ABSTRACT

Evaluating and Isolating Analog of SWEET14 Nectar Promoter from *Arabidopsis*: Towards the Development of Mosquitocidal Nectar

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Mosquito-borne diseases are a major problem, infecting hundreds of millions of people every year and leading to numerous deaths across the world. Although there have been some improvements in mortality rates from preventatives such as mosquito nets and pesticides, there is much room for improvement. Because mosquito populations have been shown to be dependent on plant nectar as an energy source, creating plants that express mosquito-specific toxins in their nectar would be a novel approach for mosquito control. In order to express mosquito toxin in the plant's nectar, a nectar-specific promoter must be isolated. One nectar-specific promoter that would be ideal to isolate is the analog of SWEET14. Construction of BLAST databases from the RNA-seq data was verified as a method to isolate SWEET14 through artificial chromosome walking. Testing the effectiveness of this promoter in *Impatiens walleriana* would be done by combining SWEET14 with enhanced green fluorescent protein (EGFP) and would be used in the transformation of *I. walleriana*. Successfully expressing mosquito-specific toxins in the nectar of *I. walleriana* can effectively control harmful mosquito populations and can further be applied as an expression model for other insect species, providing a much broader application.

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EVALUATING AND ISOLATING PROMOTERS IN *IMPATIENS WALLERIANA*: TOWARDS THE DEVELOPMENT OF MOSQUITOCIDAL NECTAR

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By

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CHAPTER ONE

Introduction

Mosquitoes infect over 300 million people per year, with 800,000 deaths from Malaria and 20,000 from Dengue (39). This does not include the effects from Zika virus, West Nile Virus, and the many other diseases that mosquitoes play an important role in. Although there have been some improvements in mortality rates from preventatives such as mosquito nets and pesticides, there is much room for improvement. Creating mosquitocidal plants that target the species of mosquitoes that are vectors for these deadly diseases would immensely reduce the mortality rates of these deadly diseases, and could one day possibly even eradica-te them.

1.1 Mosquito Biology

Aedes aegypti, a species of mosquito, is a holometabolus insect, meaning it goes through a complete metamorphosis with an egg, larvae, pupae, and adult stage. In order to survive, an adult *Aedes aegypti* needs sugar to survive, fly, and enhance its reproductive potential. The most common source of sugar for mosquitoes are floral nectaries. The adult stage can range from two weeks to a month. Female *Aedes aegypti* lay 100 to 200 eggs per clutch and can produce up to five clutches of eggs during a lifetime. The number of eggs is correlated with the amount of blood ingested. The eggs can be laid on damp surfaces in areas that are likely to flood. This includes manmade containers in and around homes, such as barrels, drums, jars, pots, buckets, vases, tanks, bottles, water coolers, as well as tree holes and other places where water could collect. The female mosquito lays her eggs at varying locations, not at a single site. Once the eggs

are submerged in water, they quickly hatch within two to seven days, making it difficult to control the population (1).

The larvae of an *Aedes aegypti* swim in water, where they feed on algae and other microscopic organisms. They go through four instars, or development stages, in about four days and become about eight millimeters long by the fourth instar, with males reaching this point faster than females. However, if the temperature is cool, *Aedes aegypti* can remain in the larval stage for months. After the fourth instar, larvae become pupae. These pupae are mobile and respond to stimuli, but they do not feed. It takes about two days for the pupae to mature into adults, which occurs by the pupae ingesting air to expand the abdomen, splitting the pupal case, and emerging headfirst (1).

1.2 Mosquito-Related Diseases

The diseases that mosquitoes carry kill nearly 725,000 people every year (6). Tropical diseases, in general, kill about 1.6 billion people annually. It is an understatement to say that these insects are harmful. Mosquitoes play active roles in transmission of diseases such as malaria, dengue, chikungunya, Zika, and West Nile.

The preliminary symptoms of malaria include fever, headache, chills, and vomiting. Complications include anemia, respiratory distress, and cerebral malaria in children as well as other types of organ failure in adults (20). The protozoan parasite *Plasmodium falciparum*, which causes malaria, is the leading cause of morbidity in some areas of Western Kenya. In addition, *Plasmodium vivax* is another protozoan parasite that frequently causes recurring malaria but is less virulent than *Plasmodium falciparum* (20).

Antimalarial medications can be used to treat or prevent malaria. Chloroquine, mefloquine, and primaquine can be taken as preventatives against *P. falciparum* and *P. vivax* infections. Doxycycline is an alternative to mefloquine. Malarone is also a useful

medication that interferes with the growth of parasites in the red blood cells of the human body (3). However, parasitic resistance can develop to one or more medications. Some patients have severe reactions to the medications and stop taking them, leading to the development of resistance. Another problem that can lead to resistance is the use of sub-curative doses of an antimalarial drug (21). Chloroquinine resistance, which has been reported in all sub-Saharan African countries, requires the use of additional antimalarial medications such as quinine. Due to these issues, there is an urgent need for new and enhanced methods to control these diseases (7).

Symptoms for dengue include fever, rash, headache, joint and retro-orbital pain. Severe forms of dengue result in vomiting, internal hemorrhaging, and death. There are about 50-100 million dengue infections per year, primarily in tropical regions (22). Four of the viruses are transmitted primarily by *Aedes aegypti*. Dengue is now a threat to the United States and Europe because of increased global temperature, resulting in more widespread presence of *Aedes* mosquitoes (23).

Chikungunya is another disease transmitted by mosquitoes. Symptoms include fever and joint pain as well as headache, muscle pain, joint swelling, or rash. Unfortunately, there is no treatment for this viral infection. Chikungunya is transmitted to people by *Aedes aegypti* and *Aedes albopictus*, which are the same mosquitoes that transmit dengue virus. Typically, Chikungunya occurs in Africa, Asia, and Europe, but with infected travelers carrying the disease, Chikungunya is becoming more widespread, with the first outbreak in the Americas occurring in the Caribbean in 2013 (24).

Zika infection results in fever, rash, joint pain, red eyes, and neurological conditions in adults. Zika is caused by a virus that asymptomatically infects mosquitoes, with the primary vector being *Aedes aegypti* (25). The virus goes through the

development stage in the mosquito for about a week, during the extrinsic incubation period. When the female mosquito bites an infected human, it can transmit the virus potentially to all bite victims for the rest of its life, which is 2-4 weeks. Zika can then be spread via sexual transmission from a male partner to a female. Pregnant women transmit the virus to their offspring, and even prior to transmission, Zika causes birth defects such as microcephaly, where the brain does not fully develop, during pregnancy (26).

Most people, about 8 out of 10, who are infected with West Nile virus do not develop symptoms. Only 1 in 5 who are infected develop a fever, headache, body aches, joint pains, vomiting, diarrhea, or rash. There is no vaccine or medication to treat West Nile, either. West Nile has been detected in several mosquito species, including *Aedes aegypti* (4).

1.3 Established Protective Measures

There are several protective measures that can be used to prevent disease transmission by mosquitoes. Insecticides, for instance, can be a useful resource. This includes indoor residual spraying (IRS) of insecticides. There is also long lasting insecticidal nets (LLINs) which are insecticide treated bed netting. This is effective for malaria but not dengue fever due to the times that they bite (27). *Aedes aegypti*, in particular, mainly bite during the day, which means that methods like mosquito nets are not useful (28). An issue with outdoor spraying is that it has led to long-term damage to natural resources. Pesticides kill more than mosquitoes. They kill beneficial insect species and vertebrates that eat those species (29). This disturbs the ecosystem. Pesticides also promote pesticide resistance, which eliminates their usefulness (29).

Another option for preventative measures includes using genetically modified mosquitoes. This seeks to suppress the population by injecting mosquito eggs with DNA

containing lethal genes, releasing only the genetically modified males to mate with wild females, and producing offspring that will not be capable of surviving into adulthood (30). An advantage to this option is that less monitoring is needed and insecticidal spraying inside and outside homes would be reduced. One disadvantage is the concern that the genetically modified mosquitoes could generate beneficial mutations that cause them to produce offspring that are resistant to the lethal genes or environmental dangers. Other disadvantages to this technique are the cost and time spent to reach success (31). Though there have been successful projects in the Cayman Islands, Brazil, and Panama, the public is still strongly concerned about introducing GMO animals in the environment. Because of this and the enormous price tag for this project to reach success globally, the likelihood of the fruition of genetically modified mosquitoes eradicating mosquito-transmitted disease is far from reality (32).

Container control is another option that is used for prevention. One empties containers of standing water that serves as larval-rearing sites (33). Poor water and sewage availability exacerbates this issue. Removal of open water containers is a strongly encouraged tactic in the United States and Europe and is also taking hold in India and other parts of South Asia. Larvicide packets have been used in collected water as well, but this is not a cost-effective method to get rid of mosquito larvae since it has to be applied every year (34).

What many people use as another method to prevent mosquitoes from entering homes is screened windows, air conditioning, and enclosed walls and roofs. Placing a mesh screen over all doors and windows allows fresh air in but prevents pests from entering. Although the air conditioning will not physically repel the mosquitoes, running

the air conditioning tends to stop one from opening up windows or leaving doors open (8).

As indicated, there are issues with the methods currently being used to reduce the number of people affected by these diseases. Whether it be an economical problem, environmental hazard, non-encompassing solution, or any of the vast number of other reasons why these methods have not been working as well as initially perceived, it is just not enough to get the job done. There must be another more solution for this dilemma, and, in fact, there is such a solution well on its way in fulfilling this niche.

1.4 Mosquitocidal Nectar System

Mosquito populations have been shown to be dependent on plant nectar to sustain themselves. It has even experimentally proven that nectar is preferred over a sucrose solution. Females require it to power their "blood quests" in order to produce eggs while males sustain themselves almost completely on this substance (9, 10). Nectar is composed of carbohydrates and sugars as well as several important proteins. Nectarin I, for example, is a germin-like proteins (GLPs) with superoxide dismutase activity which protects the plant from microbial attacks (40). Other proteins, such as Nectarin III, play a role in buffering the pH of nectar through carbonic anhydrase activity of *NEC3* (40). This role is important in providing a pH-balanced meal for visiting pollinators (40). Nectarin IV has been shown to be an inhibitor of xyloendoglucanase (XEG), which is a hemicellulose-degrading enzyme expressed in many fungi to colonize and infect plant tissues (40). As an inhibitor of this enzyme, Nectarin IV prevents degradation of the nectary cell walls and, therefore, decreases susceptibility of pathogenesis of fungal cells (40). There are also a family of proteins, called the SWEET family, found in nectar that have shown to be key players in transporting sugars in the nectar. SWEET8 and

SWEET13 provide nutrients to the pollen by mediating uptake of sugar across the plasma membrane (35). SWEET12 assists in transferring sugars from the seed coat to embryos and plays a role in phloem loading (36). SWEET9 is essential for nectar secretion. The SWEET family has also been shown to play a role in pathogen resistance in rice and cassava (15)(16). Because mosquitoes rely so heavily on nectar as a food source, expressing a toxin in one of these SWEET proteins could have major effects in killing the mosquitoes as they ingest the substance.

Figure 1. Aedes aegypti mosquito feeding on plant sugar source

An obvious choice in the plant to genetically modify with mosquitocidal nectar is *Impatiens walleriana*. The nectar of *Impatiens walleriana* has been shown to be attractive to *Aedes aegypti* mosquitoes, meaning that the nectar from this particular plant is the most preferred food source to this mosquitoes species. *I. walleriana* have also been shown to produce an mRNA analog to an Arabidopsis protein. Because SWEET14 is a native promoter, it will be easier to use and lead to more guaranteed expression of the foreign toxin proteins in the nectar. Since *I. walleriana* originates from a similar environment as the mosquitoes' prevalence, Sub-Saharan Africa, the genetically modified form would grow well in mosquito-infested areas (13, 14).

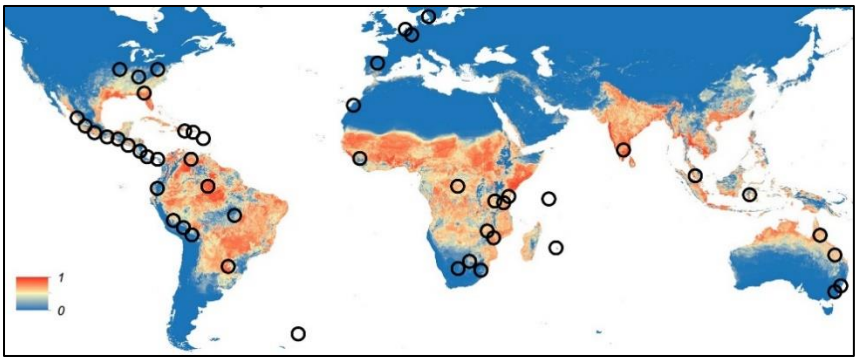

Figure 2. Global distribution of Aedes aegypti with Impatiens walleriana. The colors represent the probability of occurrence of these mosquitoes from 0 being blue to 1 being red. The black circles indicate the known locations of Impatiens walleriana that have been identified in the wild.

Impatiens walleriana is also a desirable plant species to express a toxin because it can produce large quantities of protein in nectar. In fact, this Impatiens species produces the highest level of protein in nectar compared to the nectar of other attractants, as shown in Figure 2, which will allow high levels of toxins to be secreted. Furthermore, *I. walleriana* have been shown to be readily transformable using *Agrobacterium tumefaciens* with a variety of protocols, as shown in Figure 1 (10)(11)(12). Therefore, *I. walleriana* is the perfect model system for transgenic nectar-protein delivery to *A. aegypti*.

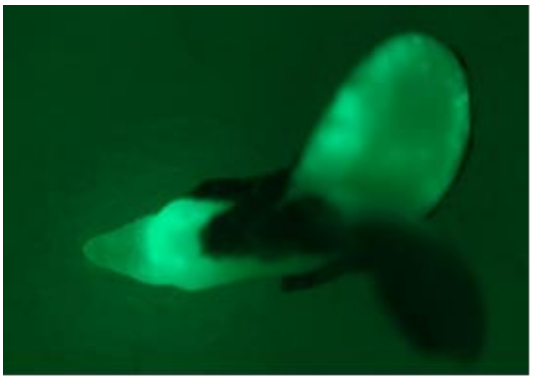

*Figure 3: **Agrobacterium transformation of impatiens seeds.** This image demonstrates successful transformation of impatiens carmine seeds with the seed transformation protocol.*

Phylloplanin has been determined by Dr. Kearney's lab to be the most highly expressed protein in nectar by the use of mass spectrometry. Phylloplanins are known to be potential antimicrobial compounds, specifically fungicidals, since they work on inhibiting pathogen germination once contact has been made with the plant tissue (37). In fact, they are the first site of contact in the host-pathogen interaction (37). Secretion of phylloplanin into the nectary, therefore, keeps the fungi from stealing the plants' sugar. Because Phylloplanin is a native protein, is necessary for the survival of the plant, and has been shown to be highly expressed, this is another ideal candidate as a promoter to express the foreign toxin protein in nectar.

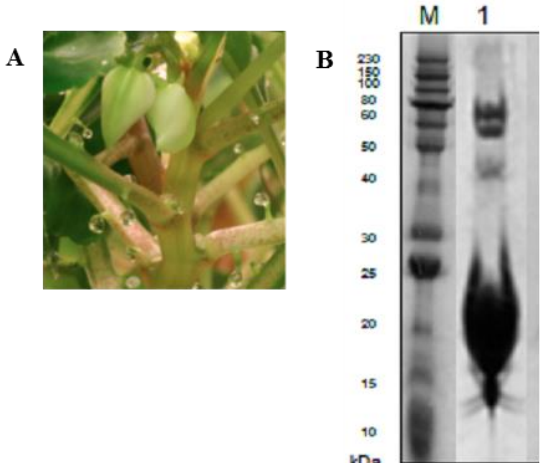

Figure 4: Contents of nectar from Impatiens walleriana. (A) Picture of impatiens extrafloral nectar. (B) SDS-PAGE of impatients nectar with high concentrations of proteins.

I propose to use SWEET14 promoter to express foreign proteins in *Impatiens* nectar. To achieve this, the promoter must first be isolated. Molecular biology techniques can be utilized to isolate and use SWEET14 and other high-expressing promoters, such as Phylloplanin. A successful toxin delivery system to specific species of mosquitoes such as *Aedes aegypti* will have significance in allowing not only third world countries but

also nations like the U.S. to be better equipped for targeting the mosquitoes that vector various tropical viruses that will be moving northward due to global climate change (13). It is also a more cost effective, sustainable solution compared to previous alternative methods such as pesticide sprays, which are extremely harmful to the environment (17)(18)(19). In addition, this delivery system will be specific to *Aedes aegypti* and will not produce these lethal effects in other species of mosquitoes as of now. The cry toxin that will be utilized for this system will kill the mosquito once they have ingested the toxin, which will then paralyze the digestive tract and cause the mosquito to stop eating and starve to death (38).

CHAPTER TWO

Materials and Methods

Primers for SWEET14

Since SWEET14 is a native promoter, we confirmed the sequence of SWEET14 using cDNA sequencing. Once the sequence was identified, we amplified it via PCR. Then, we selected the first 30 base pairs of the desired sequence and the last 30 reverse complement base pairs of the sequence for the forward and reverse primers, making sure that the complement primer contained at least 15 base pairs after trimming to ensure it met the proper melting temperature and other parameters as discussed below. Using software from https://www.idtdna.com/calc/analyzer, we tested our primers to make sure they did not self-dimerize, heterodimerize, or fall below or above the desired melting temperature. If the melting temperature was not between 55-60°C, up to 6 random base pairs were appended to the beginning of the primers to change the melting temperature. Finally, we searched the original template to ensure that the primer would bind to only one location. Deduced mRNA sequences were used to design primers for larger fragments of cDNA to perform inverse PCR to amplify the gene from nectary organ mRNA.

iwSWEET14 Sequencing Primers

Primers for verification of iwSWEET14 promoter:

iwSweet14dn2
6 random – 15 bp of iwSweet14 ORF
CCGCCA TCGGTAAAAATTGCC

iwSweet14dn3
6 random – 15 bp of iwSweet14 ORF
CCGCCG TAATTCCGAAGATGA

iwSweet14dn4
6 random – 15 bp of iwSweet14 ORF
CCGCCG AGGAATGTAATGAAA

Figure 5: Primers for PCR confirmation of iwSWEET14 promoter obtained from iPCR

Upstream Sequence of iSWEET14

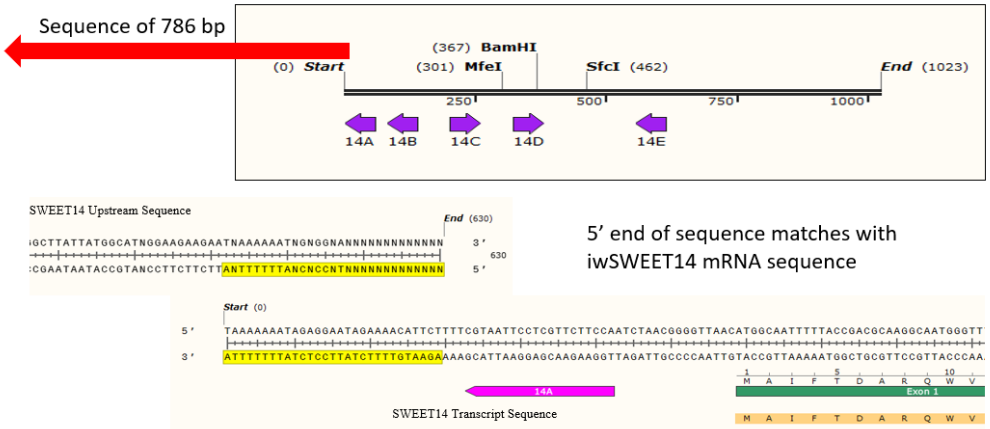

Figure 6: Alignment of upstream region of SWEET14 generated from iPCR to transcript sequence of SWEET14.

Making a BLAST Database

To make a local database in Windows, I clicked on the windows icon in the toolbar and then typed "system". I clicked on "advanced system settings", which required administrator privileges. On the bottom right of the window, I clicked on environmental variables. Under the user variables table, I clicked "new" and made a new user variable. I named it "BlastDB", and for the value it should be C:\Program Files\NCBI\blast-

2.7.1+\db, although the address will change depending on the version of Blast downloaded. I hit "Ok" and exited. I then restarted my computer. To create the databases, I ran Powershell, the administrator version, in order to add/edit the local database created.

Artificial Chromosome Walking

To run BLAST locally, we first downloaded the BLAST+ Command Line Applications. Once downloaded, we ran "blastn" on command line with a query address and an output address. After it finished running, we checked the text files that were generated that contained the matches. We looked through the matches and selected those that had a strand = plus/plus and started at base pair 1. We copied the Sequence ID of the sequence and then queried again with a database address and pasted the SequenceID as the entry.

We then copied the output, which was the full nucleotide sequence, and compared it to what we already had of the SWEET14 promoter in ApE. If there was overlap, we copied the part that was new to the SWEET14 fasta file. We continued this procedure until we did not get any good matches in any of the databases or there were no more helpful overlaps.

Genomic DNA Extraction

Before beginning the DNA extraction, we first set the water bath to 65 degrees Celsius. We then weighed out 1 gram of Impatiens walleriana leaf tissue, froze the tissue with liquid nitrogen, and ground into a fine powder in a mortar and pestle. We stored the tissue in a freezer at -80 degrees Celsius until further use to prevent the tissue from thawing. We then transferred the fine powder into a 30 mL Oak Ridge Tube and added

15 mL of EB1(Qiagen) and RNase. We also added 1 mL of 20% SDS to each tube. After screwing the lids on tightly, we mixed the solution thoroughly by shaking and then incubated the tubes in the 65-degree Celsius water bath for 10 minutes. After the bath, we added 5 mL of 5M potassium acetate. We mixed the solution thoroughly again by shaking and incubated the tubes at 0 degrees Celsius or on ice for 20 minutes. We then spun the tubes at 13K rpm for 20 minutes in a centrifuge at around 20,000 g to 25,000 g. We poured the supernatant through cheesecloth, Miracloth, but another alternate option is to pipette the supernatant through cotton into a 30 mL Oak Ridge Tube containing 15 mL of cold isopropanol. We then inverted the tubes 20 times to mix it well and incubated it at -20 degrees Celsius for 30 minutes. We spun the tubes at 12K rpm for 15 minutes in a centrifuge at around 20,000 g. We then poured off the supernatant and dried the DNA pellets by inverting the tubes on paper towels for 10 minutes. The DNA pellets were dissolved with 0.7 mL EB2(Qiagen) and transferred it to a 1.7 mL Eppendorf tube. The Eppendorf tubes were spun in a microcentrifuge for 10 minutes at maximum speed to remove insoluble debris. The supernatant was then transferred to a new Eppendorf tube, and we added 75 microliters of 3M sodium acetate and 500 microliters of isopropanol. We inverted the tubes 20 times to make sure they were mixed thoroughly, and we pelleted the DNA for 30 seconds in a microfuge on maximum speed. We washed the pellet with 500 mL of 80% ethanol 5 times (41). We then used the DNeasy Plant Mini Kit (Qiagen) steps 8-12 of the protocol. We placed the spin column into a 2-mL collection tube, added 500 microliters of Buffer AW2 to DNA, transferred the buffer and pellet to a spin column, and centrifuged for 1 minute at >= 6000 g. We then discarded the flow-through. We added another 500 microliters of buffer AW2 and centrifuged for 2 minutes at 20,000 g. The spin column was removed carefully so the column did not meet

the flow-through. We then transferred the spin column to a new 1.5/2 mL microcentrifuge tube. We added 100 microliters of Buffer AE for elution and incubated for 5 minutes at room temperature followed by centrifugation for 1 minute at around 6000 g. The column was transferred to another 1.5/2 mL microcentrifuge tube, another 100 microliters of Buffer AE were added for elution, incubated for 5 minutes at room temperature, and centrifugated for 1 minute at 6000 g to complete the process (42).

Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280
21.687	1.963	2.356	0.434	0.221
5.375	2.140	-3.628	0.107	0.050
10.874	2.147	3.416	0.217	0.101
1.266	3.780	-0.416	0.025	0.007
54.941	1.817	2.163	1.099	0.605
8.353	1.812	2.582	0.167	0.092

Figure 5: IW gDNA Nanodrop.

Inverse PCR

Inverse polymerase chain reaction (iPCR) allows us to amplify unknown sequences of DNA adjacent to known sequences of DNA with the use of specific restriction enzyme sites and ligation protocols. This protocol works well for identifying upstream sequences of a promoter, especially if there is no reference genome to follow. Restriction enzymes are used in the known sequence of the DNA. Genomic DNA is digested with these enzymes, and then the fragments are ligated to form a circular fragment of DNA. This DNA is then used as PCR template for amplification using outward-facing primers that are complementary to known sections of DNA.

Digestion reactions were a total of 100 µl total volume. 10 µg of Impatiens walleriana genomic DNA was used in 1 x NEB Cutsmart buffer with 50 µl of enzyme

and incubated for 18 hours. The enzymes were heat inactivated for 15 minutes in a water bath. Ligations were then set up using 500 ng of cut genomic DNA in a 500-µl reaction. NEB ligation buffer was added to a 1 x concentration, and 1,600 µl of T4 DNA ligase from NEB was added to the ligation reaction. The reaction lasted 18 hours overnight in 15 degrees Celsius and 2 hours at 24 degrees Celsius. T4 DNA ligase was inactivated by incubating the ligation reaction mixtures in a 65 degrees Celsius water bath for 10 minutes.

This protocol for iPCR requires the Q5 buffer, the two primers, template DNA, dNTPs, and Q5® DNA Polymerase (NEB). First, PCR reactions are done to create overlap equivalent portions to join. Then the thermocycler program will run with conditions listed below These samples are run on a gel to isolate and purify bands. These bands are then combined with overlap templates and run through a different program in the thermocycler. Flanking primers are then added to amplify the product. The second phase of the overlap thermocycler program is run. This final product is run on a gel to isolate and purify these bands (Wizard® SV Gel and PCR Clean-Up System). This promoter will replace the plant promoter in the plant transformation vector to produce the nectar.

The iPCR conditions were as follows. A program of 5 minutes at 95 ºC followed by 30 cycles of: 95 ºC for 10 seconds; 50-57 ºC for 30 seconds; 72 ºC for 2 minutes. Annealing temperature depended on primers used. All reactions were 50 µl and contained 5-20 ng template; 500 nM of each primer; 250 µM dNTPs; 1 µl of Q5 polymerase. iPCR products were either gel purified or diluted in 1:100 in water and used as template for a second round of iPCR.

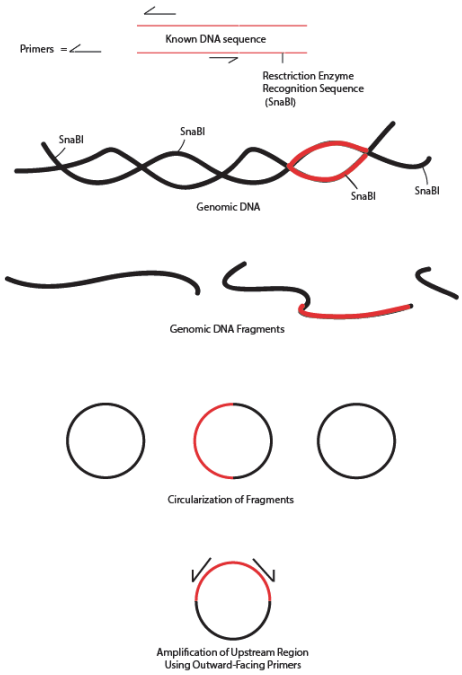

Figure 7: Illustration of Inverse PCR to Isolate Unknown Upstream DNA

CHAPTER THREE

Analysis of RNA Sequencing Data for *Impatiens walleriana*

RNA sequencing is a valuable tool for organisms that do not have a reference genome. This approach to transcriptome profiling allows for more precise measurement of levels of transcripts than other methods. In addition, this method is relatively inexpensive. However, there are a few limitations with using RNA sequencing, including high background levels due to cross-hybridization, limited dynamic range of detection due to background and saturation of signals, and difficulty comparing expression levels across different experiments (43).

RNA sequencing was done on Impatiens leaf, stem, and nectary tissue utilizing Trinity(45), which assembles the RNA sequencing reads without a reference genome. *Impatiens walleriana* SWEET14 was one of the transcripts that was found to be highly abundant in the nectary tissue as well as isolated in this tissue since both the leaf and stem tissue has very low levels of this transcript.

This candidate was identified through two thresholds. The first threshold was ensuring that a minimum number of 1,000 reads was present, and the second was that the nectary to non-nectary tissue read ratio was at least 10:1. If a transcript had a read value less than 1,000, it might not be worth isolating since it would not be considered a highly-transcribed gene. The ratio also ensures that the transcript is nectary-specific.

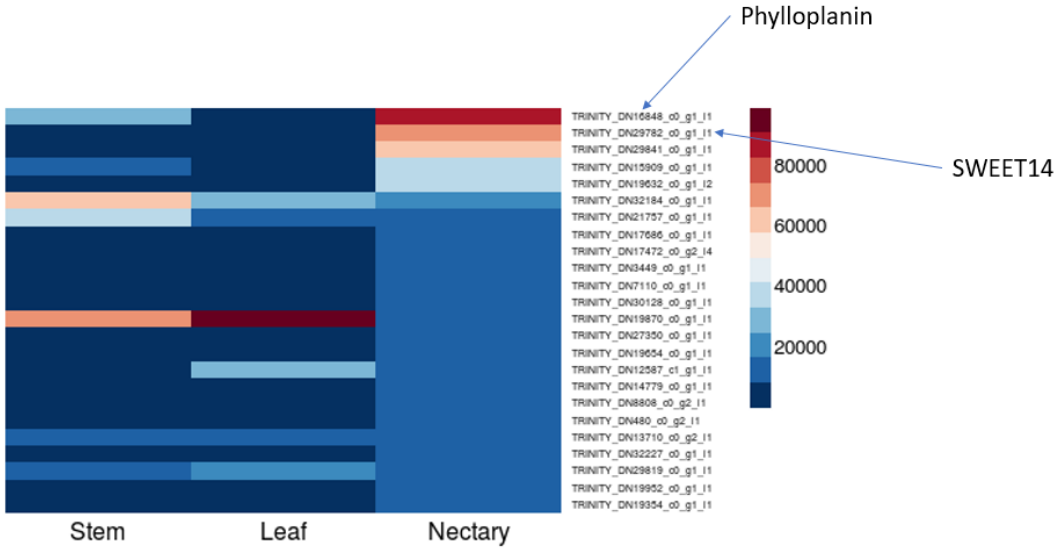

Figure 8: Heatmap representation of RNA Sequence analysis in the stem, leaf, and nectary tissues of Impatiens walleriana.

CHAPTER FOUR

Cloning and Analysis

Inverse PCR was the earliest genome walking method developed (44). It orients primers in the reverse direction, amplifying DNA in the opposite direction to normal PCR reactions and identifying the unknown sequence. This method can be utilized to clone genomic DNA. Cloning is important because it allows scientists to identify DNA sequences as well as their function when inserted into an organism that does not make this sequence of DNA. Cloning allows scientists to apply their research to large-scale endeavors such as the study of diseases and pathogens. The ability to express certain antimicrobial peptides in plants can give them antibiotic resistance. If a toxin specifically targeted towards a mosquito species responsible for carrying harmful diseases could be expressed in plants, then this could eradicate these mosquito species and, eventually, eliminate the chance of humans becoming infected with disease such as malaria, dengue, and chikungunya. To express a protein in the nectaries of plants, a nectary specific promoter is required, which is SWEET14 in this study. In addition, a protein that has a signal peptide that will allow it to be expressed in the tissue will be needed. Phylloplanin is the most highly expressed protein in the nectaries and has been designated for this role.

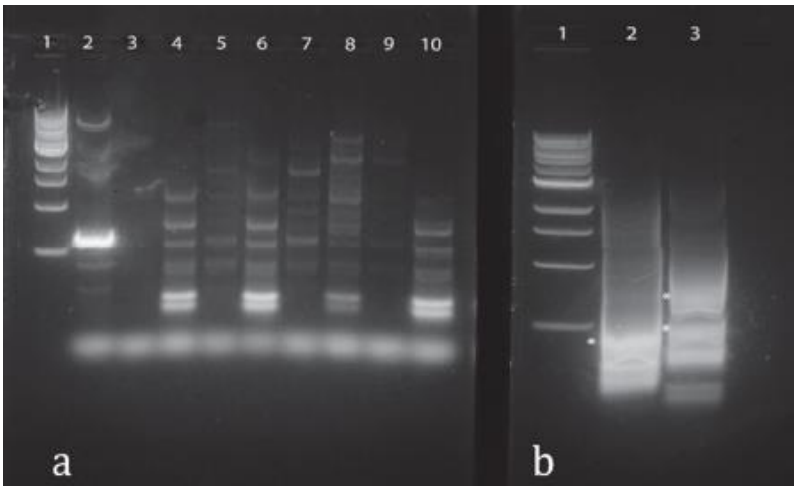

Figure 9: Agarose gels for 1st and 2nd round of iPCR for iwSWEET14. A) 1st round of iPCR for SWEET14. B) 2nd round of iPCR. Three bands were purified and submitted for DNA sequencing.

Through the chromosome walking procedure that was implemented to find the promoter, we aligned the hits with the iwSWEET14 promoter that had already been previously determined from the genomic DNA and continued aligning upstream the known region of the promoter until no more hits were found. In total, there were 708 base pairs of iwSWEET14 promoter that were located. Once the promoter had been isolated with this method, cloning was implemented to put the promoter in a vector.

Figure 8: Comparison of Original SWEET14 Promoter Sequence (Left) with New sequence of SWEET14(Right) which contains an additional 708 base pairs upstream

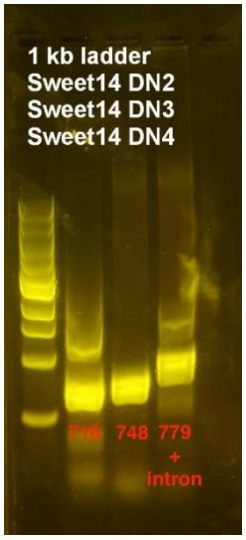

Figure 9: PCR confirmation of the iPCR product, confirming the upstream sequence of the iwSWEET14 promoter was about 750 base pairs.

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CHAPTER FIVE

Cloning Steps

A plant-optimized gBlock was constructed to fuse EGFP with the signal peptide (MASKSHVFIFSLVIILTMEMTIG) plus the subsequent 5 amino acids (EFDSI) of the impatiens phylloplanin homolog. This synthesized gBlock sequence was amplified with sequence-specific primers designed to add the restriction enzyme sites for XmaI and EcoRI, respectively, using the NEB Q5® High-Fidelity DNA Polymerase (46). These PCR products were run on a 1% agarose gel and were gel-purified with the Promega Wizard® SV Gel and PCR Clean-Up System (47). The purified PCR product and PLBW5 pORE vector were digested with the restriction enzymes XmaI and EcoRI for 1 hr at 37°C. These digested products were then run on a 1% agarose gel and were gel-purified as mentioned previously. The digested PCR product was ligated into the digested PLBW5 vector using the NEB T4 DNA Ligase (48). This recombinant plasmid was transformed into NEB 10-beta Competent *E. coli* via heat shock and the transformed colonies were selectively grown out overnight at 37°C on agar plates containing LB and 50 ug/ml kanamyacin (49). Positively-transformed colonies were confirmed with the previously used sequence-specific primers using the NEB Taq polymerase, inoculated into 10 ml of LB containing 50 ug/ml kanamycin, and grown out overnight on a 37°C shaker (50). The positive recombinant plasmid was purified from the LB cultures using the Promega Wizard® Plus SV Minipreps DNA Purification System and labeled PLBW5-PE (51). The SWEET14 homolog (iwS14) promoter was amplified from the previously purified impatiens genomic DNA using sequence-specific primers designed to add the restriction enzyme sites for KasI and BamHI, respectively, using the NEB Q5®

High-Fidelity DNA Polymerase (46). These primers were designed from the iwS14 promoter region confirmed by iPCR, genomic sequencing, and confirmation sequencing. After successfully amplifying the promoter, it was cloned into the PLBW5-PE vector using the above protocol and the restriction enzymes KasI and BamHI.

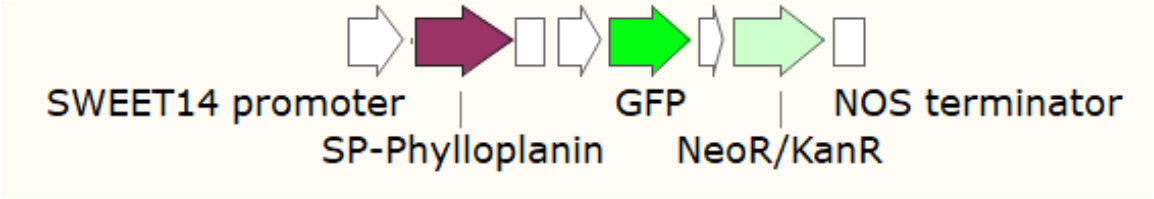

Figure 9: Diagram of construct we made for the intent in transforming Impatiens walleriana. The construct begins with the SWEET14 promoter followed by the signal peptide of Phylloplanin, CAMV 35S enhanced promoter, EGFP, Kanamycin resistance, and a terminator sequence.

CHAPTER SIX

Discussion and Conclusions

As noted in Figure 12, a digest of vector pLBW5 and pEGFP was done using restriction enzymes EcoRI and XmaI. pEGFP was successfully inserted into pLBW5, indicating that we could proceed forward in using this pORE vector. PCR reactions were then done on the promoter elements Sweet14 and Phylloplanin. Figure 13 shows another digest that was run with the promoter elements and the vector using restriction enzymes KasI and BamHI. A colony screening was then done on these digests. Finally, a plasmid screen followed by a digest of all of the elements for the construct was completed, shown by Figure 14.

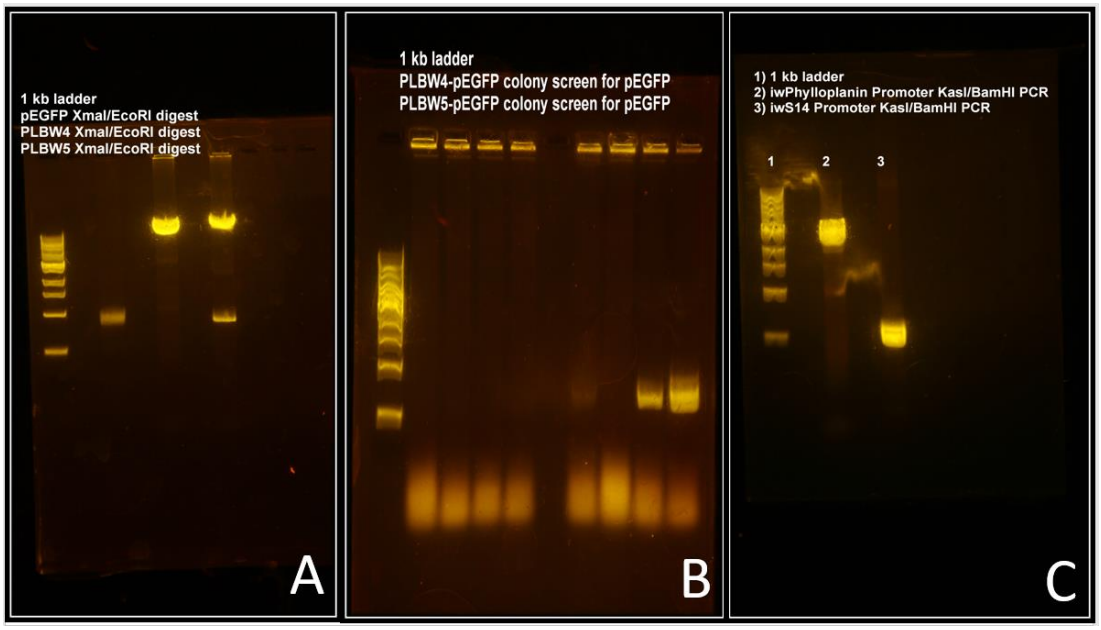

Figure 10: A) Digest of pORE vectors and EGFP using XmaI and EcoRI as restriction enzymes B) Colony screening indicating successful insertion of EGFP into pORE vector pLBW5 C) PCR reactions on promoter elements

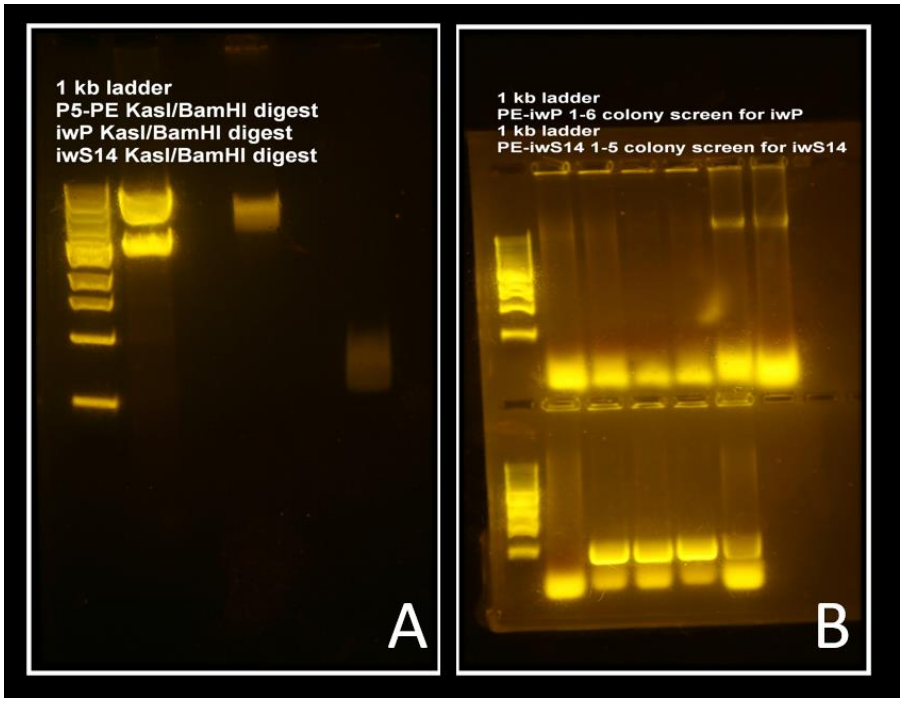

Figure 11: A) Digest with the promoter elements using KasI and BamHI as the restriction enzymes B) Colony screening for both digests shown in A

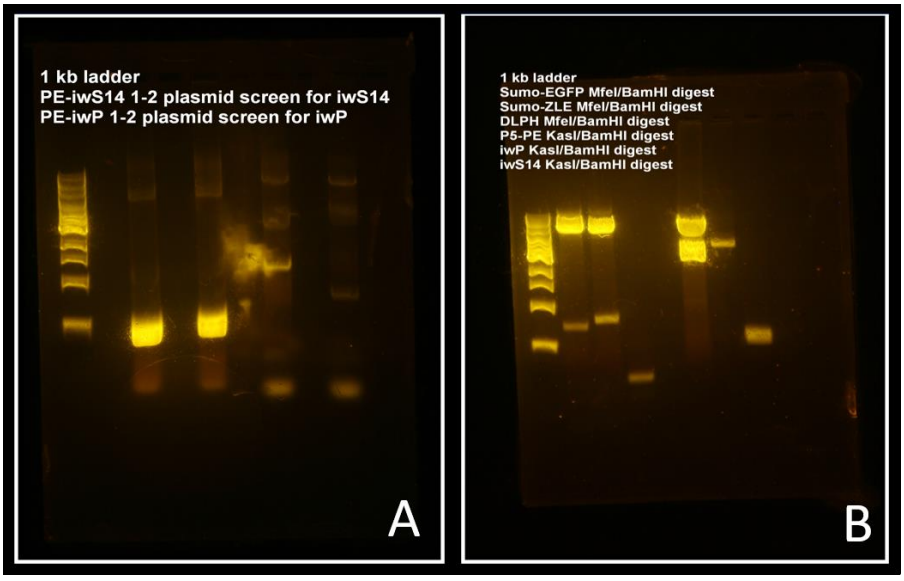

Figure 12: A) Plasmid screen for both Sweet14 and Phylloplanin promoter elements B) Digest of all of the elements for the construct

It was ultimately determined that the upstream region of the analog of Sweet14 promoter had been isolated and that promoter SWEET14 had been successfully cloned in

vector pLBW5. This construct is showing hopeful results so far with transformations in 10 beta e. coli cells. Through inverse PCR, the process took a much longer duration, but this method is a much less expensive route to utilize in isolating the promoter. Creating BLAST databases that were utilized to isolate the promoter through alignment of known regions of the promoter was much more efficient, but this process required the reference genome of Impatiens which was attained for other research purposes in the lab. The easiest method would be to have a sequencing company sequence the promoter, but the drawback to this option is that it is quite expensive.

Impatiens have not yet been transformed with this construct. The next step towards this is to fully isolate Phylloplanin and inserting this protein's signal peptide into the construct to express a toxin in the nectaries of *Impatiens walleriana*, allowing this plant species to become an effective nectar model system. Although expressing insecticides in plants has already been established in crops to avoid pests, this project will mark the first instance of such a system in nectar. Once impatiens become transformed with the pLBW5-pEGFP vector and containing the SWEET14 promoter and signal peptide from Phylloplanin, analysis can be done to quantify the fluorescence or expression of EGFP. A plausible toxin to utilize would be Cry11A, which has been found to be an insecticide specific for mosquitoes and works by destroying the lining of their gut. Using this toxin in impatiens would prevent any concerns of impacting non-target insects.

In terms of logistics, the transformed impatiens would need to be evaluated to estimate approximately how many mosquitoes are impacted by one impatiens plant to determine how many plants would be required for homes to be safe from nearby pertinent mosquitoes. Once this system has been optimized, it can be a standard that can be

integrated into other similar plant species that are native to areas that are prone to mosquito-borne diseases. A study done in Brazil estimated the maximum density of mosquitoes to be 60 to 72 mosquitoes per 100 m^2. Because the average home sits on a quarter of an acre of land, this would mean about 500 mosquitoes could potentially be near the house in one instance. Because *Impatiens walleriana* have about 200 nectaries per plant and only about 25% of them are active on average at a certain point in time, then that would mean that around 10 impatiens plants would be needed per home in order to safely extinguish the mosquitoes near each home (51, 52)

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