miniprep Kit by Qiagen. Sequences were obtained by Sanger sequencing at UCR's Gencore.

Yeast Transformation and Y2H Reporter Assays

Genes were to be cloned into yeast using Yeastmaker Yeast Transformation System 2 by Takara Bio. Yeast two hybrid assays would have been conducted following the MatchMaker Gold Yeast Two Hybrid System manual by Takara Bio.

APPENDIX

A)

Target Predicted Effector CDS Gene Sequences
>DV3007389.2 ATGTATTTCTACATTTTCATCGTGATGTATCTAACTAGTTGTCTTTCGAACGCATATCTTTTC GAATTTTCACCTTTGGACAAGGACATGGAATTGACTATGGCATCCCGAAATGTTTTGAAAA ATTTGTATATAGAGAATACAATGAATAATCAAGTAATGAAATTAACGAAATTTGATTCGCT ATTGGTTTTTTTTAGATTGGTACATAATTTGGATGATCGTGAAATTATAACATTATTCTTTG ACGAAGTTCAAAATAATAAATACAAAATTATGAATTTGGATCAATTTTTGAAACAAATGAA AAAAGTTGTACAAATAATTGAGGATCGATTAAAAACTATTTATGAAAAATATCTCAACAAA GATAAAAAGATGACTACAAACGAATTAATAAAAGCTATAGATGAAACTAGTTTCAAAATC ACGCAAGATAAAGCTACTAAGTTAATAAATAATGAAAGTATTGACAAACAAGATATCAGC TTTGAAAAATTCAGAGAAATGTACGCGAAATATTATCATGAAGTACTTGATGATTTAAAAC AAATAGAAGACCTTAAAAATTTAGCTTCTAGGTTAGCAAAATTGGAGGTTACAATTTATTG GACTAAGGCACAAGAGAGTATATGTCAACAAATAATAAATGAGAGATTAGATAGCCTTAT ATGA
>DV3023045.2 ATGGCTCATTTTAAAAGCGCAGTTGTATTGTGTTCAGTGTTCTTCATTGCCGGCGCTCTGGG TGGAATTTTAAAAGACATACAGGATGAGTACCATGTTGTTATGGATGTATTAATTAATAAT GACAAAAATAAAGATGGCCAATTTGACTATAATGAAATGGTAAAAGTATGTGATGCATTG AAGAGCAAAAATATAATCGATTTATACACGGAACGAGTTATCTTACAATATTATTGTGGTT ATTCTGAACCTAAGGCGAATTGTGTACAAACGCTTGCAACCTATCAAGTGTTGCATATACT TGGAATACACTAG
>DV3009582.2 ATGAATTTTTACTTTTTCATTGTTATGTATCTAGCAATTAACTTTTCAAATGGATTAATTTAC GATACCCCTATTACGAATAAGGATGGACAATTGACTTACAATGGCAATCTTTGTATGACTA AGCTATTTCATGATATCAGAGAGAATGACATTGGAATGAATTATGCAAAATTTTGTACGGT TTTGAAAAAATATCAAAAGATACACTGTTTAAATGATACCAAAATTCATTTATTTTTCGTTG GCATTGTTGGAAACACTGATGGATATATGACGGAAGTGCAATTTTTAAGTATAATGGCAAT AGTTCTCCATCGAGTTGAGATTTTTATTGAGAGATTTTATAATAGTTGCCTCAAAAATAATG TGATGACTAAAATTGAATTAATTGATGCTTTTAAACAATACAAAATCAATCTTAATGATTCT ACAGTTACTGAATTAATGAATCAGTTTGCAGGTACAAACGTAGAAAATATCTCCTACACAG TATTCAGAGATATTTTGGGGTACTTACTACACAAGAACAACCCTACAAAACGACAGTCGTA A

B)

Primer Set 1 (5' - 3')		
Gene	Forward	Reverse
DV3007389.2	CAG TTT TAT TCG AAA ACG TCA A	CAG TCA TAT AAG GCT ATC TAA
DV3023045.2	GTT AGC AGT AAA TTC AAA TAG	ATA TGT TAT TAC TTC AGC GTC
DV3009582.2	ATT CGA GAA CAC CGA TTT GTC	AGA GAT GTC CAC TGT TAT TCC

C)

Primer Set 2 (5' - 3')		
Gene	Forward	Reverse
DV3007389.2	ATC ATT GGC CAT GGA GGC CTT TCT TTT CCA AAA ACC A	CAG CTC GGA TCC TCA TAT AAG GCT ATC TAA
DV3023045.2	GTT CAT GGC CAT CCA GGC CGG AAT TTT AAA AGA CCA A	CAG GCT GGA TCC TAG TGT ACT CCA AGT ATA
DV3009582.2	ACT AGT GGC CAT GGA GGC CTT AAT TTA CGA TAC CCC T	ACT CAT GGA TCC TTA CGA CTG TCG TTT TGT

D)

	PCR Settings 1					PCR settings 2				
Temperature (°C)	94	94	50	72	72	94	94	55	72	72
Duration (min)	3:00	0:20	0:35	1:00	5:00	3:00	0:20	0:35	1:00	5:00
		x35					x35			

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