



Borlaug-Ruan Internship Report

Analysis of Blast Resistance of Hybrid Rice Parents by Means of PCR and Pathogenicity Assays

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Introduction

Growing up in a middle-sized city in the heart of the American south, I have never been immersed in a completely agricultural setting. While my hometown, College Station, Texas, was surrounded by farms and ranches, it was never really a part of my life, but remained something familiar to me. However, I have been passionate about food security, a major aspect of The World Food Prize's mission, for as long as I can remember. According to the most recent FAO report on hunger there are currently 868 million undernourished people globally, making up 12 percent of the world's population (Hunger 1). My passion for increasing food security worldwide and my interest in the biosciences were the driving forces in my decision to participate in the World Food Prize's Youth Institute when my high school physics teacher, Michelle Jedlicka suggested it to me. After learning more about the program and the possibility of a Borlaug-Ruan Internship, I was sold!

I presented my paper about livestock diseases in Ethiopia in September of 2011 at the Texas Youth Institute where I earned a spot at the Global Youth Institute in Des Moines, Iowa. I had an amazing experience at the Global Youth Institute. From meeting former African Presidents to talking with leading researchers in the fields of agronomy, there was never a dull moment. The talks were inspiring and informative, and I remember one in particular by the former director of the World Food Programme, Josette Sheeran, in which she gave the students the advice to "be impatient, be unreasonable, be demanding, and go for it!" It was these words that helped me to see the ability I possessed to really make a difference in the fight for food security. After my time at the Global Youth Institute I knew that the Borlaug-Ruan Internship was something I had to do.

Applying for the internship I had no idea what to expect, and from what I had read online, I was in for quite an experience to say the least. I was ecstatic when I heard that I received one of the Borlaug-Ruan Internships, and was even more excited when I found out that I would be working in Changsha, China at the Chinese National Hybrid Rice Research and Development Center (CNHRRDC). Spending a summer abroad and alone was a lot to wrap my head around at first, but the closer I got to leaving for my internship the more excited I was. By the time I boarded the plane in Houston to leave I was overflowing with excitement about working in the lab, meeting new people, and exploring the culture of China.

Chinese National Hybrid Rice Research and Development Center



The Main Research Building of CNHRRDC

The Chinese National Hybrid Rice Research and Development Center (CNHRRDC) is a national research center located in the Furlong district of Changsha, China. It was established in 1995 as an extension of a preexisting institution, the Hunan

Hybrid Rice Research Center (HHRRC), which was founded by 2004 World Food Prize Laureate Yuan Longping in 1984 and is the first domestic and foreign hybrid rice professional research institution (About 1). Yuan Longping is known as the father of hybrid rice from his work in the early 1970s developing commercial breeding systems for hybrid rice (Yuan 1). CNHRRDC and its staff of 225 now focus on developing hybrid rice with high yields, high resistance, and high quality to contribute to increasing global food security. Since the founding of hybrid rice research Professor Yuan and his teams have

developed two different breeding methods and have increased yields of hybrid rice to over 13 tons per hectare. In addition to the high-yielding varieties research, CNHRRDC also has a large effort to maintain their existing hybrid lines by developing methods to keep them safe from attacks by bacteria, pests, viruses, and fungi, as well as other natural disasters. As hybrid varieties become higher yielding, their necessity for protection increases due to their newfound economic fortitude. They also have increased hybrid rice availability to farmers in China to the point that half of all rice grown in China is a hybrid variety.

Not only does CNHRRDC do excellent research with developing new varieties of hybrid rice, they also have a program where they teach teams from countries where hybrid rice is being introduced the cultivation techniques they will need to successfully increase their rice production. This is often a three month program where the teams live on site at CNHRRDC and attend daily lectures and demonstrations to learn everything they need to know in order to return to their home countries and teach others how to grow hybrid rice. While I was on site at CNHRRDC I met many people involved in this program from countries including, Indonesia, East Amos, Chad, Angola, and Zimbabwe.

My Mentor



Dr. Xing and I at KTV

My mentor throughout my Borlaug-Ruan Internship was Dr. Xing Junjie. He is a researcher at CNHRRDC working with blast resistance in hybrid rice. He is from a northern province in China but moved to the south for school and remained to do his research. For a year and a half prior to my internship, Dr. Xing lived in Arkansas learning new research techniques for dealing with

rice blast. I worked primarily as Dr. Xing's lab assistant/technician for the duration of my internship, but he also introduced me to many other members of the research staff at CNHRRDC and I was able to participate in their experiments as well. Dr. Xing was also my guide around Changsha and the Hunan Province. Together, he and his coworker Dr. Song took me to various restaurants and sightseeing locations throughout my eight weeks in China.

My Project: Genetic Resistance to Rice Blast

I was assigned the task of cataloging blast resistance in multiple varieties of rice from the Hunan province in order to aid in future breeding efforts to allow blast resistance to be part of the hybrid's heterosis. Rice blast is a devastating disease caused by the



Blast Legions on Infected Leaves

filamentous ascomycete fungus *Magnaporthe oryzae*. This disease occurs in 85 countries worldwide, and each year destroys enough rice to feed more than 60 million people,

making it one of the most economically devastating diseases in the world (Suparyono 1). The physical evidence of a blast infection is legions that form where the fungus is growing on the plant. These legions can in turn infect the surrounding plants. Rice blast forms most optimally in high humidity and mild temperatures, similar to the early rainy part of the growing season. Natural resistance to rice blast does exist and is modelled by a "gene-for-gene" relationship. This interaction involves resistance (*R*) genes in the host's genome that correspond to an avirulence (*AVR*) gene in the fungal genome. Both genes must be present and unaltered for resistance to occur, and if one or both are missing or dysfunctional, the plant will be susceptible to blast.

Dr. Xing's work with rice blast originated from his time in Arkansas where he began most of the experiments I was assisting him with. My work involved using the polymerase chain reaction (PCR) to identify resistance genes in various rice varieties to form a database for blast resistance. This will eventually lead to the incorporation of rice blast resistance as a criterion for parental line selection in hybrid rice breeding. I also assisted with inoculating young rice plants with *M. oryzae* spores solution and recording the infection result to judge their disease reactions. This helped us to verify the results of the PCR screening and to determine what if any affects multiple resistance genes in one plant had on blast resistance.

Materials and Methods

Rice varieties were grown in 4 by 9 pot trays with each pot holding a different variety. The plants were grown under white fluorescence for 3-5 days and then were transferred to natural conditions until they were ten days old. At that point, leaves were collected from each variety for DNA extraction. After the leaves were collected the remaining plants were subjected to a standard pathogenicity assay. We inoculated the seedlings with *M. oryzae* isolates that were growing under blue and white fluorescence at 25°C for seven to ten days by spraying a conidial suspension of the fungus that had been adjusted to 2×10^{-5} conidia/mL with an airbrush sprayer onto the seedlings. The inoculated plants were then kept in a black plastic bag for twenty four hours at 25°C. The next day the inoculated plants were



Inoculating the Rice Seedlings

removed from the bags and transferred to a humidity chamber with fluorescent lighting for

seven days. After the allotted time had passed, we rated the plants' disease reactions on a scale of 0-9 with 0-3 being resistant and 4-9 being susceptible. We repeated each variety twice for a total of three trials with each of our three blast race isolates taken from the Hunan Province. The isolates names were 318-2, 195-2-2, and 193-1-1.

For DNA extraction we homogenized our samples with liquid nitrogen and used the CTAB extraction method with chloroform. Purified DNA samples were stored at 5°C for



Performing the Final Steps of a DNA Extraction

the duration of the project. PCR was performed with a set of primers (Table1) designed to amplify various resistance genes found in rice varieties in Hunan.

Standard manufacturer suggested PCR protocols were used to amplify the resistance genes. Each screening

was repeated twice with all varieties, and the varieties

that had no amplification product or a weak band were screened again to verify the results.

Table 1. List of Primers used in PCR screening of rice varieties to amplify resistance genes Primers were stored at -20°C and were used at a concentration of 10µM

Primer	Target Gene	Sequence (5' to 3')	TM (°C)	Fragment Size (bp)
JJ-11	<i>Pi-ta</i>	CTACCAACAAGTTCATCAAA	54.65	1042bp
JJ-12		AGCAGGTTATAAGCTAGGCC	57.86	
JJ-15	<i>Pib</i>	GAACAATGCCCAAAGTTGAGA	56.06	365bp
JJ-16		GGGTCCACATGTCAGTGAGC	61.90	
JJ-19	<i>Pi9</i>	CCGGACTAAGTACTGGCTTCGATA	61.97	500bp
JJ-20		CCCAATCTCCAATGACCCATAAC	60.17	
JJ-25	<i>Pi25</i>	GGACAGAGCAGGAAGTTCAGATG	61.95	1800bp
JJ-26		TTCTGGGTCTCAGGCGTACT	59.85	

PCR results were visualized with agarose gel electrophoresis and imaged with ethidium bromide and a UV transilluminator. Positive and negative controls were included

in each trial to insure validity of results.

Results

I was also able to do complete PCR screening on varieties X13, X21-X87, X174, and X175 for a total of 66 varieties. I also assisted in the inoculation of 58 varieties. I was unsuccessful at amplifying any *Pi25* genes from the varieties that I screened. I found three varieties that possessed three distinct resistance genes. These varieties were X23, X38, and X55. I also found nine additional varieties that had two distinct resistance genes. The inoculation results show that plants with a greater number of resistance genes have a higher chance of being resistant to blast infection than those plants with fewer or no resistance genes. For example Variety X55 had an average disease reaction of 1, meaning it was very resistant. This can be modeled by the average disease reactions grouped by the number of resistance genes present in the host. For varieties with three identified resistance genes the average reaction was 1. For varieties with two identified resistance genes the average reaction was 1.675. For varieties with only one identified gene the average reaction was 2.9, and for varieties with no resistance genes identified the average reaction was 2.67. While the last two data points seem contradicting to the trend it is important to note that the resistance genes I identified in this study are only three of many blast resistance genes. There are quite possibly other resistance genes present in many of these varieties interacting with the blast isolates we used.

Discussion

Based on this evidence alone I would suggest that the best candidates for blast resistant parental lines for hybrid rice production are X28, X33, and X55 because they possess three

separate resistance genes. This will give them a great advantage over varieties with only one or two resistance genes because they will be able to resist a broader range of blast isolates because they can interact with more avirulence genes. However, there are still more factors to consider when selecting a parental line, including grain quality, high yield, high salinity tolerance, and desired morphology. It is only when all of these factors are present and strong in a rice variety that a great parental line is developed. Throughout my time working in the lab I faced many challenges, one of the largest being the language barrier. Many times I had to rephrase my questions multiple times in order to help others understand. After some time and many laborious exchanges I finally developed a method of questioning that was successful where I stuck to simple phrases using almost completely scientific terms. Another challenge that I faced was contamination during PCR. A few times my results would show that every sample contained the gene of interest, but the negative control would indicate that contamination occurred. This was very frustrating because it caused me to have to repeat an entire PCR which took upwards of 4 hours to perform in the first place. Every time there was a contamination I had to replace the water I was using, and if that didn't fix the problem, I had to replace the primers, and as a last resort the Taq Mix. While these contaminations happened more often than I would have liked, I eventually overcame them and finished all of the PCR screening in time.

Food Security

Rice blast currently destroys enough rice to feed more than 60 million people annually. While this number seems large, it may help to know that in 2010 the FAO estimated that there were 925 million hungry people in the world (Hunger 1). While this number is far

greater than the 60 million people that would have access to food if not for rice blast, eradicating rice blast still means that 60 million people will be fed. With the implementation of blast resistance in parental line selection criteria for hybrid rice breeding this becomes possible. We will be able to take a stand against this devastating disease once and for all and with the constant improvements in hybrid rice high yielding technology the 60 million new people that will be fed after the control of rice blast could increase to a far greater number.

My Experience

My time in China was an absolute blessing. I met so many interesting people and learned so much about their rich cultural history. I got to meet and talk with Professor Yuan a few times during my internship and sit in on a few of his delegations with visiting professionals of hybrid rice technology. Professor Yuan's knowledge of hybrid rice technologies far exceeded any and all of my expectations and every meeting I was able to attend held a new and exciting experience. Apart from working in the lab, I was able to help Dr. Xing with the revision of a paper he was trying to publish in an American journal since his reviewer suggested that he have a native English speaker revise it. We worked together for almost three entire days going through the comments from the reviewers with me trying to explain to him what everything meant and why I made the changes that I did. It really taught me how difficult English is for non-native speakers and even helped me learn some new grammar rules that I just adhered to naturally. The food was difficult to adjust to at first because everything was spicy and I was eating unfamiliar pieces of meat frequently. So, during my first few weeks I would often be told "eat more." This became a phrase that I

heard every time I thought I was done with a meal. After a few weeks Dr. Xing and I went out for dinner on a Saturday and ordered many different dishes that I had discovered I liked and I finally ate enough to prevent the ever occurring remark. The second weekend I was in China was the dragon boat festival, where men race long rowboats with dragons carved on them and the people eat a traditional snack called Zongzi, a pyramid shaped cluster of rice and meat wrapped in leaves.



The Dragon Boat Festival

Throughout my internship, I got to know Xingyu Zhang, Dr. Xin's son, who had just returned from spending his senior year in Virginia. Spending time with Xingyu was nice because I could talk with him about my excitement about going off to college and things that were happening back home in the United States, and he could relate.

From the festivals to the food, every experience was eye-opening and full of lessons to learn. While in America we often attribute success to how big our house is and how fast our cars go, we are missing out on the main point of life: happiness. I found just that in China. Everywhere I looked there were people smiling and having a good time. They are not constantly worried about trying to impress everyone around them, they just live their lives like they see fit. The balance between work and relaxation is very important. In the afternoons they took a break after lunch until 2:30 or 3:00. At first this was very hard to get accustomed to and I found myself antsy the entire time waiting to get back to work. But after a few weeks I began to appreciate the time to rest during the day and soon found

myself more energized and focused in the afternoons. I even had trouble adjusting to not having my afternoon nap time when I returned home and back to school. This is just one way I have learned to slow down my life and just enjoy my time while I've been in China.

While I was staying at the center there was a volleyball tournament that I got to



Playing Volleyball with
Professor Yuan

participate in. I played on Dr. Song's team and while we never won an entire match, we had a great time. Professor Yuan even played on our team for a couple of games and gave us all a lesson in wellness as he dominated the court. I enjoyed learning about

many Chinese characteristics on my internship as well

as visiting many historic places in Hunan Province. The most interesting place I visited was Zhangjiajie National Forest Park. It was breathtaking and awe-inspiring in every way while filled with a rich history of China's past. Everyone should have a chance to see something as beautiful as Zhangjiajie sometime in their lifetime. The views from the top of the park are well worth the 3,974 stairs it takes to get back to the base.



A Beautiful View at Zhangjiajie

I am extremely grateful for everyone that worked with me during my time at CNHRRDC; they worked hard to make sure that I felt welcome and didn't have any trouble. It seemed that after a few weeks everyone knew my name and I was greeted in the halls with "Hello Peter" and "Good Morning Peter" on a regular basis. This was a huge comfort for me in my daily routine because I did not frequently hear

English unless I was speaking. I cannot thank everyone at CNHRRDC enough for giving me this amazing opportunity and working so hard to help me while I was here. I have special thanks for Dr. Xing who graciously gave up his time to mentor me during my stay. He was extremely helpful and was always easy to work with. I enjoyed the time we spent together talking about topics ranging from American basketball to Japanese foreign policy. He helped me reach my full potential as a Borlaug-Ruan Intern and a citizen of the world and for that I am truly grateful. This summer has been some of the best months of my life and I am sure it will continue to impact me throughout the rest of my life.

Appendix I: Protocols

DNA Extraction Protocol

1. Homognize leaves with liquid nitrogen for 2 minutes in 2ml microcentrifuge tubes using a grinder.
2. Add 700µl of CTAB (preheated to 65°C)
3. Heat samples a 65 for 30 minutes inverting to mix every five minutes
4. ***UNDER THE FUME HOOD*** Add 500µl of Chloroform:isoaml alcohol (24:1) solution and invert to mix
5. Centrifuge for 10 minutes at 4°C and 13200rpm
6. ***UNDER THE FUME HOOD*** Transfer 400µl of the supernatant to a new 1.2ml microcentrifuge tube and add 450µl of Isopropyl Alcohol and invert to mix
7. Store tubes at -20°C for 1.5 hours to precipitate DNA
8. Centrifuge for 10 minutes at 4°C and 13200rpm and discard supernatant
9. Add 70% Ethanol to wash precipitate then pour out tubes
10. Dry precipitate at 37°C for ten minutes
11. Add 100µl DNase and RNase free water and vortex to resuspend pellet.
12. Extraction quality can be determined through electrophoresis.

PCR Protocol

1. Add the following reagents in the given order to a 1.5µl microcentrifuge tube to create a reaction mix multiplying the given volumes by the total number of samples + 2 (1 for a negative control and another to account for any pipetting errors)

PCR Reaction	1 Reaction
2X Taq Mix	12.5µl
Forward Primer (10µM)	1µl

Reverse Primer (10 μ M)	1 μ l
Water	10 μ l

2. Add 24.5 μ l of the reaction mix to each being used in a 96 well PCR plate.
3. Add 0.5 μ l of DNA from each variety to its respective well being careful not to contaminate any samples (make sure to change pipette tips between each sample)
4. Place a silicone cover on the well plate and make sure it seals on every well.
5. Insert the well plate and insert into the thermocycler on the following protocol

Cycle	Temperature	Duration (min)
Initial Denaturation	95°C	2:00
Denaturation	94°C	0:30
Annealing	Dependent on Primers	0:30
Extension	72°C	1:00/2kbp
Final Extension	72°C	10:00
Cooling	4°C	∞

6. After reaction is completed store the products at 4°C

Gel Electrophoresis Protocol

1. Weigh out 1.000g agarose powder and add it to a 250ml flask
2. Add 100ml of 1x TAE buffer to the flask and swirl to dissolve
3. Microwave the solution until it becomes clear indicating that the agarose is fully dissolved
4. Pour solution into the gel mold and wait approximately 30 minutes for the gel to set.
5. Remove the gel carriage from the well with the gel inside it and place in the PCR chamber
6. Fill the chamber with 1x TAE buffer until the gel is completely submerged
7. Load each sample into a different well in the gel making sure to change pipette tips between each sample.
8. In the first well of each row add 3 μ l of 1kb DNA Ladder
9. Run the gel at 85 volts for 30min (Pib/Pi9) or 50min (Pita/Pi25)
10. ***WEARING GLOVES*** Remove gel from carriage and submerge in ethidium bromide solution for five minutes for staining
11. Remove the gel from the ethidium bromide solution and rinse with water
12. Place gel on the transilluminator for imaging
13. Using the controls on the computer adjust the camera settings until the picture of the gel is clear
14. Export the picture as a .jpg file and save to the experiment folder
15. Discard gel and gloves in their respective ethidium bromide waste receptacles

Inoculation Protocol

1. Add 0.2% gelatin solution to a Petri dish of *M. oryzae* and scrape the fungus to suspend the conidia in the solution
2. Filter the suspension with three sheets of cheese cloth to produce the appropriate conidial suspension

3. Using a hemecytometer and a light microscope adjust the conidial concentration 2 X 10⁻⁵ conidia/mL
4. Pour the suspension into an airbrush sprayer and connect it to an air compressor
5. Place the trays of rice seedlings into large trash bags and spray the suspension on them to sufficiently coat all of the leaves
6. Quickly tie the bag shut and tape the top to the wall to keep the bag upright
7. Let the bag sit for 24 hours to complete the inoculation process
8. Open bag and move the plants under a florescent light and keep them under high humidity conditions for seven days.
9. Rate the disease reactions of a scale of 0-9 with 0 being completely resistant and 9 being completely susceptible

Appendix II: Data

Inoculation Results

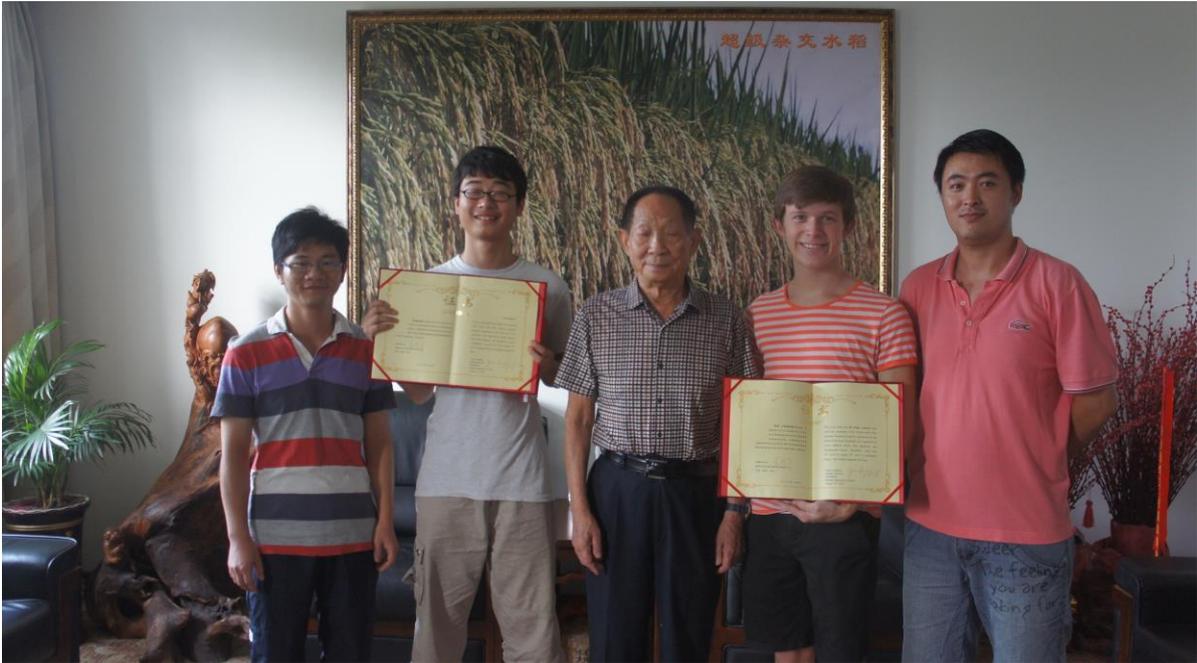
Variety	Average Disease Reaction	X59	1.80	X80	2.13
X41	5.33	X60	5.50	X81	0.75
X42	2.33	X61	1.75	X82	2.25
X43	2.75	X62	2.25	X83	0.00
X44	2.80	X63	3.00	X84	2.38
X45	3.50	X64	3.00	X85	3.13
X46	2.25	X65	0.25	X86	0.38
X47	7.00	X66	3.13	X87	1.88
X48	2.00	X67	1.50	X88	2.00
X49	1.25	X68	0.50	X89	0.75
X50	3.60	X69	5.13	X90	9.00
X51	6.20	X70	4.75	X91	3.50
X52	1.40	X71	2.75	X92	6.00
X53	8.33	X72	3.38	X93	5.00
X54	7.66	X73	3.00	X94	1.66
X55	1.00	X74	0.00	X95	0.00
X56	1.80	X75	1.25	X96	5.00
X57	0.60	X76	0.00	X97	1.75
X58	0.80	X77	1.13	X98	1.75
		X78	1.25		
		X79	0.25		

PCR Screening Results

Variety	Resistance Genes Present					
		X41		Pi9	X64	Pi9
		X42			X65	Pi9
X13		X43	Pita		X66	
X21	Pib Pi9	X44			X67	
X22	Pita Pib	X45			X68	
X23		X46	Pita		X69	Pi9
X24	Pita	X47		Pi9	X70	Pi9
X25		X48	Pita		X71	Pita Pib
X26	Pita Pib	X49			X72	Pib
X27	Pita Pib	X50			X73	
X28	Pita Pib Pi9	X51		Pi9	X75	Pita
X29	Pita Pib	X52	Pita		X76	
X30	Pita	X53		Pi9	X77	
X31		X54			X78	
X32	Pita Pib	X55	Pita Pib	Pi9	X79	
X33	Pita Pib Pi9	X56	Pita		X80	
X35		X57		Pib Pi9	X81	Pita
X36		X58		Pib	X82	
X37		X59	Pita		X84	
X38		X60			X85	
X39	Pita Pib	X61		Pi9	X86	Pita
X174		X62			X87	
X175	Pib	X63				

Appendix III: Pictures





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