ABSTRACT

Evaluating and Isolating Promoters in *Impatiens walleriana*: Towards the Development of Mosquitocidal Nectar

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Mosquito-borne diseases, such as malaria, are a persistent problem, leading to numerous deaths across the world that could be easily prevented by better control of mosquito populations. Because the main source of energy for many mosquitoes are the sugars present in nectar, a novel approach for mosquito control that would complement methods already in use would be to develop plants that express mosquito-specific toxins in their nectar. Nectar-specific promoters would be a necessary step in developing plants that express mosquito toxin solely in their nectar. One nectar-specific promoter that has been found and sequenced is the pNEC promoter of *Nicotiana langsdorffii* X *N. sanderae*. To test the effectiveness of this promoter in *Impatiens walleriana*, pNEC was combined with enhanced green fluorescent protein (EGFP) and used in the transformation of *I. walleriana.* Kanamycin resistance was used to select transformants; presence of EGFP in nectar would then prove successful promotion of expression by pNEC. When no EGFP was found to be present, further investigation determined that the transformations were not successful, despite surviving the kanamycin selection step. A means for potentially isolating the nectar-specific promoter endogenous in *I. walleriana* was also tested. Thermal asymmetric interlaced PCR (TAIL-PCR) was used to successfully isolate the 26S rRNA promoter of *I. walleriana*, verifying it as a method to eventually isolate the IW23 promoter of *I. walleriana*.

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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

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*, the most deadly of which is *P. falciparum* (Antinori et al., 2012). The malaria parasite has two main life-cycle stages: the endogenous, asexual stage and the exogenous, sexual stage, with only the asexual stage being pathogenic. The *Anopheles* mosquito is the major vector, or means of transmission, for malaria and plays a key role in the sexual stage of the parasite by being the location of fertilization and maturation before infection of a new host (Greenwood et al., 2008). Upon entering a host, the *Plasmodium* parasite infects red blood cells, leading them to become stuck to the linings of smaller blood vessels and allowing the parasite to reproduce hidden within the body (Miller et al., 2002). This build up of nonfunctioning red blood cells eventually leads to such issues as acidosis and anemia and can be fatal if untreated.

Malaria is one of the top ten leading diseases for global disease burden and is more deadly in children five years of age or younger (Lopez et al., 2006). Millions suffer and die from malaria all across the world annually, though it is most rampant in sub-Saharan Africa with Southeast Asia being second in prevalence (Snow et al., 2005). The number of cases and deaths had steadily increased until it reached a peak in 2004 with an estimated total of 1.817 million deaths and has since steadily declined to 1.238 million estimated deaths in 2010 with a more marked decrease found in children ages five and younger (Black et al., 2010; Murray et al., 2012). This decline can be readily attributed to the implementation of protective measures against mosquitoes such as the widespread

use of bed netting, though there is still much room for improvement in a disease that can be easily prevented through more comprehensive protective measures.

The use of bed netting has had a significant effect on decreasing the number of indoor nocturnal feedings, which in turn lowered malarial infection rates, but it has also affected the behavior of mosquitoes. One study conducted in Tanzania found that the implementation of indoor protective measures has actually selected for *Anopheles* mosquitoes that have the altered behavior of seeking hosts outdoors at earlier hours in the evening as opposed to the usual behavior of nocturnal indoor feedings (Russell et al., 2011). This study is further corroborated by studies conducted in Kenya, Senegal, Uganda, and Equatorial Guinea revealing the need for new additional protective measures that target these behaviorally different mosquitoes and act as a complement to current protective measures (Cooke et al., 2015; Ndiath et al., 2014; Ojuka et al., 2015; Reddy et al., 2011). Furthermore, a study in the Sudan-Savanah zone found an outdoor host-seeking subgroup of *Anopheles gambiae* that is more susceptible to the malaria parasite as compared to the subgroup found predominately indoors (Riehle et al., 2011). Even though this trend may not be true for all new outdoor host seeking subgroups, it does stress the importance of not only having protective measures indoors but outdoors as well, especially in conjunction with the two studies that found an outdoor shift in *Anopheles* feeding habits.

One outdoor protective measure that can be utilized is attractive toxic sugar baits (ATSB), which can be either placed where desired or sprayed onto vegetation. These ATSB can be strategically placed so as to have the greatest impact, for instance targeting newly hatched mosquitoes by spraying ATSB around larval habitats such as ponds and

other bodies of water (Müller et al., 2010). The use of ATSB was proven to be very effective in one study in Israel with a 10-fold decrease in human-landings of mosquitoes after their implementation (Müller and Schlein, 2008). When this method was applied in a study in Mali similar results were achieved, proving the efficacy of ATSB as an outdoor protective measure (Mueller et al., 2010a). However, a disadvantage to this method is the fact that the ATSB must be regularly and consistently refilled or re-sprayed in order to ensure the maximum protection possible. This method can be improved upon by finding a more permanent means of controlling outdoor mosquito populations.

ATSB work because mosquitoes discern where to land and feed by odor. Nectar feeding is key for the survival of both male and female mosquitoes as it is the main source of energy for the females of most species and the only source for males, and it is only reproducing females that have blood feedings in order to develop eggs (Foster, 1995). In fact, nectar feeding is so important that mosquitoes must drink nectar within hours of hatching and females often choose to drink nectar before drinking blood. Furthermore, mosquitoes have demonstrated a preference for some nectars over others, which has been attributed to volatile semiochemicals emitted by nectar that mosquitoes can detect and use to discern which plant to drink from (Nikbakhtzadeh et al., 2014). Multiple studies have used plants native to various parts of Africa that are found near both human and mosquito larval habitats to demonstrate mosquito preferences for certain plants over others (Gary and Foster, 2004; Impoinvil et al., 2004; Manda et al., 2007). A result in common to each of these studies was that mosquitoes were found to drink predominately if not exclusively from the extra-floral nectaries instead of from floral nectaries like bees do. Some additional studies have sought to determine which nectars

and odors are the most attractive and why they are the most attractive, with the purpose of utilizing this knowledge to make more effective ATSB (Mueller et al., 2010b; Schlein and Müller, 2008). This raises the idea of using the plants themselves to deliver a toxin to mosquitoes by engineering mosquito attracting plants to express the toxin in their nectar. Factors defining the ideal plant for such a toxin delivery system would be the presence of extra-floral nectaries for mosquitoes to feed from, a natural habitat that overlaps with that of both humans and newly hatched mosquitoes, and existence of a working transformation protocol for genetically engineering the plant species. These requirements were all met in a study that found *Impatiens walleriana* to be the most attractive of the five plants studied and also discussed the additional benefit of *I. walleriana* as a model plant because of its easy propagation and high protein content in the nectar boding well for expressing a protein toxin (Chen and Kearney, 2015).

The individual technologies that would be needed to produce a mosquitocidal plant have already been developed and used successfully in other applications. The idea of engineering a plant to express an insecticide is not new, having been utilized before in a variety of crops since 1996, such as maize, cotton, and castor bean, to express a Cry protein to make the plant resistant to pests (Malathi et al., 2006; Romeis et al., 2006). Furthermore, nectar has been engineered to stably express a desired product before as demonstrated in *Nicotiana langsdorffi* x *N. sanderae* using the CARN2 promoter to express human epidermal growth factor to be taken up by bees for incorporation into honey for use as an ointment (Helsper et al., 2011). Nectar is naturally composed of carbohydrates and amino acids which serve a dual purpose of attraction and protection (Heil, 2011). It is the proteins within the nectar that play the largest role in protecting the

plant against microbes and fungi. Five nectar proteins that have been thoroughly characterized are Nectarin I-V, all of which have been proven to play a protective role (Park and Thornburg, 2009). Nectarin I proved to be the most abundant in tobacco species and so proved to be of great interest, prompting the isolation of the NEC1 promoter (pNEC1) in order to be further studied (Carter and Thornburg, 2003). This promoter is specific for expressing protein in nectar at high levels and offers the potential for being used to express different proteins, such as a mosquitocidal toxin, in the plant nectar of choice at a level that will prove deadly to mosquitoes.

Transformation is the process by which a new gene is introduced into a genome and expressed in an organism in which it is not otherwise found. One method of transformation in plants is through the use of *Agrobacterium tumefaciens*. Normally, *Agrobacterium* causes tumorous growths, or galls, to develop at the base of a plant by inserting *vir* genes into a chromosome of the plant (Gelvin, 2000). This insertion is achieved by the Ti (tumor inducing) plasmid which contains the T-DNA that is inserted. The T-DNA has inverted terminal repeats that act as borders for the *vir* genes between and it is these borders that allows for integration into the plant chromosome by being homologous to that portion of the genome. However, by removing the *vir* genes and replacing them with an antibiotic resistance gene and a DNA segment that contains multiple restriction sites, one makes *Agrobacterium* into a non-virulent, binary vector (Bevan, 1984). The restriction sites are significant because they are what allow the insertion of DNA fragments that contain the gene of interest (GOI) so that the *Agrobacterium* is now capable of transforming plant cells to contain the GOI while no longer being able to cause tumors.

In transformation, it is desirable for every cell within the transformed plant to contain the GOI and not just a region of cells as in the normal manner of *Agrobacterium* infection. This requires infecting a special type of cell, one that is capable of becoming an entire plant, with *Agrobacterium*. This involves plant tissue culture, which can take multiple forms. One commonly used method is taking cut leaf squares and inoculating them with *Agrobacterium* before placing them on an antibiotic containing medium on which only the successfully transformed cells that are now antibiotic resistant will be able to propagate and eventually develop into a full plant (L. M. Winkler, 2002; Monteiro-Hara et al., 2011). Another similar method uses isolated cotyledonary nodes in place of cut leaf squares which otherwise undergo the same procedure (Aslam et al., 2009; Dan et al., 2010). By using cotyledonary nodes, it is possible to successfully regenerate transformed plants in species that otherwise experience poor regeneration using the traditional cut leaf squares, such as soybeans and melons (Hinchee et al., 1988; Zhang et al., 2013).

Hormones play an important role in tissue culture, encouraging the growth of a particular cell type depending on the presence and levels of various hormones. They are what make it possible to grow an entire transformed plant from a small group of cells that started out as a single cell type. One commonly used hormone is 6-benzylaadenine (BA), which is a synthetic cytokine that promotes plant shoot regeneration (Malik and Saxena, 1991). Thidiazuron (TDZ) is another synthetic cytokine for shoot regeneration that can be used in conjunction with BA (Guo et al., 2011). Indole-3-butyric acid (IBA) is in the auxin family of phytohormones and promotes root growth (Ludwig-Müller, 2000). Shoot growth is normally accomplished first with BA and TDZ before transplanting the shoots

to a new medium containing IBA to induce root growth before transplanting the small yet fully differentiated plant to soil where it is allowed to mature.

In transformation utilizing *Agrobacterium*, it is the binary vector, a circular molecule of DNA, that enters the plant cell and inserts the new genetic information. The binary vector is a shuttle vector between *Escherichia coli* and *Agrobacterium* and is constructed in *E. coli*, where it is easier to assemble the vector that contains the T-DNA plus the desired genes and elements intended for insertion. *Agrobacterium* then receives this vector where it resides alongside the disarmed Ti plasmid that is already present within the *Agrobacterium* until it is time to transform. One binary vector that can be used is the JL22 plasmid (pJL22), which contains the 35S promoter, a termination sequence, and multiple restriction sites for allowing insertion of one or more DNA fragments that contain the GOI or other elements of interest (Lindbo, 2007). By digesting the pJL22 and the ends of the DNA fragment with the same restriction enzymes it is possible to insert the DNA fragment into the plasmid in the correct orientation and position relative to the promoter and termination sequences to support expression in transformed cells. It is important to insert an antibiotic resistance gene as well which can be used for selecting cells that were successfully transformed. One places all cells that were exposed to *Agrobacterium* on medium containing an antibiotic that kills non-transformed cells and allows only the transformed cells to survive. One possible gene for this is kan^R, which codes for kanamycin resistance. Another gene that is occasionally inserted as well is an RNA silencing suppressor, such as p19, that ensures that the plant's natural defense mechanism against foreign double-stranded RNA (dsRNA) from viruses does not silence the expression of the gene of interest (Mérai et al., 2006).

For the present experiment, it is the pNEC1 nectary-specific promoter that is of interest and so restriction digest was used to replace the 35s promoter in pJL22 with pNEC1. In order to track the expression pattern of pNEC1 in *I. walleriana* a reporter gene needs to be placed downstream of the promoter. An example of a reporter gene is the *egfp* gene which results in the production of the enhanced green fluorescent protein (EGFP), whose presence can easily be confirmed in a number of ways (Yang et al., 1996), the simplest being through fluorescence. When hit with a particular wavelength of light, a fluorescent molecule will become excited and then emit another wavelength of light as it returns to its unexcited state (Turner Designs, 2015). These wavelengths are referred to as the excitation and emission spectra and are unique for every fluorescent molecule. For EGFP, the excitation wavelength is 488nm, which is in the blue light range, and the emission wavelength is 510nm, which is in the green light range (Yang et al., 1996). Knowing this, one quick and simple way to verify the presence of EGFP in the appropriate location within the plant, in this case in only the nectar, is to use a blue LED light that will cause the plant to glow green wherever EGFP is present. If EGFP is not present in high enough concentrations to be detectable by the naked eye, more sensitive means of detection are available. Use of a fluorometer is one method that can detect EGFP concentrations as low as one part per trillion (Turner Designs, 2015). The additional benefit of using a fluorometer is that only a small sample is required.

However, it may not always be possible to detect EGFP using fluorescence no matter its concentration. This is because EGFP ($pK_a = 5.8$) denatures in an acidic environment and so will not fluoresce as expected (Malik et al., 2005). In such instances it is necessary to use an alternate method to verify the presence of EGFP.

Mass spectrometry is commonly used to identify proteins. In particular, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is used to determine the peptide mass fingerprint of an isolated protein that has been digested by a protease (Domon and Aebersold, 2006). The protein is often digested using trypsin, which enzymatically cleaves after every arginine or lysine present in the peptide chain, except when followed by proline. Each protein will have a unique cleavage pattern and fragment sizes due to its unique amino acid sequence. The resulting fragments of the digested protein then undergo liquid chromatography-mass spectrometry (LC-MS). The fragments are first separated by elution time in LC and are then ionized for MS. The mass-to-charge ratio for each of the ionized protein fragments is then determined based on the flight time of the ionized protein fragment in the mass spectrometer, with a longer flight time corresponding to a larger fragment. This data is then processed into a mass spectra that is a unique pattern for each protein, representing the protein's peptide mass fingerprint (PMF). The PMF is then compared to genomic and proteomic databases to determine the sequence of the protein and so identify it. Even if EGFP is denatured by an acidic environment, it will still have the same PMF, allowing it to be easily identified by mass spectrometry.

In addition, a method for finding an alternative promoter to pNEC1 that will work in *I. walleriana* was also tested. The method tested was thermal asymmetric interlaced PCR (TAIL-PCR), a means of isolating segments of a genome for which the sequence of only one end is known (Liu et al., 1995). Furthermore, this method has been proven to be efficient in isolating promoters for sequencing in yams (Terauchi and Kahl, 2000).The promoter of ultimate interest in this case is the promoter for IW23 (pIW23). *IW23* is a

gene endogenous to *I. walleriana* that codes for a protein that has been demonstrated to have high levels of expression in the nectar of *I. walleriana* (Chen and Kearney, 2015). It is hoped that isolating and sequencing pIW23 will produce a promoter that will work reliably in *I. walleriana* for expressing mosquitocidal toxin. However, the gene sequence of *IW23* remains to be determined. The likely sequence for *IW23* is currently being resolved using *de novo* mass spectrometry to first determine the amino acid sequence of the IW23 protein. This amino acid sequence will then be used to determine the most likely DNA sequence of *IW23*, taking into consideration the codon bias of *I. walleriana*. This DNA sequence will be further verified by comparison with RNA-Seq data that has been obtained using mRNA isolated from *I. walleriana*, leading to a more accurate gene sequence to base TAIL-PCR primers upon. Yet because this sequencing is still a work in progress, the 26S rRNA gene will be used as a positive control of TAIL-PCR in *I. walleriana*. The 26S rRNA gene has highly conserved core sequence among plant species (Kuzoff et al., 1998), allowing PCR primers to be based upon the known 26S rRNA gene sequence from *Arabidopsis thaliana* to isolate the gene promoter in *I. walleriana*. Thus, the protocol for TAIL-PCR will be validated in *I. walleriana* using the 26S rRNA gene in order to facilitate more rapid isolation of pIW23 once the sequence of *IW23* is verified.

TAIL-PCR works by using nested primers of known sequence in conjunction with an array of random 10mer primers. Nested primers are primers whose sequences are based on a gene of known sequence and act as the reverse primer for PCR (Figure 1A). There are three stages to TAIL-PCR: primary, secondary, and tertiary PCR (Figure 1B). The secondary and tertiary PCRs use the product from the previous stage as a template to

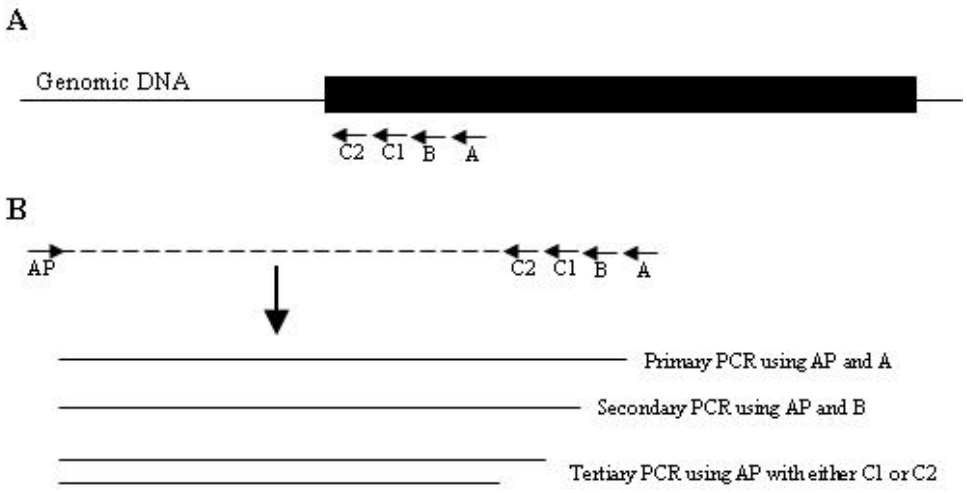

Figure 1: Schematic outline of TAIL-PCR. (A) Placement of nested primers. (B) Results of primary through tertiary PCR using nested primers in conjunction with arbitrary primer (AP).

ensure that only that desired portion of the genome is amplified throughout all three stages. In primary PCR, an arbitrary primer (AP), one of the random 10mer primers, is paired with the first of the nested primers (A) that is set furthest into the gene and away from the promoter region. The product of primary PCR is then used in secondary PCR, which uses the same AP paired with the second nested primer (B) that is slightly closer to the promoter region. This results in a secondary PCR product that is slightly shorter than the primary product. Finally, the secondary product is used in tertiary PCR, which consists of two different PCR reactions. One reaction is the initial AP paired with the third nested primer (C1) while the second reaction is the initial AP paired with the fourth nested primer (C2) that is closest to the promoter region. It is desirable to have two different tertiary products because then the relative lengths of the products can be compared and the difference in lengths should correspond to the different placements of C1 and C2, confirming isolation of the desired promoter region.

In summary, malaria is an ongoing problem that can be readily prevented by gaining better control over mosquito populations by implementing protective measures in

addition to those already in use, such as bed netting and ATSB. It is proposed that it is possible to engineer a plant to express mosquitocidal toxin in its nectar, providing a new outdoor protective measure that can be allowed to grow naturally and does not need to be refilled or resprayed like ATSB. The plant that will be studied is *I. walleriana* because of its attractiveness to mosquitoes and the existence of an effective method for transforming it. It is hoped that pNEC1 from tobacco can be used to stably express mosquitocidal toxin in the nectar of *I. walleriana*. For the first half of this thesis the objective was to determine if pNEC1 will be effective in *I. walleriana* and demonstrate the same expression pattern and levels as in tobacco. The success will be determined by the presence of EGFP in just the nectar and nectaries of *I. walleriana*. The second half of this thesis was to verify TAIL-PCR as an effective method for isolating a promoter in *I. walleriana*, using the known 26S rRNA gene as a positive control. The success of the TAIL-PCR method was determined by the presence of bands after tertiary TAIL-PCR.

CHAPTER TWO

Materials and Methods

Vector construction of pNEC-EGFP and pNECS-EGFP

In order to determine whether pNEC can be expressed in *I. walleriana*, a vector containing pNEC in front of a reporter gene, in this case *egfp*, was constructed by Dr. Kearney. Via the restriction sites HindIII and XbaI, pNEC was cloned from pRT260 into JL22, replacing the 35S promoter and creating JL22/pNEC. PCR, digestion, and ligation were then conducted with one set of JL22/pNEC to add the restriction sites ApaI, XbaI, and ClaI between pNEC and the terminator, creating JL22pNECApaCla. The restriction sites AscI, XbaI, and AvrII were added in a similar fashion to the second set of JL22/pNEC to create JL22pNECAscAvr. The pNECApaCla cassette was then added to JL22pNECAscAvr via the restriction sites KpnI and SalI creating JL22/2xpNEC. Next, *egfp* was added to the pNECAscAvr via AscI and AvrII and kanamycin resistance (*kan*^{r}) was added via SalI and HindIII creating pNEC-EGFP (Figure 2A). Finally, p19 was added to the pNECApaCla cassette via ApaI and ClaI creating pNECS-EGFP (Figure 2B). Sequence verification was then performed on both pNEC-EGFP and pNECS-EGFP.

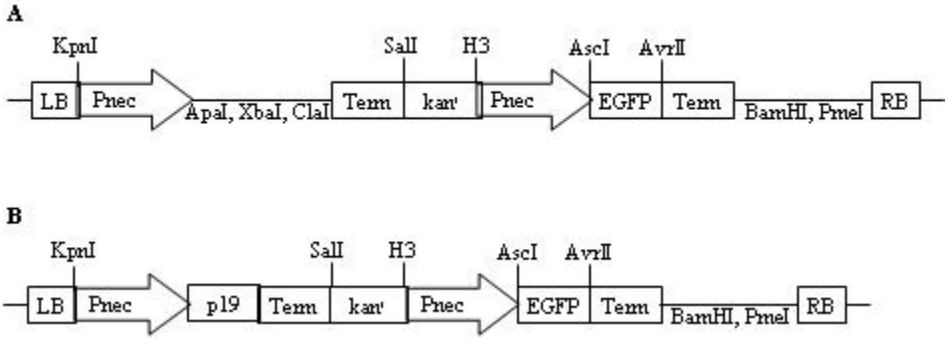

Figure 2: Final pNEC-EGFP (A) and pNECS-EGFP (B) vectors

Transformation of I. walleriana *with pNEC-EGFP and pNECS-EGFP*

The pNEC-EGFP and pNECS-EGFP vectors were introduced to *Agrobacterium tumefaciens* strain GV3101 by electroporation using a BTX 600 Electro Cell Manipulator (BTX Inc., San Diego, CA, USA). Transformed *A. tumefaciens* were selected for using plates containing kanamycin and then used in the agroinoculation of *I. walleriana*.

I assisted Nina Yu and Thuy Nguyen in the transformation of *I. walleriana*, which was conducted as described (Dan et al., 2010) with some modifications. Seeds were sterilized using the following gas sterilization technique. *I. walleriana* seeds were placed in open eppendorf tubes. These eppendorf tubes were then placed on their sides so as to create a single layer of seeds and placed in a petri dish with a lid. A small beaker containing 30 mL bleach was set in a chamber jar in a fume hood. Next, 5 mL of 12 M HCl was added to the bleach before covering the beaker with the petri dish (lidded but unsealed) and sealing the chamber jar to trap the released chlorine gas. The seeds were allowed to sterilize for 45 min before being placed on agar medium (Table 1). Hypocotyl segments containing cotyledonary nodes were then prepared by dissecting seedlings grown on the agar medium. These hypocotyl segments were then placed on induction medium 1 (IM1) without kanamycin and timentin (Table 1) where they were allowed to develop into multiple bud clusters (MBC). MBC approximately 4-6 mm in size were inoculated in 30 mL *Agrobacterium* solution at OD_{600} = 0.5 and incubated for 30 min at room temperature on a shaker. The MBC were then placed on agar medium topped with filter paper that was wetted with inoculation medium and cultured in the dark at 24 ^{o}C for approximately 96 hrs. Afterwards, the MBC were transferred to IM1 containing kanamycin and timentin for selection and shoot induction. Clumps of multiple shoots that formed were

Table 1: Components of media used for transformation of I. walleriana

Medium component	Medium name and concentration			
	Agar medium	Induction medium 1 (IM1)	Induction medium 2 (IM2)	Induction medium 3 (IM3)
MS Basal medium with vitamins (PhytoTechnology Laboratories)	-	4.43 g/L	4.43 g/L	4.43 g/L
MES (Alfa Aesar)	-	1.95 g/L	1.95 g/L	1.95 g/L
Sucrose (PhytoTechnology Laboratories)	-	30 g/L	30 g/L	30 g/L
Agar (PhytoTechnology Laboratories)	12 g/L	8 g/L	8 g/L	6 g/L
Thidiazuron (PhytoTechnology Laboratories)	-	0.05 mg/L	0.05 mg/L	-
6-benzyladenine (Sigma)	-	0.4 mg/L	0.05 mg/L	-
indole-3-butyric acid	-	-	-	0.5 mg/L
Kanamycin	-	50 mg/L	50 mg/L	50 mg/L
Timentin	-	300 mg/L	300 mg/L	300 mg/L
pH	-	5.7	5.7	5.7

then transferred to IM2 (Table 1) for shoot elongation. Elongated, individual shoots were then dissected from the clumps and transferred to IM3 (Table 1) for root induction.

Finally, fully developed rooted shoots were rinsed of agar and transferred to pots, which were watered until the soil was moist. The pots were moved to trays that were then covered with plastic wrap in order to allow the shoots to slowly acclimate to non-sterile conditions. The plastic wrap was gradually loosened over a two week period until the plants were fully exposed and allowed to grow freely.

Determination of EGFP presence in the nectar by de novo *mass spectrometry*

For protein isolation, nectar was then collected and dissolved in 10 mM sodium phosphate buffer, pH 7.0. The nectar solution was spun twice through a size exclusion

centrifuge spin column (Vivaspin 5 kda cutoff) at 4,000 g for 20 min at 20 ^{o}C to isolate the protein from the nectar. The remaining solution then underwent tryptic digestion in preparation for protein identification through LC-MS. EGFP was tested for using a Waters Synapt G2 mass spectrometer (Data Dependent Acquisition [DDA] + MS^e).

Determination of success of transformation by PCR

PCR was performed using genomic DNA isolated from the PNEC and PNECS plants; DNA from an untransformed *I. walleriana* plant and from a 35S-*egfp* plasmid served as a negative and positive control respectively. Genomic DNA for PCR was isolated from leaves of the plants using Plant DNAzol Reagent (Thermo Fisher Scientific Inc., Waltham, MA). The forward primer and reverse primers used were 5'–TTTTTTGCGGCCGCTTTTTTACTAGTATGGAGATCTTAGGCCTG–3' and 5'–TTTTTTTCTAGATTTTTTCCTAGGTCACGCGTCGACGGACTT–3' respectively. The polymerase used was Taq polymerase. The thermocycler was programmed to run for initially 30 sec at 95 ^{o}C, then to run for thirty cycles of 30 sec at 95 ^{o}C, 30 sec at 55 ^{o}C, 1 min at 68 ^{o}C, and finally run for 5 min at 68 ^{o}C before holding at 4 ^{o}C. PCR products were then run on a 1% agarose gel using a 1kb ladder (New England Biolabs).

Use of TAIL-PCR to isolate the 26S rRNA gene promoter from I. walleriana

The sequence of the highly conserved 26S rRNA gene was used as a starting point from which we "walked" upstream seeking to determine the *I. walleriana* promoter for this constitutive gene. Thermal asymmetric interlaced PCR (TAIL-PCR) was used to amplify the portion of the sequence upstream of the 26S rRNA gene containing the

promoter. Genomic DNA for TAIL-PCR was isolated from leaves of *I. walleriana* using Plant DNAzol Reagent (Thermo Fisher Scientific Inc., Waltham, MA). Specific primers were designed based on the DNA sequence, and were A (5'-ggggTTTGCTATCGGTCT CTCGTCAATA -3'), B (5'-ccttGACGAAATTTACCGCCCGATTGG-3'), C1 (5'-taggC AAACAACCCGACTCGATGACAGC-3'), and C2 (5'-atacGGACTCTCACCCTCTCT GGAGCC-3') with the complementary sequences depicted in upper case (Figure 3). Arbitrary 10mer primers were obtained (Carl Roth, random-primer kits) after ensuring

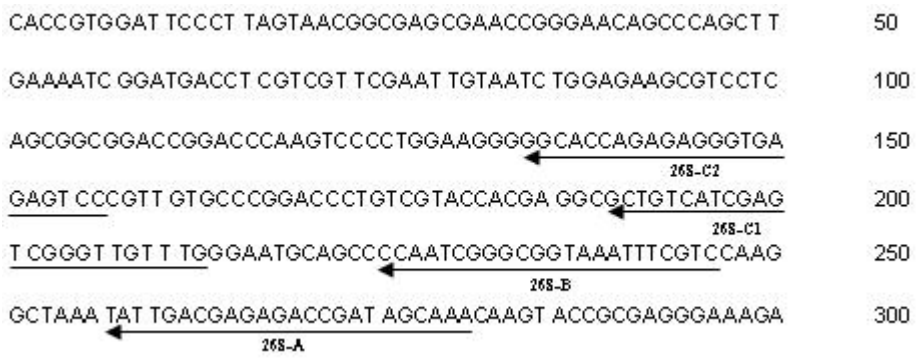

Figure 3: Localization of the gene specific primers for the 26S rRNA gene of I. walleriana

each 10mer primer would not form stable duplexes with the specific primers using OligoAnalyzer 3.1 (Integrated DNA Technologies). TAIL-PCR was carried out as described (Terauchi and Kahl, 2000). Three rounds of PCR (Table 2) were carried out. Primary PCR consisted of a 20 µL volume containing 100 ng genomic DNA, 0.2 µM specific primer (primer A), 2.0 µM arbitrary primer, 200 µM dNTP, 0.2 U Taq polymerase, and 1x Taq polymerase buffer. Secondary PCR was carried out similarly to primary PCR except that 1 µL of a 1/50 dilution of the primary PCR product was used as the template in place of genomic DNA and primer B was used as the specific

Table 2: PCR parameters for TAIL-PCR to amplify upstream region of 26S rRNA *gene*

Reaction	Primer combination	Number of cycles	Cycle parameters
Primary PCR	Primer A/AP	1	93 °C, 1 min; 95 °C, 1 min
		5	94 °C, 30 s; 62 °C, 1 min; 72 °C, 2.5 min
		1	94 °C, 30 s; 25 °C, 3 min; ramping to 72 °C over 3 min; 72 °C, 2.5 min
		15	94 °C, 10 s; 68 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 68 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 29 °C, 1 min; 72 °C, 2.5 min
		1	72 °C, 5 min
Secondary PCR	Primer B/AP	12	94 °C, 10 s; 64 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 64 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 29 °C, 1 min; 72 °C, 2.5 min
		1	72 °C, 5 min
Tertiary PCR	Primers C1-C2/AP	20	94 °C, 15 s; 29 °C, 30 s; 72 °C, 2 min
		1	72 °C, 5 min

primer. Tertiary PCR was again similar but used1 μL of a 1/10 dilution of the secondary PCR product as the template and 0.2 μM specific primers C1 and C2 were used separately resulting in two different tertiary PCR, though the arbitrary primer was kept the same for each tertiary PCR reaction. Primary through tertiary PCR was carried out for each of the arbitrary primers. PCR products were then run on a 1% agarose gel using a 100bp ladder (New England Biolabs).

CHAPTER THREE

Results

Verification of pNEC and pNECS directed expression of EGFP in nectar

Multiple bud clusters (MBCs) were cultivated from *I. walleriana* seeds and grown on induction medium 1 (IM1) without antibiotics in preparation for transformation. These MBCs were then transformed using *Agrobacterium* containing either the pNEC-EGFP vector or the pNECS-EGFP vector, which contains the additional component of p19, an RNA silencing suppressor to help ensure expression of foreign RNA. These MBCs were then placed on IM1 containing kanamycin and timentin, with kanamycin serving to select for MBC that had been successfully transformed and so contained *kan*^{r}, and timentin serving to kill any *Agrobacterium* that remained on the MBCs, preventing bacterial overgrowth. Clumps of multiple shoots that formed were then transferred to IM2 for shoot elongation. Elongated, individual shoots were then dissected from the clumps and transferred to IM3 for root induction. Finally, rooted shoots were transferred to soil and carefully monitored as they reached adulthood. From the MBC transformed by *Agrobacterium* containing pNEC-EGFP, five regenerated after kanamycin selection and survived until adulthood. These plants were labeled PNEC1-5. From the MBC transformed by *Agrobacterium* containing pNECS-EGFP, three regenerated after kanamycin selection and survived until adulthood. These plants were labeled PNECS1-3.

No fluorescence was detected in the nectar of any of the five PNEC and three PNECS plants using either a UV light or a fluorometer. When the pH of the nectar was checked, it was found to be acidic, potentially denaturing any EGFP ($pK_a = 5.8$) that may

have been present and explaining the lack of fluorescence. For this reason, it was decided to use mass spectrometry to detect any EGFP that may be present in the nectar of the PNEC and PNECS plants.

Protein was isolated from the nectar and underwent tryptic digestion in preparation for liquid chromatography-mass spectrometry (LC-MS). The resulting peptide fragments were unique to those proteins present in the nectar, allowing for definitive identification of proteins that have been previously characterized by comparing the measured peptide mass fingerprint to those in genomic and proteomic databases. EGFP is present in these databases and so was readily compared to data obtained from nectar samples from the PNEC and PNECS plants. From *de novo* mass spectrometry, it was determined that none of the PNEC and none of the PNECS plants were expressing EGFP in their nectar.

To determine whether this absence of EGFP was due to unsuccessful transformation or due to improper expression, PCR was conducted on the genomic DNA of the PNEC and PNECS plants to determine if the *egfp* gene sequence was present in the regenerated plants. The 35S-*egfp* plasmid and DNA from an untransformed *I. walleriana* served as the positive and negative control, respectively. As the PCR results revealed no bands (Figure 4) corresponding to the *egfp* band of the 35S-*egfp* plasmid in the regenerated plants, it was determined that the transformations were unsuccessful, causing the observed lack of EGFP in the nectar.

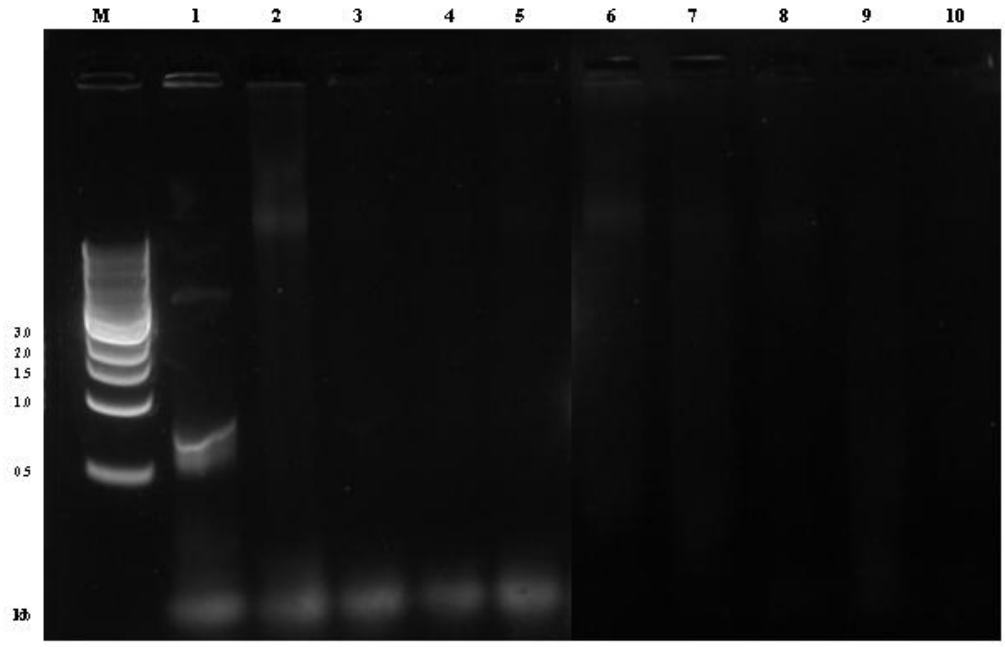

Figure 4: Electrophoresis gel for verification of egfp *presence. Lanes 3-5 represent the three replicates for the plants transformed by the pNECS-EGFP vector, plants PNECS 1 through 3. Lanes 6-10 represent the five replicates for the plants transformed by the pNEC-EGFP vector, plants PNEC1 through 5. M: marker lane; 1: 35S-*egfp *plasmid; 2:* I. walleriana *DNA; 3: PNECS1; 4: PNECS2; 5: PNECS3; 6: PNEC1; 7: PNEC2; 8: PNEC3; 9: PNEC4; 10: PNEC5.*

Use of TAIL-PCR to isolate I. walleriana *promoters verified with the 26S rRNA gene*

TAIL-PCR was conducted on the 26S rRNA gene to isolate the 26S rRNA gene promoter from *I. walleriana* and verify TAIL-PCR as a method for eventually isolating the *IW23* promoter once the *IW23* gene sequence has been accurately determined. Specific primers that would act as reverse primers for TAIL-PCR were designed based on the conserved and thus known 26S rRNA gene sequence and nested so that the specific primer for primary TAIL-PCR (26S-A) was nested furthest into the gene sequence while the specific primers for each of the following TAIL-PCR rounds (26S-B for secondary TAIL-PCR, 2S-C1 and 26S-C2 for tertiary TAIL-PCR) was nested progressively closer to the start of the gene sequence (Figure 5). This allowed for the selection of only the desired segment as it survived each round of TAIL-PCR, meaning that an arbitrary

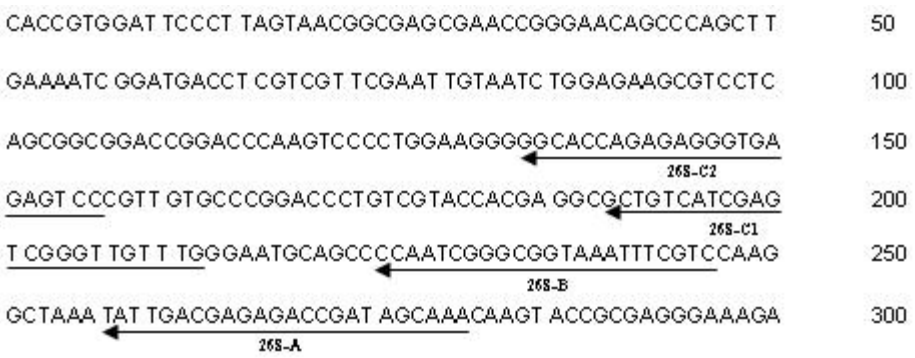

Figure 5: Localization of gene specific primers for the rRNA gene in I. walleriana

primer was capable of forming a product with the specific primer 26S-A in primary TAIL-PCR, with 26S-B in secondary TAIL-PCR, and with both 26S-C1 and 26s-C2 in tertiary TAIL-PCR (Figure 6). Off target amplifications were minimized by using the product from the previous round of PCR as the template, ensuring a higher concentration of the desired segment compared to any genomic DNA that may have remained. TAIL-PCR was conducted using 13 arbitrary primers (AP), of which only four (AP1, AP5, AP8, AP13) demonstrated successful amplification in both primary and secondary TAIL-PCR and so were used in tertiary TAIL-PCR. There was one positive result found with AP1 after tertiary TAIL-PCR (Figure 7), verifying successful use of TAIL-PCR to isolate the promoter of the 26S rRNA gene in *I. walleriana*. A band was found at approximately

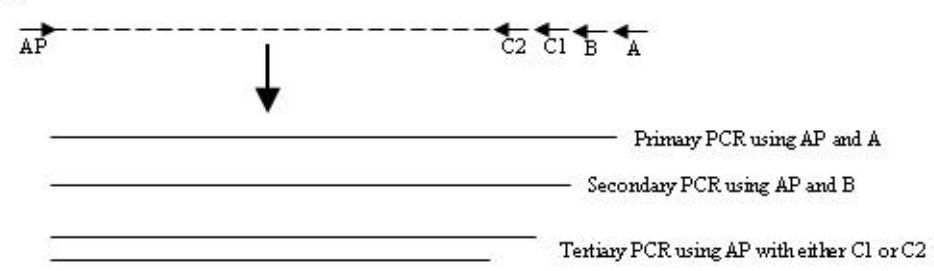

Figure 6: Schematic outline of TAIL-PCR. Results of primary through tertiary PCR using nested primers in conjunction with arbitrary primer (AP).

1100 bp for AP1 paired with 26S-C2 and another band appeared at a slightly larger bp for AP1 paired with 26S-C1. This difference in bands corresponded with the 32 bp difference in specific primer placements within the 26S rRNA gene (Figure 5), further confirming successful identification of *I. walleriana* gene promoters via TAIL-PCR. Although two light bands appeared for AP5 paired with both 26S-C1 and 26S-C2 (Figure 7), these bands are a false positive. These products are not considered as positive results

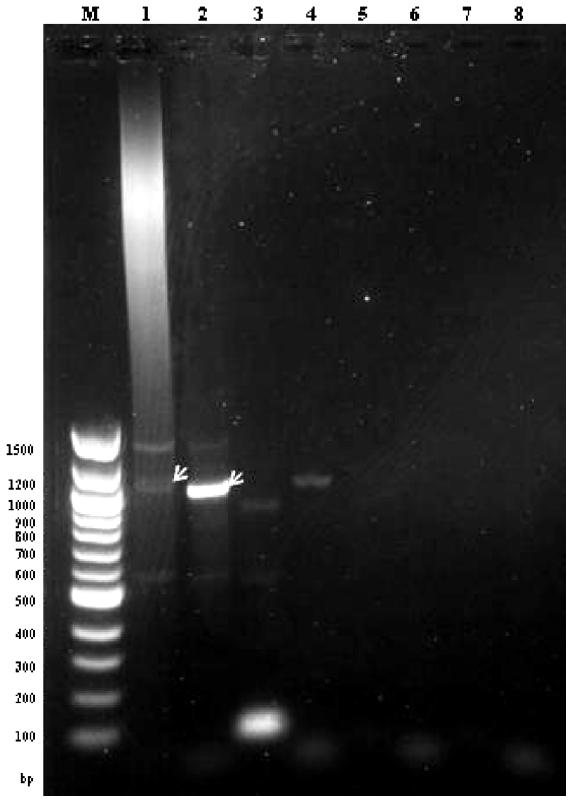

Figure 7: Electrophoresis gel of tertiary TAIL-PCR results. M: marker lane; 1: Arbitrary primer 1 (AP1) with 26S rRNA specific primer C1 (26S-C1); 2: AP1 with 26S rRNA specific primer C2 (26S-C2); 3: AP5 with 26S-C1; 4: AP5 with 26S-C2; 5: AP8 with 26S-C1; 6: AP8 with 26S-C2; 7: AP13 with 26S-C1; 8: AP13 with 26S-C2. After each round of TAIL-PCR, the set of arbitrary primers (AP) was narrowed to those capable of producing a product when paired with the 26S rRNA specific primer A in primary TAIL-PCR and then with rRNA specific primer B in secondary TAIL-PCR. The APs that proved productive in both primary and secondary TAIL-PCR were AP1, AP5, AP8, and AP13. These APs were then paired with two different 26S rRNA specific primers (C1 and C2) for the final tertiary TAIL-PCR, depicted above.

because the difference in strand lengths is greater than rather than consistent with the base-pair difference in placement of 26S-C1 and 26S-C2 within the 26S rRNA gene (Figure 5). Furthermore, the longer product is not AP5 paired with 26S-C1 as would be expected since 26S-C1 is nested further than 26S-C2 into the 26S rRNA gene (Figure 5). It is more likely that AP5 bound elsewhere in the genome using genomic DNA that was present as a template, pairing with the different specific primers in different locations resulting in two apparent products.

CHAPTER FOUR

Discussion

It was ultimately determined that the attempted transformations of *I. walleriana* using pNEC-EGFP and pNECS-EGFP were unsuccessful, meaning no conclusions can be drawn on the effectiveness of pNEC-driven expression in *I. walleriana*. Escapes from kanamycin selective media are not uncommon and may be due to either chimeric transformation or residual *Agrobacterium* (Estopà et al., 2001; Ghorbel et al., 1999). However, timentin was included in the selection media in order to kill any remaining *Agrobacterium*. It is more likely that *I. walleriana* has some low level of inherent resistance to kanamycin. Escapes from inherent resistance to kanamycin have been reported in plant species as diverse as citrus trees (Jain and Minocha, 2013) and alfalfa crop (Araujo et al., 2007), with alfalfa being capable of escaping kanamycin concentrations as high a 100 mg/L. Evidence of some levels of kanamycin resistance inherent in some *I. walleriana* strains is also found in the study from which the *I. walleriana* transformation protocol was obtained (Dan et al., 2010). Kanamycin levels were varied (25, 35, and 50 mg/L) and tested for selection efficacy. It was found that 50 mg/L kanamycin most significantly limited the new growth of untransformed MBC by 91.8%, yet was unable to completely prevent new growth, allowing for potential escapes.

To avoid escapes in the future, it is recommended that another form of positive selection be used in conjunction with kanamycin resistance. In the original paper from which the method for transforming *I. walleriana* was taken the second selective marker used was EGFP (Dan et al., 2010). However, the present research objective was to

demonstrate nectar specific expression using EGFP, making it unsuitable to use constitutively expressed EGFP for early-stage verification as a second screenable marker in our own experiment. Therefore, another marker should be used that is still expressed constitutively throughout the entire transformant but will not interfere with interpreting successful expression of a nectar specific promoter. One such marker could be an enzyme in the anthocyanin synthesis pathway, which is responsible for the purple pigmentation of certain plants and has been previously used in apple, strawberry, and wheat plants as a marker of successful transformation (Kortstee et al., 2011; Mentewab et al., 1999). Furthermore, this experiment did reveal that the nectar of *I. walleriana* is acidic meaning EGFP ($pK_a = 5.8$) may be denatured and not easily visualized even though it may be present. For this reason, it is recommended that an alternative fluorescent protein be used. There are a wide selection of fluorescent proteins with pK_as ranging from 5.0 to lower than 4.5 (Chaner et al., 2005). Examples are the orange class mKO protein with a pK_a 5.0 and the red class mStrawberry protein with a pK_a lower than 4.5. Preferred emission and excitation spectra and brightness may be used to select between the various available fluorescent protein choices.

Finally, this thesis confirmed that TAIL-PCR is a useful method for isolating the promoter from a targeted *I. walleriana* gene of known sequence, demonstrating such by isolating the 26S rRNA gene promoter. This further means that once the sequence for the nectar-expressed *IW23* gene has been determined the isolation and sequencing of pIW23 can quickly follow. The DNA sequence for *IW23* will be based on the amino acid sequence for the IW23 protein as determined by *de novo* mass spectrometry, taking into consideration codon bias. This putative DNA sequence will be made easier to determine

by comparison with RNA-Seq data of mRNA isolated from *I. walleriana*. Nested primers for TAIL-PCR can then easily be designed based on the doubly confirmed sequence of *IW23*, increasing the likelihood of successful TAIL-PCR by having specific primers based upon an accurate sequence of *IW23*. These specific primers will then be tested against an array of arbitrary primers that after tertiary TAIL-PCR will result in isolation of the *IW23* promoter that can then be sent off for sequencing by commercial company.

The expected efficacy of endogenous pIW23 in *I. walleriana* can then be verified using the transformation method that was conducted using pNEC1 with the added improvement of using anthocyanin as a second selection for transformation, and a different fluorescent protein capable of fluorescing at the *I. walleriana* nectar pH level. Furthermore, alternative nectary-specific promoters are being considered. One promoter being considered is the promoter for SWEET9, a nectary-specific sugar transporter that has been identified in *Arabidopsis thaliana*, *Brassica rapa*, and *Nicotiana attenuata* (Lin et al., 2014). Transformation of *I. walleriana* with SWEET9 is already in process. With MBC now on selection medium, regeneration of transformed plants is anticipated with two weeks. Another is the promoter for CELL WALL INVERTASE 4 (CWINV4) that has been identified in *A. thaliana* with an orthologue in *B. rapa* (Ruhlmann et al., 2010). CWINV4 catalyzes the hydrolysis of sucrose into glucose and fructose and has been shown to have an enriched expression in nectaries and plays a key role in successful nectar production. The third promoter being considered is the promoter for CRABS CLAW (CRC), which has been identified in *A. thaliana* with orthologues found in three Brassicaceae species (Lee et al., 2005). CRC is a member of the YABBY family that

encodes for a transcription factor that plays a crucial role in nectary development. Both CWINV4 and CRC are already in transformed *I. walleriana* that will be allowed to mature, at which point the expression of the reporter gene *GUS* will be determined. The presence of GUS can easily be verified with the addition of X-Gluc because GUS is β-glucuronidase that will cleave X-Gluc into a blue product.

Even though these alternative nectary-specific promoters are not endogenous to *I. walleriana*, it is still expected that they will function properly. One example of a promoter working across different species is the promoter for CARN2, the most highly expressed nectar protein in carnations (*Dianthus caryophyllus*) (Helsper et al., 2011). The promoter for CARN2 was used to successfully express human epidermal growth factor in *Nicotiana langsdorffii* x *N. sanderae*. Even though carnations and tobacco are two distantly related species, the *Dianthus* promoter allowed proper expression of its associated gene in *Nicotiana*. For this reason, it is expected that the promoters for SWEET9, CWINV4, and CRC will function in *I. walleriana* like the promoter for *IW23* does. However, it remains to be demonstrated which promoter will have the highest efficacy in *I. walleriana*.

When an effective, nectar specific promoter is identified, it will be used in the development of *I. walleriana* that will express mosquitocidal nectar. The idea of engineering a plant to express an insecticide is not new, having been utilized before in a variety of crops since 1996, such as maize, cotton, and castor bean, to express a Cry protein to make the plant resistant to pests (Malathi et al., 2006; Romeis et al., 2006). It has been determined that Cry11A is specific to mosquitoes and blackflies (Höfte and Whiteley, 1989). Cry11A is one of several insectidical toxins produced by *Bacillus*

thuringiensis, a Gram-positive soil bacteria (Yamagiwa et al., 2002). It is currently thought that Cry11A works by creating pores in the membrane of the cells lining the gut of mosquitoes, leading death (Revina et al., 2004). Because Cry11A is specific for mosquitoes, using it as the mosquitocidal toxin to be expressed in the nectar of *I. walleriana* would minimize any effects on off target species.

An alternative to mosquitocidal nectar, however, could be the expression of anti-pathogen peptides that are pathogen specific, leaving the mosquitoes unharmed so as to not disrupt ecosystems, but preventing them from spreading disease. This concept was proven feasible in one study that transformed *Metarhizium anisopliae*, a fungus capable of infecting mosquitoes, to express various anti-malaria peptides, effectively combating malaria within infected mosquitoes (Fang et al., 2011). One anti-malaria peptide that could potentially be expressed in the nectar of *I. walleriana* is scorpine, which has proven effective against different stages of the malaria parasite's life-cycle within the mosquito (Conde et al., 2000). Another anti-malaria peptide is salivary gland and midgut peptide 1 (SM1) that blocks the entry of malaria into the salivary gland of mosquitoes, preventing transmission (Ghosh et al., 2009).

In addition, anti-viral peptides for dengue virus and West Nile virus have been discovered (Chew et al., 2015; Hrobowski et al., 2005). These anti-viral peptides work by blocking the entry of the virus into human cells and have been proven most effective when administered simultaneously with the virus to *in vitro* cells. This mechanism of action can be fully utilized by having mosquitoes ingest these antiviral peptides and then administer them simultaneously as the virus when blood feeding.

Another means of targeting pathogens within mosquitoes could be the use of antibodies. Production of antibodies within plants is a growing field because plants are capable of making post-translational modifications (Schillberg et al., 2002). These antibodies can either collect within cells or be excreted (Ko et al., 2009). One anti-malaria antibody that has been found is the single chain antibody PfNPNA-1 that binds to sporozoites, a life stage that occurs prior to entry into the mosquito salivary gland, rendering the parasite sterile (Chappel et al., 2004). An anti-dengue antibody (F_{ab} 5J7) has also been discovered, which functions by coating the dengue virus, preventing it from binding receptors on human cells and fusing with the cell membrane (Fibriansah et al., 2015). Furthermore, pathogen specific antibodies could be used to guide previously mentioned toxins and anti-pathogen peptides to their respective targets. This has been demonstrated to be successful in transgenic *Arabidopsis thaliana* that expressed anti-fungal peptides targeted to the fungus *Fusarium oxysporum* using a small chain variable fragment (scF_v) of an antibody (Peschen et al., 2004). Furthermore, making target specific anti-fungal and anti-microbial peptides using either a scF_v or a microbial pheromone as a guide has proven to increase specificity and toxicity (Eckert et al., 2006; Peschen et al., 2004).

Ultimately, transgenic *I. walleriana* expressing either mosquitocidal nectar or pathogen specific peptides using a nectar specific promoter will be developed. These plants can then be planted in areas where mosquito-borne diseases such as malaria, dengue, yellow fever, and zika virus are prevalent and help control mosquito populations and transmission of mosquito-borne pathogens, reducing disease and death. However, this development of a generalized nectar delivery system is a technology expected to

have even broader impact as it is not limited to controlling mosquito populations and mosquito-borne diseases, but can be used to express any desired protein in nectar. Any organism that partakes of the nectar of plants can be influenced by controlling what is expressed in the nectar. For example, honey bee colony collapse could potentially be prevented by expressing an anti-fungal protein in the nectar that the bees then make into honey, thus ridding the colony of fungal infection. This is new technology of a nectar delivery system has far reaching implications and is waiting to be fully utilized.

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