

Making Every Seed Count

A summer of agricultural research and cultural discovery in the name of global food security



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The World Food Prize
Borlaug-Ruan Internship
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EVERY SEED COUNTS



THE WORLD FOOD PRIZE

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Introduction

Namaste! My name is Madeline Song, and I am from San Diego, California. In October of 2015, I participated in the World Food Prize Global Youth Institute (GYI). Five years earlier, my older brother, Justin, had done the same, later going on to conduct research as a 2011 Borlaug-Ruan intern at EMPBRAPA in Londrina, Brazil. As a sixth grader when he left for Brazil, I had never studied agriculture or even stepped foot on a farm. I caught glimpses into his life overseas during our many video chats that summer, but I didn't understand exactly what he was researching or the significance of his work in agriculture.

It wasn't until I started high school that I was able to make sense of his eight weeks in Brazil. As I began my freshman year, I participated as a delegate in Model United Nations, where I learned about the international policy and innovative research being used to address the world's most pressing needs. I was inspired to do more than discuss policy and research in the confines of a high school lecture hall. I wanted to be on the frontlines of research and innovation.

I joined my school's synthetic biology team when I realized they were researching solutions to issues like global health and food security. From my sophomore to junior year, we conducted research at UC San Diego, working hours each day to engineer *E. coli* strains that secreted chitinase. This discovery had the ability to eliminate Fusarium wilt, a disease devastating over 120 plant species. We brought our results to the International Genetically Engineered Machine Competition, where we won a bronze medal. I left the competition convinced this was what I wanted to do with my life: use science and technology to effect change.

What followed were months of research on issues of hunger and food production for the 2015 GYI, where I proposed the use of bioengineered rice as a solution to the unpredictable drought and flooding in Bangladesh. I discovered that technology such as bioengineered rice had already been developed by scientists and distributed in certain areas, but it had not been able to reach the most vulnerable smallholder farmers who desperately needed this technology.

After meeting hundreds of passionate hunger fighters, policymakers, and agricultural researchers at GYI, I was inspired to apply for the Borlaug-Ruan internship. In the personal essay I mailed to the World Food Prize, as a part of my application, I wrote:

“As an intern, I will use this opportunity to follow in the footsteps of Dr. Norman Borlaug—I want to start the next agricultural revolution. Science and research are the keys to opportunity, and creating opportunity is the first step to restoring justice in the world. During Sheryl WuDunn's speech at GYI, she estimated that if women were treated equally, 100-150 million individuals would be lifted out of poverty. By working directly with the most food-insecure individuals, I wish to create a path that will one day make equality and justice a reality. Norman Borlaug was not just a hunger fighter, but a champion for justice and a creator of opportunity. I would be honored to continue Dr. Borlaug's legacy as a 2016 Borlaug-Ruan Intern.”

I read these words again, months later, as I boarded a plane to London, England and then on to Mumbai, India. As I got lost in airport terminals and grew frustrated by confusing signs in foreign languages, I was empowered by these words to continue in my fight for global food

security with the same determination, strength, and passion that I, as well as so many, admired in Dr. Borlaug.

Maharashtra Hybrid Seeds Company

Dr. B.R. Barwale founded Mahyco in 1964 with two inspiring goals: to 1) fight world hunger with Indian agriculture and 2) empower smallholder farmers with agricultural research and innovative seed technology. In 1998, he was recognized as a World Food Prize Laureate for his work in revolutionizing hybrid seed technology in India. In my research paper for the 2015 GYI, I proposed the use of hybrid seeds in climate-vulnerable areas where unpredictable drought and flooding often devastated smallholder farmers as well as their entire livelihoods. I had discovered that bioengineered technology existed, but it had not been properly implemented or taught to local villagers and farmers. At Mahyco, scientists and researchers were directly involved in this seed production and implementation process.

Because Mahyco was a company, its primary goal was not research. Its main objective was, instead, to produce effective seeds that would help farmers maximize their yields. And so, Mahyco had a marketing team, an intellectual property cell, and a diverse number of research and development labs. I was assigned to the Molecular Entomology lab, but I had the opportunity to train with four other research groups: Entomology Resource Unit, Plant Virus Interactions, Plant Transformation, and Molecular Breeding and Applied Genomics. As I trained in each lab, I started to understand how findings in the Mahyco labs translated into actual seed products.

Entomology Resource Unit

Before I started brainstorming research ideas, I was given an overview of how a gene, for example, one coding for insect resistance, would be incorporated into a plant. The first step, after finding that gene, involves testing its effects on insects in the Entomology Resource Unit (ERU). This testing process is executed with bioassays. I was trained in the ERU to prepare these bioassays, which contained chickpeas, antibiotics, and different concentrations of the test protein. After approximately seven days of testing, the effect of the protein, in its corresponding concentrations, is observed, based on the number of dead and alive insects. From that point, the lab will decide whether to proceed with the gene or isolate another, possibly more effective, gene.

Plant Virus Interactions

The Plant Virus Interactions (PVI) lab researches various plant viruses in order to create resistance in plants. PVI is similar to Molecular Entomology in that it isolates a gene of interest for transformation and further breeding into a final hybrid seed product. It differs in that it achieves the final seed product through genetic engineering as well as traditional breeding practices. I worked with the lab to inoculate okra plants with the Yellow Vein Mosaic virus. Okra affected with this virus displays symptoms that include green and yellow patches, vein clearing, and vein thickening. The resulting fruits are deformed, dwarfed and of a yellow-green

color. I worked with scientists in the lab to transmit the disease to healthy plants through the whitefly insect in order to maintain a stock of infected plants for future transgenic testing.

Plant Transformation

After the Molecular Entomology and Plant Virus Interactions labs discover a gene, the researchers from each respective lab collaborate with Plant Transformation (PT). In PT, scientists use transformation, a process in which foreign genetic material is taken up by a cell, to incorporate a desired characteristic into a plant.

While training at the PT lab, I learned to perform rice shoot and okra subculturing. Because the majority of transformations are not successful, sub culturing is used to eliminate failed transformation at each stage of plant growth. Bleached leaves, false roots, and dead areas of the plant are removed during each subculturing to avoid any restrictions to plant growth. For plants like okra, approximately seven total selections are performed, in a process spanning at least 4 months. After subculturing, the transformed plants undergo further testing in the on-campus greenhouses.

Molecular Breeding and Applied Genomics

After a gene of interest is successfully transformed into a plant and tested in the campus greenhouses, the Molecular Breeding and Applied Genomics (MBAG) lab assists local breeders in backcross breeding, or backcrossing.

Backcrossing is used to transfer a desired to an elite strain of the crop. The elite strain is high yield, high quality, etc. It has characteristics that are necessary for its survival in natural conditions as well as qualities that are desirable to an average consumer. Traditional breeding requires 5-6 generations to generate results, but with genetic markers, breeders can identify progeny with 50% to 60% of the desired traits and continue backcross breeding with the highest performing progeny. Marker-assisted breeding decreases the time and guesswork involved with traditional breeding practices—MBAG-assisted breeding only requires 2-3 generations of breeding.

Because the MBAG lab screens thousands of samples in a short period of time before relaying its findings to the breeders, it contains the most advanced machines at Mahyco. I learned to operate state-of-the art machinery, such as the tissue lyser, which can complete a day's work of DNA isolation in a mere 5 minutes, as well as the chip-based capillary electrophoresis machine and the PAGE, polyacrylamide gel electrophoresis, two automated machines which can analyze DNA samples in significantly less time, without the chance of human error associated with traditional gel electrophoresis.

Research:
Silencing of Brown planthopper (BPH) Genes with the use of RNA interference technology

Abstract

RNA interference technology (RNAi) was used to suppress the Hexose transporter (HT) and Juvenile hormone (JH) genes in the Brown planthopper (BPH), *Nilaparvata lugens*, for the development of BPH-resistant rice plants. Double-stranded RNA (dsRNA) was synthesized and tested *in vitro* with rice seedling bioassays using hydroponics. The effect of feeding dsRNA to BPH was tested with real-time PCR. dsRNA feeding significantly reduced HT and JH gene expression in BPH. The HT and JH genes can be used for the development of dsRNA-producing transgenic crops, which will be resistant to BPH attack. The dsRNA delivery method described here may also be used as a valuable tool for the study of various target genes in BPH.

Introduction

Rice is one of the most important cereal crops in the world, specifically in the Asia-Pacific region. The Brown planthopper is one of the most destructive insect pests of rice, causing significant yield losses for farmers. BPH in both nymph and adult stages suck the cell sap from rice plants, causing yellowed leaves and a reduced chlorophyll and protein content (Liu *et al.*). This affects the rate of photosynthesis, and, in cases of severe attack, causes extensive plant mortality referred to as “hopper burn” (Liu *et al.*). Pesticides, such as imidacloprid, are used extensively to control the BPH population; however, their widespread use has led to environmental pollution, pesticide resistance, a decrease in the number of natural enemies of BPH, and resurgence in the BPH population (Lakshmi *et al.*).

RNA interference (RNAi) is a process in which double-stranded RNA interferes with the expression of a target gene (Agrawal *et al.*). RNAi occurs naturally in cells, but in recent years, has been developed as a novel technique to silence, or turn off, unwanted genes (Agrawal *et al.*). There are two main steps in RNAi. Dicer RNase III-type enzymes first cleave cytoplasmic dsRNAs into small interfering RNA (siRNA) duplexes composed of approximately 21 nucleotides (Agrawal *et al.*). siRNA duplexes are then incorporated into a multiprotein RNA-inducing silencing complex (RISC), which separates the two strands of RNA into a passenger and guide strand (Agrawal *et al.*). RISC leads the guide strand to the mRNA site and cleaves the mRNA, degrading it so that no target protein is formed (Agrawal *et al.*). As a result, the gene is effectively silenced.

DsRNA-mediated gene silencing has been successfully conducted in insect orders Diptera, Coleoptera, Hymenoptera, Orthoptera, Blattodea, Lepidoptera, and Isoptera (Zha *et al.*). While past methods of gene silencing have included injecting dsRNA into insects, plant-mediated RNAi remains critical for the development of insect-resistant crops. Plant-mediated RNAi has been tested and observed in Lepidopteran and Coleopteran insect pests but not in Hemipteran insects (Zha *et al.*).

Additionally, while Bt (*Bacillus thuringiensis*) toxins have been introduced successfully in plants such as cotton in order to protect plants from insect attack, there are no known Bt toxins for

hemipteran insects, such as BPH (Zha *et al.*). Therefore, there is a greater need to explore plant-mediated RNAi in BPH as well as other hemipteran insects.

RNAi-mediated gene silencing has been demonstrated in the carboxypeptidase gene (Nlcar) and protease gene (Nltry) in BPH (Zha *et al.*). Gene expression was significantly reduced in nymphs fed with the synthesized dsRNA, but there was no significant sign of mortality (Zha *et al.*). Gene silencing has also been demonstrated in the V-ATPase E gene, which is the energy source for alkaline and amino acids absorption in BPH (Li *et al.*). Gene expression was reduced by a maximum of 55% (Li *et al.*). However, no significant mortality was observed after dsRNA bioassay (Li *et al.*).

Materials and Methods

Insects and Rice Plants

BPH were procured from the Entomology Resource Unit. Synchronous late nymphal instars were selected for the feeding bioassay. One- to two-month old rice plants of the susceptible variety, TN1, were procured from the greenhouses at Mahyco.

RNA isolation from Brown planthopper

BPH were separated into different stages: male, female, adult, and nymph. Males were identified by long, thin abdomens and black coloring. Females were identified by larger abdomens. Nymphs were identified by smaller size. RNA isolation was completed using the Qiagen RNeasy Mini Kit.

BPH tissue was submerged into liquid nitrogen and ground into a fine powder with a mortar and pestle. Tissue was further disrupted with Buffer RLT to produce a homogenized mixture. Tissue lysate was centrifuged at maximum speed in a microcentrifuge. Supernatant was transferred to a new tube by pipetting. Ethanol was added to the cleared lysate and mixed by pipetting. Sample was added to an RNeasy mini column placed in a 2 ml collection tube. Buffer RW1 and Buffer RPE were added to wash the column. RNase-free water was used to elute the RNA into the collection tube.

Gel electrophoresis

Gel electrophoresis is used to separate DNA, RNA or proteins by molecular size. Negatively charged particles migrate from the cathode, the negatively charged electrode, to the anode, the positively charged electrode. Molecules travel through the gel at a speed inversely proportional to their lengths. Smaller molecules travel a greater distance than longer molecules. Samples are prepared with loading dye for visual tracking of molecule migration during electrophoresis. A DNA ladder is used to quantify size of molecules.

The quality of the isolated RNA was tested with a 1.2% Formaldehyde Agarose gel. The gel was loaded with samples, loading dye, and ladder and electrophoresed for 45 minutes.

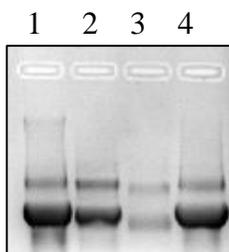


Fig. 1 RNA isolation

Lane 1: Nymph, Lane 2: Adult, Lane 3: Male, Lane 4: Female

DNase I treatment

DNase treatment was performed to remove DNA contamination from the isolated RNA. DNase I enzyme (1ul) was added to each RNA sample (9 ul). DNase I enzyme, or deoxyribonuclease, can digest single- and double-stranded DNA into single bases or oligonucleotides, therefore degrading the DNA. The enzyme is DNA-specific without causing damage to RNA. The RNA was incubated at 37 ° C for 20 minutes then at 75 ° C for 5 minutes. The concentration of purified RNA was quantified using the Nanodrop spectrophotometer.

BPH Developmental Stage	Concentration (ng/ul)
Adult	712.8
Nymph	1819
Male	453
Female	1952

Table 1. RNA concentration after DNase treatment

Reverse transcription (cDNA synthesis)

Reverse transcription uses RNA as a template to synthesize complementary DNA (cDNA). RNA is used along with a short primer complementary to the 3' end of the RNA to direct the first strand of cDNA. Reverse transcription was performed to synthesize cDNA from isolated BPH RNA. The Thermo Fisher Scientific First Strand cDNA Synthesis Kit was used to perform reverse transcription.

Sr. No.	Reaction Components	Volume (ul)
1	Reaction Buffer (5X)	4
2	dNTP mixture (10mM)	2
3	Oligo (dT) ₁₈ random primer (0.5 ug/ul))	1
4	Reverse transcriptase (200 U/ul)	1
5	Template, RNA	3
6	Ribolock RNase inhibitor (20 U/ul)	1
7	Nuclease-free water	8

Table 2. Reaction components for cDNA synthesis

PCR reaction cycles: 42 ° C for 60 min, 70 ° C for 5 min, 4 ° C for ∞. Synthesized cDNA was quantified using the Nanodrop spectrophotometer.

Insect Developmental Stage	Concentration (ng/ul)
Adult	1617
Nymph	1476
Male	1489
Female	1636

Table 3. cDNA concentration after reverse transcription of isolated RNA Polymerase chain reaction (PCR)

PCR is used to synthesize new strands of DNA from a template strand. There are three main steps: denaturation, annealing, and extension. High temperatures first denature the double-stranded DNA into two separate strands (denaturation). Two primers, short segments of DNA, attach to each end of the gene of interest (annealing). DNA polymerase, an enzyme responsible for synthesizing new strands of DNA, then attaches to the end of the primer and adds complementary nucleotides (A, G, T, C), the building blocks of DNA (extension). After 30 or 40 cycles, the reaction results in more than one billion copies of the gene of interest.

Sr. No.	Reaction Components	Volume (ul)
1	Taq Buffer (10X)	2
2	DNTP mixture (2 mM)	2
3	Forward primer (10 pM)	1
4	Reverse primer (10 pM)	1
5	Template, cDNA	2
6	Taq polymerase (1U/ul)	1
7	Autoclaved Mili-Q water	11

Table 4. Reaction components for DNA amplification using PCR

PCR reaction cycles: 94°C for 4 min, 94°C for 30 sec, 58°C for 30 sec, 72°C for 1min, 72°C for 7 min. The PCR product was loaded on 1.2% Agarose gel to check for amplification.

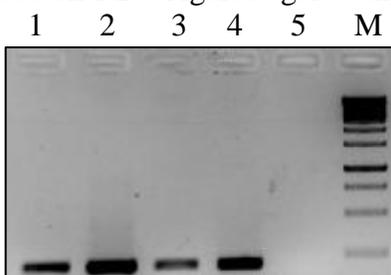


Fig. 2 PCR amplification of HT gene

Lane 1: Adult, Lane 2: Nymph, Lane 3: Male, Lane 4: Female, Lane 5: Water control, Lane M: DNA ladder

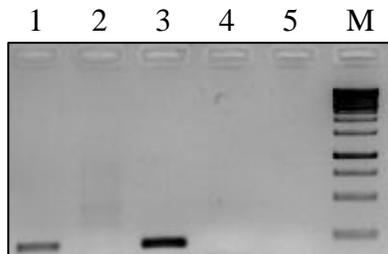


Fig. 3 PCR amplification of JH gene

Lane 1: Adult, Lane 2: Nymph, Lane 3: Male, Lane 4: Female, Lane 5: Water control,
Lane M: DNA Ladder

Real-time PCR

Real-time PCR combines PCR amplification and detection into a single step, eliminating the need for PCR and gel electrophoresis. Real-time PCR increases the accuracy and precision of traditional amplification and detection methods. Real-time PCR measures the accumulation of fluorescent signals during the exponential phase of the reaction. Intercalating dye (SyBr green) is used to label the PCR product during thermal cycling. The dye intercalates with the DNA double helix, altering the structure of the dye, which causes it to fluoresce. Fluorescence increases as the number of gene copies increase.

Gene expression was first tested for the HT gene, then the JH gene. The actin gene was used as an endogenous control for both reactions. Based on cDNA concentrations, cDNA template concentrations for each reaction were calculated.

Sr. No.	Reaction Components	Volume (ul)
1	SyBr green master mix (2X)	10
2	Forward Primer (10pM)	1.2
3	Reverse Primer (10pM)	1.2
4	Template, cDNA (500ng)	2
5	Nuclease-free water	5.6

**Table 5. Reaction components for DNA amplification using Real-time PCR
Gene amplification with T7 primer**

cDNA synthesized from RNA template was used to amplify JH and HT genes in BPH with the T7-promoter sequence primer. cDNA with T-7 primers is necessary for future dsRNA synthesis.

Sr. No.	Reaction Components	Volume (ul)
1	Taq Buffer (10X)	2
2	DNTP mixture (2mM)	2
3	Primer Forward T7(10 pM)	1
4	Reverse Primer T7(10 pM)	1
5	Template cDNA	2
6	Taq Polymerase enzyme (1U/ul)	0.5
7	Autoclaved Mili-Q water	11.5

Table 6. Reaction components for DNA amplification using T7 primers

PCR reaction cycles: 94 ° C for 5 min, 94 ° C for 30 sec, 58 ° C for 1 min, 72 ° C for 1 min (30 cycles), 72 ° C for 7 min, 4 ° C for ∞.

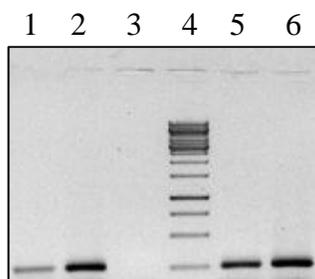


Fig. 4 Gene amplification with T7 primer

Lane 1: HT gene w/ DNase treatment, Lane 2: HT gene w/o DNase treatment, Lane 3: Blank
Lane 4: DNA Ladder, Lane 5: JH gene w/ DNase treatment, Lane 6: JH gene w/o DNase treatment

Purification of PCR product (Gel extraction)

The Qiagen Gel Extraction Kit was used to purify the PCR product. DNA fragments were extracted from the agarose gel with a scalpel using a UV trans illuminator. The fragments were stored in 1.5 ml tubes. Buffer QG was added to the gel fragments. The tubes were incubated at 50 ° C for 10 min to melt the gel. The mixture turned yellow, which indicated correct pH levels. Isopropanol was added to the sample to form a precipitate. Tube contents were placed in a spin column inside a 2 ml collection tube and centrifuged for 1 min. Flow-through was discarded. Buffer PE was added to the column to wash the DNA. Column was centrifuged for 1 min to removed residual wash buffer. Column was placed into a clean microcentrifuge tube. Buffer EB was added to the center of the tube to dissolve the DNA attached to the center of spin column, which allowed the DNA to pass through the column and into collection tube.

Production of dsRNA

dsRNA was synthesized from purified PCR amplicons using the Ambion MEGAscript T7 Kit. Nucleotides (A, C, G, U), buffer (warmed to 37°C), cleaned PCR product (product of gel extraction), and enzyme mix were added to a PCR tube in the listed order, spun down and

incubated at 37 ° C for 6-12 hours in the shaker. The samples were then incubated at 75°C for 5 min (annealing), cooled down to room temperature, 25°C, for approximately 4 hrs., and stored at -20°C. The synthesized dsRNA was quantified using Nanodrop spectrophotometer.

Target gene	dsRNA concentration
HT	295.3 ng/ul = 0.3 ug/ul
JH	112.8 ng/ul = 0.1 ug/ul

Table 7. dsRNA concentrations

BPH Bioassay

The dsRNA was tested with two types of bioassays. The dsRNA was first fed to BPH through an artificial diet, then through rice seedlings.

Artificial Diet:

Artificial diet was prepared according to existing protocol (Mitsuhashi, 1974). Diet incorporated with dsRNA was poured between two layers of parafilm in a small, plastic vial. The set-up was designed to replicate natural feeding habits of BPH. BPH feed on rice plants by first inserting stylets into the plant's phloem tissues (first layer of parafilm) and then sucking the cell sap (artificial diet). 10 BPH were placed in each tube. Vials were stored in an incubator (growth chamber) in 25-28 ° C and 60% relative humidity to replicate natural BPH feeding conditions.

BPH were discovered dead after 24 hours, suggesting that the artificial diet and bioassay set-up were unable to sustain the insects.

Rice Seedling Bioassay:

The bioassay was repeated with rice seedlings. Higher and lower concentrations of dsRNA for each gene were tested. Concentrations were calculated from the dsRNA concentrations measured with the Nanodrop spectrophotometer. Higher and lower concentrations for the two genes were replicated in 3 tubes for greater accuracy. The higher concentration for HT was 3 ug and the lower was 0.5 ug. The higher concentration for JH was 1 ug and the lower was 0.2 ug. 3 tubes, in which dsRNA was not added, were used for control.



Fig. 5 BPH bioassay, dsRNA delivery through rice seedlings (root uptake)

Water was pipetted into 1.5 ml tubes. Tubes were suspended in a thin disk of Styrofoam placed inside a plastic container. Concentrations were pipetted into corresponding tubes and mixed by pipetting. Individual rice seedlings were placed in each tube and covered with plastic to prevent BPH from escaping. 5 BPH were placed inside each plastic covering. The BPH were kept in the bioassay for 7 days, then removed from the bioassay and stored into corresponding HT 3 ug, HT 0.5 ug, JH 1 ug, JH 0.2 ug, and Control tubes. RNA was isolated from the insects and used to synthesize cDNA. cDNA was used as a template to amplify HT and JH gene with the T7-promoter sequence.

Results

State-Specific Gene Expression:

Real-time PCR was used to quantify gene expression in different stages of BPH, adult, nymph, male, and female.

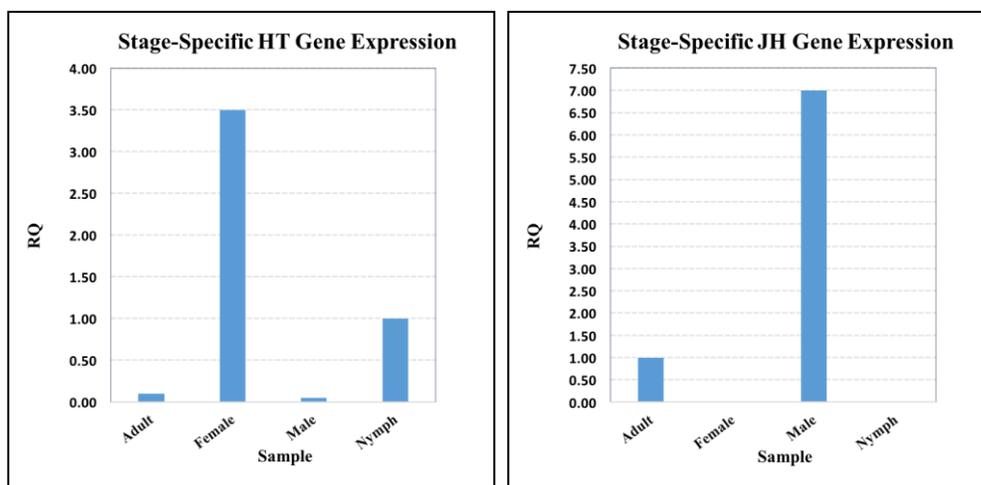


Fig. 6 Gene expression of HT (Left) and JH (Right) in Adult, Female, Male and Nymph

The highest gene expression of HT gene was observed in female, then nymph, then adult, then male. The highest gene expression of JH gene was observed in male then adult. No expression of the JH gene was observed in female and nymph.

Real-time PCR results confirmed gel image results for HT gene and JH gene.

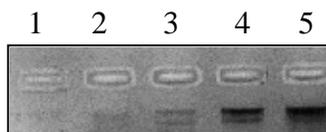


Fig. 7 RNA isolation from BPH, after bioassay

Lane 1: HT 0.5 ug, Lane 2: HT 3 ug, Lane 3: JH 0.2 ug, Lane 4: JH 1 ug, Lane 5: Control

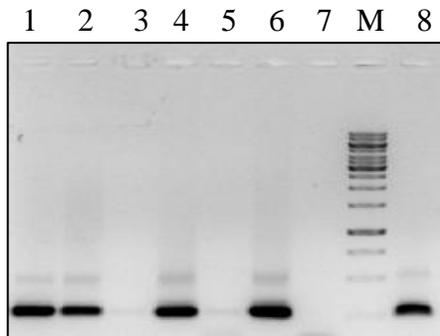


Fig. 8 cDNA amplified with T7-primer sequence

Lane 1: HT 3 ug, Lane 2: HT control, Lane 3: HT water control, Lane 4: JH 0.2 ug, Lane 5: JH 1 ug, Lane 6: JH control, Lane 7: JH water control, Lane M: DNA ladder, Lane 8: HT 0.5 ug

Amplification was observed in HT 0.5 ug, HT 3 ug, HT control, JH 0.2 ug, JH control. No amplification was observed in JH 1 ug, HT water control, and JH water control. The results were expected with the exception of JH 0.2 ug. Amplification in JH 0.2 ug indicated correct synthesis of cDNA from RNA template. Therefore, amplification in both JH 1 ug and JH 0.2 ug was expected. The result may have been caused by a pipetting error when amplifying the JH gene with the T7-primer.

Real-time PCR results after bioassay

HT gene:

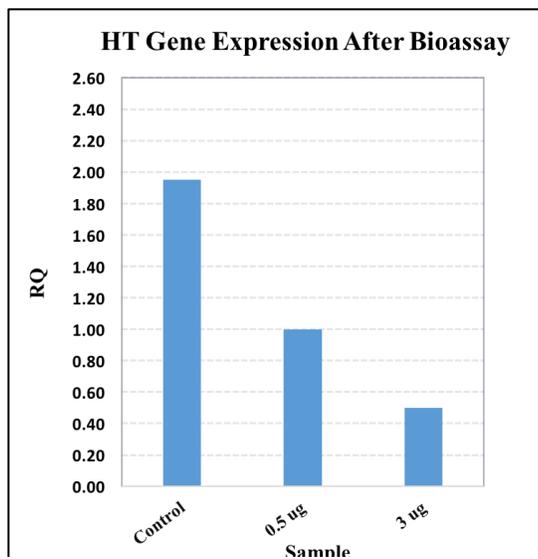


Fig. 9 Relative Quantification of gene expression for HT gene after bioassay

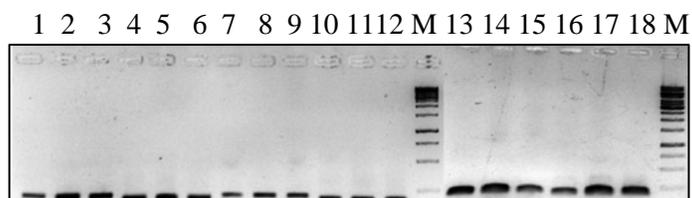


Fig. 10 Real-time PCR product, HT gene

Lane 1-3: 0.5 ug, Lane 4-6: Actin, Lane 7-9: 3 ug, Lane 10-12: Actin, Lane M: DNA ladder,
Lane 13-15: Control, Lane 16-18: Actin, Lane M: DNA ladder

Insects fed with low concentration of dsRNA had higher gene expression levels than insects fed with high dsRNA concentration. Control had highest gene expression.

JH gene:

JH gene bioassay was repeated with higher (4 ug) and lower (0.3 ug) dsRNA concentrations. The same protocol used in the previous experiment was repeated.

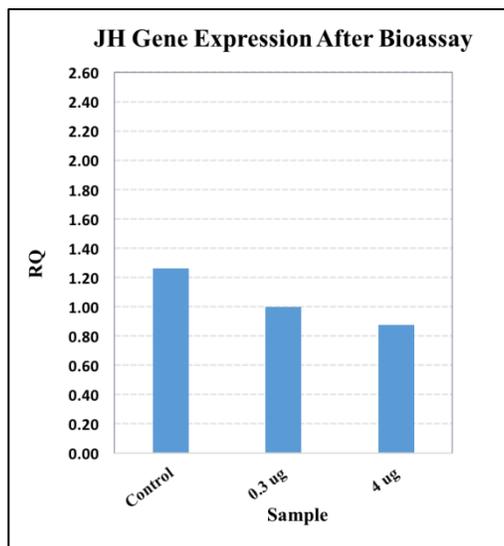


Fig. 11 Relative Quantification of gene expression for JH gene after bioassay



Fig. 12 Real-time PCR product, JH gene

Lane 1-3: Control, Lane 4-6: Actin, Lane 7-9: 0.3 ug, Lane 10-12: Actin, Lane 13-15: 4ug, Lane 16-18: Actin, M: DNA ladder

Insects fed with low concentration of dsRNA had higher gene expression levels than insects fed with high dsRNA concentration. Control had highest gene expression.

Discussion

Stage-specific gene expression was tested with real-time PCR in order to determine which stages displayed the highest gene expression of HT and JH. As shown by Figure 3, there is significantly greater expression of the HT gene in the Female and Nymph stages and the JH gene in the Male and Adult stages. RNA interference can be tailored to these specific stages in order to maximize the effects of gene silencing.

RNA interference of the HT gene was confirmed with real-time PCR results, as shown in Figure 9. The highest transcript reduction was found in the treatment with higher dsRNA concentration followed by the treatment with lower dsRNA concentration. Gene expression was highest in the control. The highest expression of the HT gene was observed in the control because it was not treated with dsRNA and therefore RNA interference did not occur. Gene expression was lowest in BPH treated with dsRNA, which confirms that the dsRNA was effective in RNA interference. BPH treated with low concentration of dsRNA displayed higher gene expression than BPH treated with high concentration of dsRNA, confirming that higher amounts of dsRNA were more effective in reducing gene expression of HT in BPH.

The bioassay was repeated to test for JH gene expression. The dsRNA generated for the JH gene was not effective in the first bioassay. Gene expression was observed in the lower concentration of the JH gene and also observed in the control, but in a lower amount. No expression was observed in the higher concentration of the JH gene. The highest gene expression should have been observed in control, then JH low concentration, then JH high concentration. The control bioassay was not treated with dsRNA and should have displayed higher concentration of the JH gene in comparison to the treated bioassays. Therefore, dsRNA was synthesized again before testing the second bioassay.

RNA interference of the JH gene was confirmed with real-time PCR results after the second bioassay, as shown in Figure 11. Gene expression was highest in control, second-highest in low concentration, and lowest in high concentration. The correct trend was displayed, confirming the effectiveness of the dsRNA in silencing the JH gene in BPH.

Conclusions

Prior to this research, RNA interference had not been used to silence the HT and JH genes in BPH. Researchers used RNA interference, in the past, to silence the carboxypeptidase protease, and V-ATPase E gene. While these genes did lead to a decrease in gene expression, they did not result in significant signs of insect mortality.

The dsRNA, discovered in this research, can be used to produce BPH-resistant rice plants that result in significant insect mortality.

This research has also identified BPH stages with the greatest gene expression of HT and JH. This information can be used to tailor RNAi technology and insect-resistant seeds to certain stages of BPH development, allowing farmers to maximize their use of hybrid seed technology.

RNA interference is a powerful technique that can be used by farmers to protect their crops against deadly BPH attack and eliminate the need for harmful, costly, and ineffective pesticides of the past. Because RNA interference is incorporated into the plant, itself, the insect will automatically ingest the dsRNA once it feeds on the plant. The dsRNA will then target the messenger RNA responsible for the expression of the gene of interest, effectively silencing the gene in the insect and causing insect mortality.

This novel application of RNA interference can be used to breed BPH-resistant rice strains that farmers can use to save resources, reduce environmental pollution, decrease pesticide-related health risks, and protect their crops against deadly BPH, ultimately maximizing their yield.

Personal Reflection

A Better Researcher

After an unfortunate case of food poisoning, my stomach could only tolerate a diet of dry toast and bananas for weeks on end. In response, my host mom brought home every possible variety of the fruit she could find at the local market. I discovered that her husband, who was from the southernmost part of India, only ate the brown, longer type of banana, while she, raised in the north, preferred the smaller, yellow type. I had been oblivious to the fact that there were such strong regional and cultural ties in agriculture.

I worked at Mahyco to conduct research on hybrid seeds, focusing on RNA interference technology and its use in fortifying crops against BPH. My novel application of RNAi could be used by local breeders to develop BPH-resistant rice strains. However, after learning of these significant differences in banana, I realized it wasn't enough to create a high yield, insect-resistant crop—it had to be the exact variety familiar to local villagers and farmers.

A Better Communicator

After the first few weeks at Mahyco, my mentor and I had made progress in communicating with each other, foregoing the initial, all-too-frequent “I’m sorry, what did you say?”, but we still lacked a cultural understanding. I came to learn that teenagers in India were generally discouraged by their families from traveling far—and that even my mentor, an adult, had never left his state. What I had initially interpreted as a dismissiveness towards me for my age and gender, I came to learn was a feeling of protectiveness.

I began to pay more attention to these behaviors, asking my colleagues to expound on these cultural and societal differences, as I shared my own background. I realized that these slight differences, in their totality, had contributed to our initial barriers in communication. Instead of feeling frustration, I worked to learn from my mentor, teach him about my culture, and

ultimately prove to him that I was beyond my years in knowledge, experience, and passion for research.

I realized, while working at Mahyco, exactly how important communication was to scientific research. Cross-cultural, international collaboration is necessary in the fight against world hunger. By researching in India and interacting with local scientists and farmers, I was able to understand regional and cultural preferences associated with agriculture. I learned new techniques and protocols that I had never been trained in back home, but that, I discovered, were more suitable for the research and field work conducted in India. I also discovered that, for two people who didn't share the same cultural and societal backgrounds, there were many opportunities for misunderstandings. I learned to be open to new ways of thinking in order to understand and address the reasons behind our initial frustrations and misunderstandings.

A Bolder, Stronger Individual

I challenged myself to explore India as a bolder, stronger version of myself. I was eager to experience every opportunity at my grasp, included the chance to spend a week training and helping out at Shri Ganapati Netralaya (SGN). SGN is an eye hospital built by the founder of Mahyco, Dr. B.R. Barwale, to provide affordable, quality eye care to the people of India.

As someone who wore glasses and contact lenses since the second grade, I was familiar with the lenses and alphabetical charts in the optometry department at SGN, but I was unprepared for the poverty and illiteracy of the patients who came to SGN for treatment. Many of these patients traveled to SGN for help because SGN offered affordable prices to individuals who could not pay full rates for treatment.

For illiterate patients in the optometry department, the doctors used charts with images of flowers and animals, instead of the traditional letters and numbers. These patients required a great degree of patience from the doctor as they would many times misunderstand requests to cover a specific eye or fail to answer the doctor's questions. As I continued to work in the optometry department, I became more comfortable interacting with these patients, realizing that I could communicate and connect with them in more ways than language.

I was inspired to learn more about SGN, traveling days later, on a 3-hour journey to one of the SGN extension centers in the Malkapur village. Because the center was located in a rural village, the majority of the patients had never seen a foreigner before that day.

One woman continued to ask the doctor in Marathi, "Who is she?" while pointing to me. She thought her condition was so severe that the center needed the opinion of an American doctor. I was, of course, confused why she was so anxious after seeing me, until the doctor translated her words and we both could not help but burst into laughter. The staff gradually filed into the room, later in the day, excited by my presence. Besides the two doctors and sole administrator, the staff did not speak English and had likewise never seen a foreigner, but we communicated with laughter and interesting Marathi-English translations.

I realized that this was no longer something I had to remind myself to do: to be bolder and stronger. It was someone I had become, someone who India had shaped me into as a person and as a researcher. A few days before I left India, I visited the Mahyco test farms in Jalna with researchers from the Molecular Biology lab. After a morning of jotting down observations and collecting plant samples treated with different microbial solutions, we started our drive back to the research center.

We stopped on the side of the road after a scientist, Ram, spotted a grove of pomegranates. We exited the car, so that Ram and Pritesh, another Mahyco scientist, could brainstorm possible routes from the car to the pomegranate fields. After exhausting a few options, they decided to jump to the other side of a river bank. By the time I finally understood their intentions, Ram had already followed Pritesh by leaping across the water and safely landing on the other side.

They motioned for me to follow suit, and I surprised myself, starting to jump before I even considered what I was doing. I took a literal leap of faith and trusted two scientists who I had met all but two hours ago.

We reunited near the pomegranate fields where the farm manager, who had learned we were scientists at Mahyco, allowed us to pick the fruit free of charge. I was amazed by his generosity, but I eventually came to understand the reasons behind his kind gesture—Mahyco had revolutionized seed technology in India and empowered many farmers, like himself, to maximize his yields and achieve food security.

If I hadn't taken a leap of faith, I would have never experienced that inspiring moment between the grove owner and myself, Ram, and Pritesh.

After months of work and experience, failure and success, I was no longer just a scientist. I had become a scientist and hunger fighter with the means to work on the frontlines of research and innovation in the most vulnerable, developing countries across the world. I had fought through language and cultural barriers, unfamiliar environments, and intimidating situations, gaining the experience and strength necessary to effect real change and difference in the world, especially in the fight for global food security. I had learned to take leaps of faith, both literal and figurative, and to, most importantly, make every seed count.

Pictures



Works Cited

- Agrawal, Neema et al. "RNA Interference: Biology, Mechanism, and Applications." *Microbiology and Molecular Biology Reviews* 67.4 (2003): 657–685. *PMC*. Web. 17 Aug. 2016.
- Beers, Elizabeth H., and Geraldine Warner. "Insect Growth and Development." *Tree Fruit Research & Extension Center Orchard Pest Management Online*. Washington State University, n.d. Web. 17 Aug. 2016.
- Brar DS, Virk PS, Jena KK ., Breeding for resistance to planthoppers in rice. Planthopper: new threats to the sustainability of intensive rice production systems in Asia. IRRI. (2009) p 401-427
- Fernando G. Noriega, "Juvenile Hormone Biosynthesis in Insects: What Is New, What Do We Know, and What Questions Remain?," *International Scholarly Research Notices*, vol. 2014, Article ID 967361, 16 pages, 2014. doi:10.1155/2014/967361
- Ge, Lin-Quan et al. "Silencing a Sugar Transporter Gene Reduces Growth and Fecundity in the Brown Planthopper, *Nilaparvata Lugens* (Stål) (Hemiptera: Delphacidae)." *Scientific Reports* 5 (2015): 12194. *PMC*. Web. 17 Aug. 2016.
- Ghaffar, Mohamad Bahagia AB, NorlizaTendot Abu Bakar, Jeremy Pritchard, and Brian Ford Lloyd. "Identification of Candidate Genes Involved in Brown Planthopper Resistance in Rice Using Microarray Analysis." *Journal of Tropical Agriculture and Food Science* 44.1 (2016): 49-62. Web. 17 Aug. 2016.
- J.L. Liu, J.F. Yu, J.C. Wu, J.L. Yin, H.N. Gu Physiological responses to *Nilaparvata lugens* in susceptible and resistant rice varieties: Allocation of assimilates between shoots and roots *J Econ Entomol*, (2008), 101 (2): 384–390
- Jie Hu, Cong Xiao, Yuqing He Recent progress on the genetics and molecular breeding of brown planthopper resistance in rice., *Rice* (2016), Volume 9, Number 1, Page 1
- Lakshmi VJ, Krishnaiah NV, Katti G et al., Development of insecticide resistance in rice brown planthopper and whitebacked planthopper in Godavari Delta of Andhra Pradesh. *Indian J. Plant Prot* (2010) 38:35–40
- Li, Jie, Qihong Chen, Yongjun Lin, Tingru Jiang, Gang Wu, and Hongxia Hua. "RNA Interference in *Nilaparvata Lugens* (Homoptera: Delphacidae) Based on DsRNA Ingestion." *Pest. Manag. Sci. Pest Management Science* 67.7 (2011): 852-59. Web.
- Zha, Wenjun, Xinxin Peng, Rongzhi Chen, Bo Du, Lili Zhu, and Guangcun He. "Knockdown of Midgut Genes by DsRNA-Transgenic Plant-Mediated RNA Interference in the Hemipteran Insect *Nilaparvata Lugens*." *PLoS ONE* 6.5 (2011): n. pag. Web.