

Optimization of CAPS assay for selected *Milagrosa* accessions



Cassandra Proctor

Trumansburg, NY

The World Food Prize Foundation

2016 Borlaug Ruan International Internship

International Rice Research Institute (IRRI)

Los Baños, Laguna, Philippines

Table of Contents

	<u>Page Numbers</u>
I. Who I Am	2 - 3
II. My Internship Facility	3
III. My Program	4 - 5
IV. The People	6 - 9
i. I Worked With	6 - 7
ii. At IRRI	7 - 8
iii. Outside of IRRI	8 - 9
V. Goals and Methods	9 - 10
VI. My Responsibilities	10 - 13
VII. Impact on Food Insecurity	13
VIII. Overall Experience	13 - 14
IX. Works Cited	15



Who I Am

My name is Cassandra Proctor and I grew up outside a small town in Central New York called Trumansburg. My parents are small business owners, running an excavation company started by my grandfather. Trumansburg is located near Ithaca New York, home to Cornell University where I am a freshman Plant Science major. I attended the Global Youth Institute during my senior year of high school; in 2015 where I presented my paper on how foreign aid in Nepal has contributed to its food insecurity. Figure 1 shows my fellow classmate and I in front of the capital building in Des Moines just before the start of last year's GYI. Figure 2 is a photo of me on the final day of GYI, at DuPont Pioneer, before my presentation.



Figure 1



Figure 2

My interest in agriculture came from this experience. Before the conference, I knew the bare minimum about poverty and food insecurity. When I heard the word “agriculture” all I thought of was farming but those four days in Iowa made me realize just how diverse the word is. Agriculture encompasses a vast variety of subjects from farming, to policy making, to infrastructure and transportation, to research. The one key factor is feeding people. The area is so much more complicated than we like to believe and it is easy to ignore all the work that goes into bringing food to our table. Whether one will admit it or not, food is the most important aspect of our world. We cannot survive without it! We have the ability to produce enough food to feed everyone, and yet there are children who go to bed hungry, parents who are terrified, not knowing where their next meal will come from, even those who die of starvation. I found myself passionately wanting to do my part in ending the issue, and so I applied to be a Borlaug Ruan Intern.

Growing up in a middle-class family in Central New York, I had never seen nor experienced extreme poverty, but the Borlaug Ruan internship granted me this opportunity. I had already decided that I wished to follow a career path in which I could combat food insecurity but I

wanted to see the problem first hand. I had have seen pictures and watched videos, but something you see with your own two eyes always has a more profound effect.

My Internship Facility

When I learned I had gotten the internship, I was ecstatic. I would spend an entire two months exploring my newfound passion. I waited in anticipation for the email telling me where I would be going. I was excited but shocked when I found out it would be the Philippines. I knew hardly anything about the country. But the shock lasted for only seconds and then I was sitting in front of my computer doing all the research I could on it. The more and more time I spent researching, the more and more excited I became. Since New York high schools do not finish until the end of June, I left later than most interns. In fact, I flew out of the country the morning after my high school graduation. I landed in Manila around 10:00 pm the following day. With exactly a 12-hour time difference from my home, I was exhausted. I remember being a bit disappointed that it was so dark so I was not able to see much of this place that would be my home for eight weeks.

My internship facility, the International Rice Research Institute, was located about an hour and a half drive away, without traffic, from Manila, in a city called Los Baños. The International Rice Research Institute, IRRI for short, was founded in 1959. The center has five main goals : “Reduce poverty through improved and diversified rice-based systems; ensure that rice production is stable and sustainable, does minimal harm to the environment, and can cope with climate change; improve the nutrition and health of poor rice consumers and farmers; provide equitable access to information and knowledge on rice and help develop the next generation of rice scientists; and provide scientists and producers with the genetic information and material they need to develop improved technologies and enhance rice production”. IRRI is located near the University of the Philippines in Los Baños (UPLB), which is one of the best agricultural universities in Southeast Asia. Many of the researchers at IRRI also study at UPLB. In addition, there are a great number of international researchers, creating a unique and interesting dynamic; I could experience parts of cultures from all over the world! Figure 3 shows a photo of IRRI facing the front entrance. Figure 4 is a photo of me standing in front of some of the oldest rice fields at IRRI. The experiment has been going on for years, testing the impact of having three harvests in one year.



Figure 3



Figure 4

My Program

IRRI is divided up into organizational units called divisions. I worked in the Genetics and Biotechnology (GB) Division. Until recently, the GB and Plant Breeding divisions were one and the same. However, due to increased demand for both aspects of research, the two were split up. I was placed in the Plant Molecular Biology Lab (PMBL), run by Dr. Ajay Kohli. I was placed in PMBL because I knew that I wanted to do research on plant genetics. I had an internship during my senior year at the Boyce Thompson Institute for Plant Research and I loved it. I have always been interested in genetics, and the Borlaug Ruan internship allowed me to continue learning more about the field in a setting much closer to the poverty I would be working against.

PMBL mainly focused on two subjects, drought resistance and African rice; I worked on the drought resistance. My lab mentor was a Masters student named Jose Abucay, but everyone called him Joe. Joe's project was on a tetrafunctional protein called OsGLP8-2. Tetrafunctional means it has four functions. OsGLP8-2 has a unique and highly stable structure and it is only upregulated in the spikelets (grains) of rice during development. The four functions all occur at different times during grain development. They include working as a superoxide dismutase (SOD) to catalyze the harmful chemical superoxide into regular oxygen and hydrogen peroxide, an oxalate oxidase (OxO) to produce carbon dioxide and hydrogen peroxide, a serine protease inhibitor which regulates the protease's activity, and a nucleotide generic pyrophosphatase (NGPPase) which is a starch degrading enzyme. Figure 5 is a photo of me working in the PMBL and Figure 6 shows me working at my desk. The green notebook besides me was my official lab notebook. Everyone in the lab was expected to keep it up to date and submit it to Phoenix, Dr. Ajay's secretary, every Monday. Dr. Ajay would review and return them throughout the week. In addition, we submitted over email a summary of the research and results we had gotten that week.

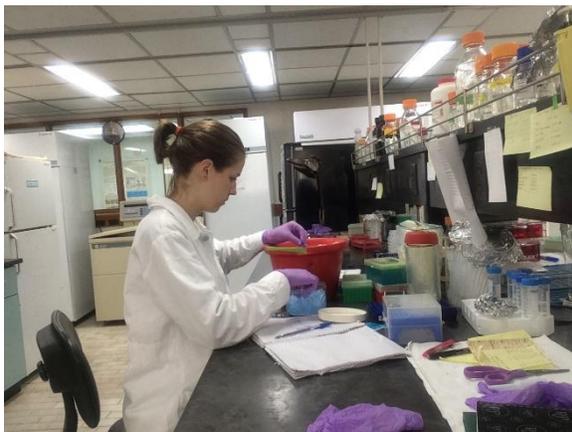


Figure 5

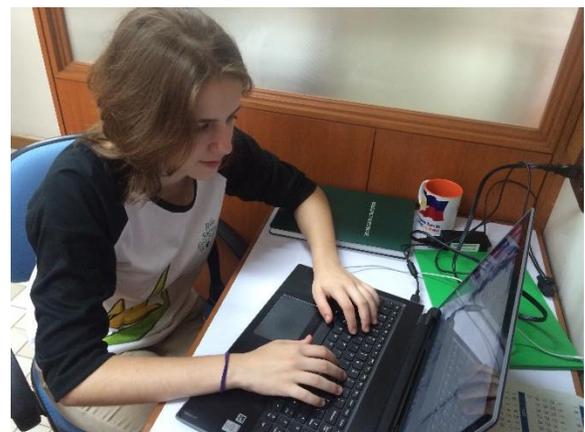


Figure 6

Every month, there would be a lab meeting in which everyone had to present the research they had done during that month. Since everyone in the lab had to present, the meetings usually lasted a day and a half. There was a lab meeting during my first week in the lab, so I just observed, but I participated in the following one. In addition, Dr. Ajay scheduled an additional meeting for one of the lab members who had been sick the previous time, and since it was a week before my departure, he asked me to present an overall summary of the work I had done. Figure 7 shows me presenting at the final meeting. At the end, Dr. Kohli presented me with a gift from the entire lab, shown in Figure 8. The mug was designed by one of the lab members, Beng! On the other side, she had placed everyone's names, seen in Figure 9. It was so touching!



Figure 7



Figure 8



Figure 9

The People I Worked With:

When I arrived at PMBL, there were nine researchers, Dr. Naoki, Jenny, Richard Cely, Beng, Weng, Vincent, Joe and Danny, plus two other interns, Henna and Arshiya, both of whom were from India, almost everyone else was Filipino. Dr. Naoki was the only post-doctorate in the lab. We also had two lab assistants, Helen and Francis, which helped with washing lab supplies, creating stock solutions and generally just aiding the other researchers as needed. Phoenix was Dr. Ajay's secretary and kept all of us on track, making sure we submitted our lab books on time and such. Dr. Ajay himself was a great help to me. He dedicated time to sit with me and insure not only that I understood what I was doing but also to help me work through the errors in my research. A few weeks before I left, a new researcher from Iran joined PMBL named Mahdi. I worked mostly with Joe and one of the other interns, Henna, who was also mentored by Joe. Everyone in the lab was friendly and open to questions. When I struggled with my experiments, I felt comfortable asking any one of them for advice. On my last working day at IRRI, some of the lab invited me out to do karaoke and have dinner. Since they were mostly Filipino, they also took me to try some street food. It was strange but delicious. Figure 10 shows me trying grilled chicken intestines. Figure 11 is a photo of most of the lab members. Starting from the top right it goes, Joe, Jenny, Beng, Weng, Arshiya, Richard and the next row is me, Henna and Vincent.



Figure 10

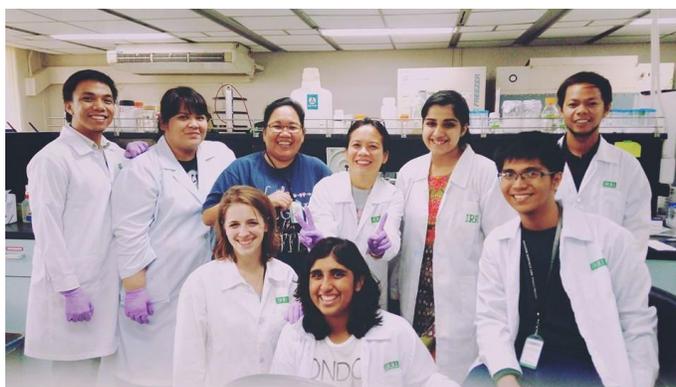


Figure 11

Ate Weng (Ate is a Tagalog term of respect for an older woman) took me to a nearby city with her husband and son to watch a presentation of the traditional dances from all over the Philippines. The city, Tagaytay, also overlooks the Taal Volcano, one of the most active volcanos in the Philippines. The show was in a hotel that overlooked the crater volcano however the weather was terrible and the fog so thick that we could not see the volcano. Since it was raining, instead of hiking into the crater, we visited with some of their family friends who lived in the area. We stayed for supper and I got to try some traditional Filipino dishes. In addition, we spoke about the various issues both of our countries are facing. It was eye opening to learn about how others view the policies and ideals of my country. I found myself looking at the USA with an entirely new perspective. It was also rewarding for me to be able to dissuade some typical stereotypes others held about Americans. The view from the hotel can be seen in Figure 12 and Figure 13 shows one of the traditional dances presented in the show.



Figure 12



Figure 13

At IRRI

In addition to my lab mates, I meant a great many interesting people inside the institute, both foreigners and locals. While there were some other Americans there, I was the youngest and my fair skin and blue eyes made me stand out. As I said before, I grew up in Central New York, so I had never experienced the feeling of being “exotic”. Inside of IRRI, I would walk into the cafeteria and the workers would immediately be pleased. People would come out from the kitchens just to stare. Heads would turn and blatantly stare at me as I got my food. It was a bit nerve-wracking at first, but no one did anything other than stare. In fact, the most uncomfortable thing about the situation was that I felt a bit alienated. No one was intentionally doing so of course, but because they always treated me with special treatment, it was harder to make connections. However, as time passed, I realized it was just part of the culture. Whereas in the USA, we all wish to be tan, in the Philippines most wish they were fair skinned. Where I took for granted blue eyes and was fascinated by the deep, almost black, brown eyes that are so common there, they felt just the opposite. After I reached this understanding, I felt a lot more comfortable and as my internship progressed, my novelty mostly wore off. It was a rather nice feeling to walk into a room and have everyone turn with a smile and say “Ma’am Cassie” with delight. Filipinos are some of the most joyful people I have ever meant. They always seem to go around with a smile on their face and a kind word to say.

In addition to the Filipinos, I made a lot of “foreign” friends as well. Just outside of my dorm there was a soccer field where three days a week, some of the IRRI staff and locals from outside the institute would come and play a match. When they discovered that I played, they allowed me to join in. It was so much fun! I had not brought any cleats with me so after it rained, the field would be a mud pit and I would be covered at the end of the game. In addition, tons of cane toads would hop across the field and we would have to dodge or run over them. Liana, also an American, would also sometimes play. She had graduated from Cornell the year before and she gave me all sorts of advice to help me along my freshman year. Figure 14 is a photo taken on my last day playing soccer with some of who usually participated. Figure 15 shows Liana and I after a particularly muddy game.



Figure 14



Figure 15

In addition to soccer, I made several friends in the dorm as well. Shamik, an intern from Indian became a good friend to me as well as a bit of a mentor. When I was struggling to understand some complicated papers or key concepts in lab, he never hesitated to come to my aid. Even though we came from very different backgrounds and countries, we had a lot in common. It is strange how that happens. In addition to Shamik, I also became good friends with another American named Alex and Henna, from my lab. Shamik actually played soccer with me; he is the man in the white and blue striped shirt in Figure 14.

My roommate, Nhien, was Vietnamese and a member of AFSTRI, and IRRI sponsored program that planned events for the internationals at the institute. She and other higher ups in AFSTRI would alternate weekends in which one of them would cook a dish from their country. I first attended Nhien's where she made a Vietnamese soup and another where I tried Iranian barbeque, as seen in Figure 16. AFSTRI also organized hiking trips and shopping trips to Manila, both of which I attended. Figure 17 shows the view from the Filipino Arts Center located on one of the mountains near IRRI, the destination of the AFSTRI hike.



Figure 16



Figure 17

Outside of IRRI

I did not travel very much outside of IRRI during my internship because lab work took up a lot of my time as well as the researchers who could take me places. However, even from just going out to eat in the restaurants of Los Baños, I found that the happiness and friendliness of Filipinos was mostly the same. There was the typical Southeast Asian laid-backness, so foreign to us Americans. However, I also saw extreme poverty. People living in shacks composed of precariously balanced sheets of metal and wood, as many as seven or eight people crammed together. On the streets of Manila, I walked past several starving people, sleeping under dirty cardboard boxes as if they were blankets. I was followed by two malnourished little girls, no more than 7 or 8 years old, who pulled on my arms and begged for money. I never wanted anything more than to pull out my wallet at that moment but on the crowded streets, it would not have been safe. The people I was there with pulled me along, but I felt sick the rest of the day. I do not think I will ever be able to forget those two dirty little faces. As distressing as the experience was, it was my favorite part of my travels in the Philippines because it was the real reason I had gone. Yes, the lab work was important and I enjoyed it, but I could have stayed in the US to do so. By becoming a Borlaug Ruan intern, I wanted to see poverty first hand. I wanted to see the suffering with my own two eyes to ensure myself that I would never be able to fully put it out of my mind again. Those faces will haunt me but they shall give me the motivation I need to overcome any barriers that present themselves in my own path towards fighting food insecurity.

Goals and Methods

Due to its unique structure and small size, *OsGLP8-2* is highly stable, even under extreme conditions such as desiccation and pH extremes. Because of its stability and importance in the grain development, this gene could potentially replace other proteins labile under stressful conditions and it is therefore important to understand its functions. Its utility in plant response to drought could also be explored due to its response to desiccation of the grain. The project I worked on focused on the effects of mutating the N54 glycosylation site in the protein. Glycosylation is the process in which polysaccharides bind to the protein. Our hypothesis is that by mutating this site, the protein's efficiency will deteriorate and affect the grain structure. Based on previous results we expected that only one of the protein's functions will be affected by this change, and that is the NGPPase activity. Previously, NGPPase activity had only been found in the leaves of barley (Rodriguez-Lopez et al.). Since NGPPase is a starch degrading enzyme, by proving that lack of it causes malformed starch and hence malformed grains, we will add a mechanistic explanation to the hypothesis that starch synthesis and accumulation during grain development is actually an equilibrated process of synthesis and breakdown, with the equilibrium shifted towards synthesis and accumulation. This discovery will further our understanding of rice spikelet development and change our methods of breeding for specific grain sizes and textures.

Our project focuses on exploring natural variants of the gene where N54 site may be naturally mutated. To do this, the 3K rice genotype was searched for accessions that would have naturally mutated *OsGLP8-2* genes. The accession Milagrosa showed data that suggested it had such natural mutants, so 35 different accessions of it were grown and sequenced. Sequencing data

showed eight accessions where *OsGLP8-2* is mutated. These accessions contain single nucleotide polymorphisms (SNPs), which can change an amino acid in the protein sequence. Of the eight, only three showed a SNP in the actual N54 site. The SNP, located at the 335 base pair, changed an A to a G, thus changing the amino acid asparagine (N) to serine (S). This mutation would shut off the N54 site, which should, in theory, affect the NGPPase activity and thus the grain development. Because sequencing data is not always accurate, the existence of the SNP had to be confirmed. In addition, the phenotypes of all the accessions had to be quantified to prove a decrease in fertility and grain structure among the mutants. Assays, tests that show the concentration of specific enzymes in a sample, also had to be run to demonstrate that only the NGPPase function decreased due to the mutation.

My Responsibilities

My first couple of weeks at IRRI, I shadowed Joe in the lab and was taught various techniques such as running assays for NGPPase, SOD and O_xO. However, because of my previous lab experience, I was given the task of proving that the 335 base pair SNP actually existed. I could have cloned the samples again and sent them for sequencing, however, that was expensive. Instead, I used a cleaved amplified polymorphic sequence (CAPS) assay. The first step of the assay was to amplify the *OsGLP8-2* gene, which was done using polymerase chain reaction (PCR). Primers specifically designed for the gene bind to the ends of the gene and through a series of temperature changes, again based upon the primers, the DNA is cut at the gene which is then replicated multiple times. The result is a sample with a much higher concentration of the DNA of the wanted gene. After the amplification, I had to confirm that the gene was indeed amplified, using gel electrophoresis. We knew that the gene was 840 base pairs long. By running the samples on the gel, the DNA travels down the gel based on its size. A ladder containing DNA fragments of known sizes is included so that after your samples are run, you can tell if your samples are the needed size. After, I had to digest the amplified samples using a restriction enzyme. Restriction enzymes have sites with highly specific sequences to which they bind, cutting the DNA. Restriction enzymes are selected so that there should be a size difference in the fragments between the wildtype and mutated samples. Figure 18 shows the positive results of a PCR amplification of *OsGLP8-2*. Figure 19 is a photo of me taking a photo of a gel using the special UV machine.

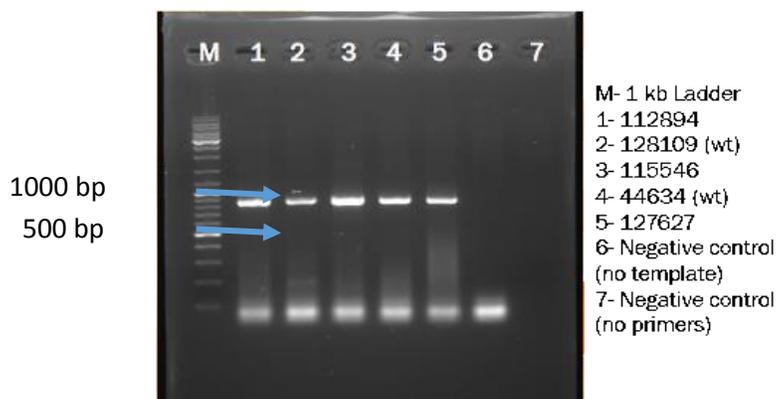


Figure 18 (*wt = wild type samples)

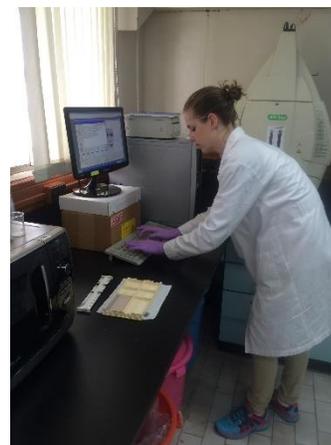


Figure 19

When I was given the project, Joe had already found an enzyme to use for the digestion; *TspRI*. The mutated samples actually had two SNP mutations, the one we desired in the N54 site as well as one occurring right after. The *TspRI* site was formed by the second mutation. Figure 20 shows the theoretical fragment sizes in both the wildtype and the mutant samples. Figure 21 shows my actual results which were inconclusive.

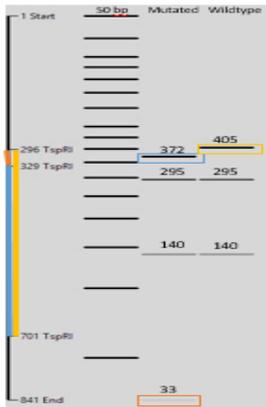


Figure 20

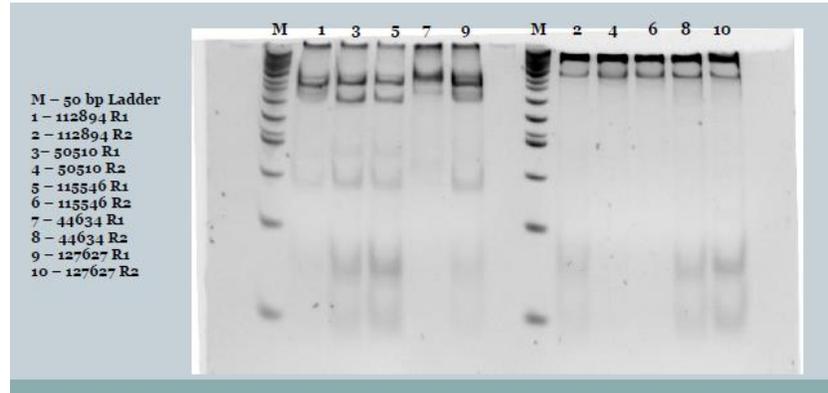


Figure 21

The problem with *TspRI* is that the size difference between the DNA fragments was only 33 bp, which is very hard to see on a gel. After adjusting the conditions for the restriction, I still was not getting the results I needed so I used a free online program called Restriction Mapper to find an enzyme that would make it easier to see a size difference in the bands. I ended up finding two enzymes; *HinPII* and *HhaI*. Both enzymes only had a cut site caused by the SNP mutations therefore the wildtype samples would show only the 840 band. However, when I tested both, the results showed no fragmentation in any of the samples; all samples showed only the 840 bands.

After speaking with Joe, Dr. Ajay and a few other lab members, I had a list of reasons why the digestion was not working: there could be contamination that was impeding the restriction enzyme activity, the concentration of my amplified DNA samples could be too low, or the amplified DNA could also be contaminated. The final possibility was that the mutation did not exist. However, I spent weeks troubleshooting both the amplification and the digestion. I attempted several different PCR techniques, including leaf disk PCR which involves taking a leaf sample and using it directly in the PCR instead of extracting the DNA first. After amplification, instead of using the samples directly, I tried gel purification. This involved excising the bands of the amplified DNA and running a protocol that removed all the contaminants, leaving just the DNA. Figures 22 and 23 show me excising the bands from a gel, which must be done in UV light.



Figure 22

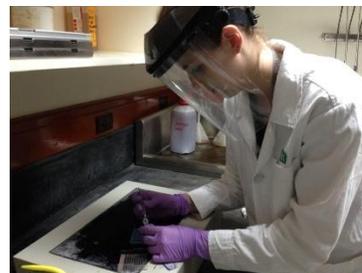


Figure 23

Despite my efforts, I was unable to produce any conclusive results that supported the existence of the SNP mutation before my departure. Joe or someone in the lab will either attempt to optimize the CAPS assay or clone and send the samples off for sequencing.

In addition to my own project, I also helped briefly in the quantification of fertility in the 35 accessions of Milagrosa. Panicles from each of the rice plants were collected and mounted on pieces of paper as seen in Figure 24. After they were mounted, the number of filled and unfilled spikelets had to be counted, as well as the number of primary and secondary branching. The quantification was not yet complete when I left IRRI, however I received the final data from Joe, which showed no significant decrease in fertility among the mutated samples, as shown in Figure 25.



- | • Mutant | • Wild Type | | |
|----------|-------------|---------|----------|
| • 5159 | • 5826 | • 97821 | • 109115 |
| • 44633 | • 26967 | • 97822 | • 109116 |
| • 44636 | • 38807 | • 97895 | • 109117 |
| • 97899 | • 44634 | • 97896 | • 109118 |
| • 97903 | • 44635 | • 97897 | • 109133 |
| • 112894 | • 50510 | • 97898 | • 111055 |
| • 115546 | • 53182 | • 97900 | • 112893 |
| • 127627 | • 84132 | • 97901 | • 115033 |
| | • 97820 | • 97902 | • 128109 |

Figure 24

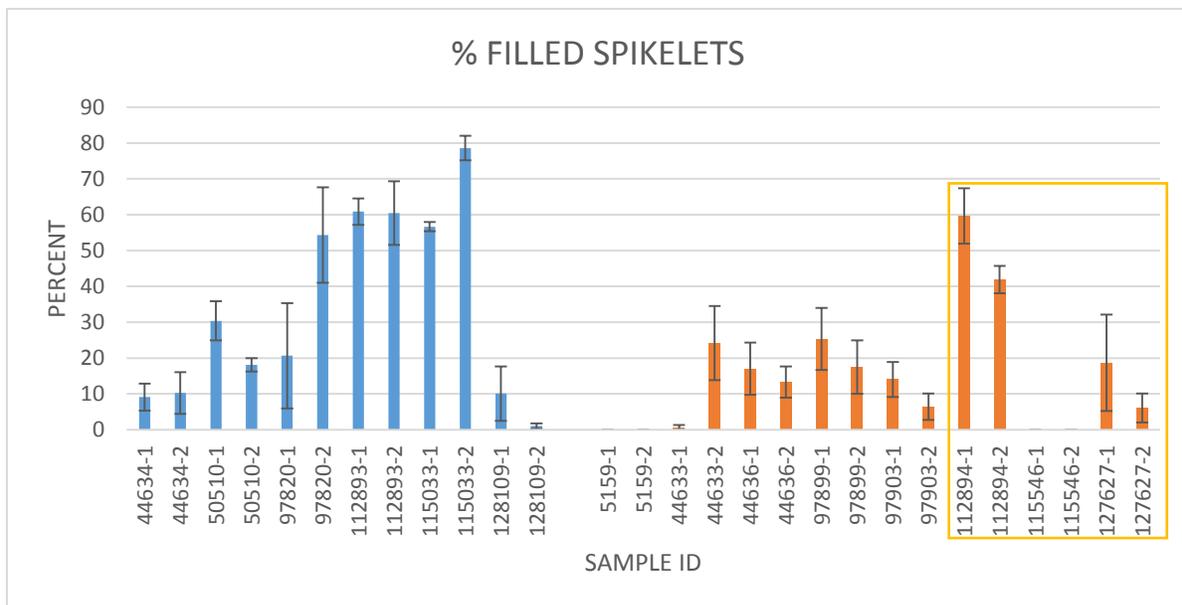


Figure 25

Our hypothesis remains unproven at this point in time. The data has shown that the mutation in the N54 site may not actually exist in the chosen variants which would then explain why there is no decrease in fertility. However, if it is found that the SNPs do exist, then our hypothesis will be proven to be false. Only further research into the matter will provide the correct conclusion.

Impact on Food Security

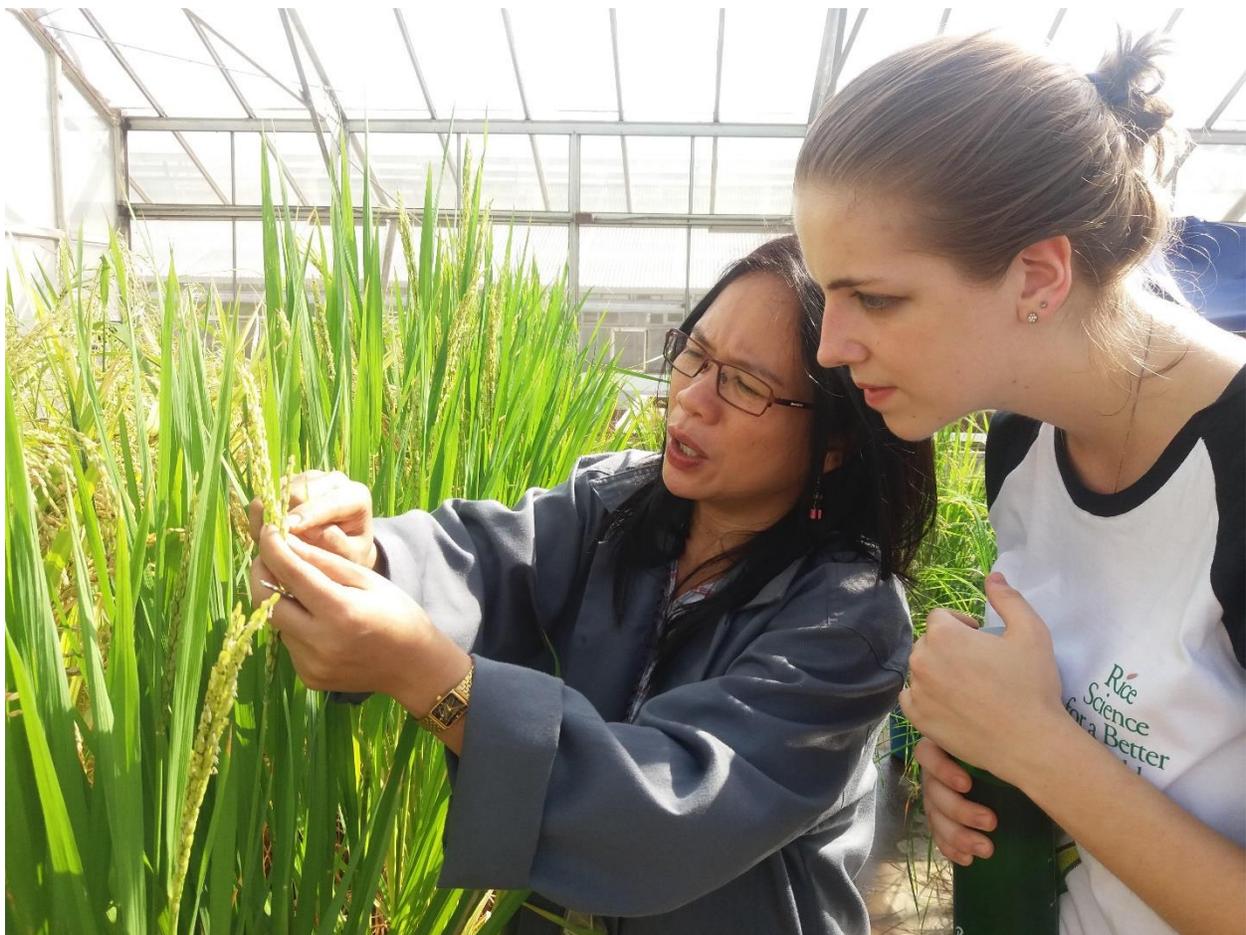
Rice is one of the most important staple crops in the world; “In Asia, where 90% of rice is consumed, ensuring there is enough affordable rice for everyone, or rice security, is equivalent to food security. In Africa and Latin America, rice is becoming a more important staple too”. (Our Impact). With the increasing world population, it is becoming more and more important to insure we can feed everyone. To do this, we must further our understanding of how rice and other staple reproduce and deal with stressful situations such as drought or flooding. With this understanding, we can breed more successful plants, better adapted to the environment available in the area. Though my research did not provide any conclusive results, it still has the potential to aid in food security. Whether we prove NGPPase is behind the development of rice grains or not, we still know that some part of the OsGLP8-2 protein is involved in grain development. With it being as stable as it is, it has the potential to increase drought resistance and even perhaps pathogen resistance. That is the beauty of research, even if you do not receive the results you hoped for, or any results at all, you always learn something and that information opens doors to new ideas!

All the troubleshooting I did over the summer taught me that it does not necessarily matter if you fail, if fact sometimes failing at something increases your understanding. If I had not had to go back and figure out where my experiments went wrong, then I would not have learned exactly what I had been doing. It is easy to blindly follow directions given to you in a lab, especially if you are getting great results. But by stepping back and trying to understand, you can find answers. Maybe they were not the answers you were hoping for, but that is how the world works. When we are faced with a problem, instead of blindly going through the motions dictated by other people, we must educate ourselves and we must think for ourselves. Food security cannot be reached unless we make an active effort to understand the problems, not only those on the surface but the underlying ones as well. If one solution does not work, then it is our duty to figure out why and fix it or even come up with a totally different approach. We cannot just give up—we must trouble shoot!

Overall Experience

I cannot thank the World Food Prize Foundation enough for this amazing opportunity. My Borlaug Ruan internship fostered my passion for fighting food security and cemented in me the knowledge that I wish to dedicate my career to solving the problem. People like those little girls on the streets of Manila or the starving men sleeping in cardboard boxes have a right to food, an unalienable right. I discovered this passion at the 2015 GYI, but it seemed like a naïve and frankly unoriginal idea. “I want to feed the world” seemed like a phrase people used to make

themselves look good on a college application. But, after seeing poverty on that scale for the first time, I realized that the phrase “I want to feed the world” is one of the most noble goals one can fight for! I am optimistic now that I can in fact make a difference. Language, cultural and economic barriers may be hard to overcome but it is not impossible, and neither is ending food insecurity. In addition, I was able to create strong and diverse friendships that I know will last a lifetime. I envy and yet am also so excited for next year’s Borlaug Ruan interns. They will be able to have an illuminating experience that I am positive will change their world, as it did mine. It will kindle the passion that begins to grow in them at the GYI and it will explode out of them, out into the world and start to change it. So I will say it again, thank you so much for this amazing opportunity! It will shape the remainder of my life, and hopefully many young people after me.



Works Cited

Our Impact - Increasing food security. Retrieved November 10, 2016, from <http://irri.org/our-impact/increase-food-security>

Our Mission. Retrieved November 10, 2016, from <http://irri.org/about-us/our-mission>

Rodríguez-López et al. (2001). Two isoforms of a nucleotide-sugar pyrophosphatase/phosphodiesterase from barley leaves (*Hordeum vulgare*L.) are distinct oligomers of HvGLP1, a germin-like protein. *FEBS Letters*, 490(1-2), 44-48. doi:10.1016/s0014-5793(01)02135-4