Breathtaking Brazil

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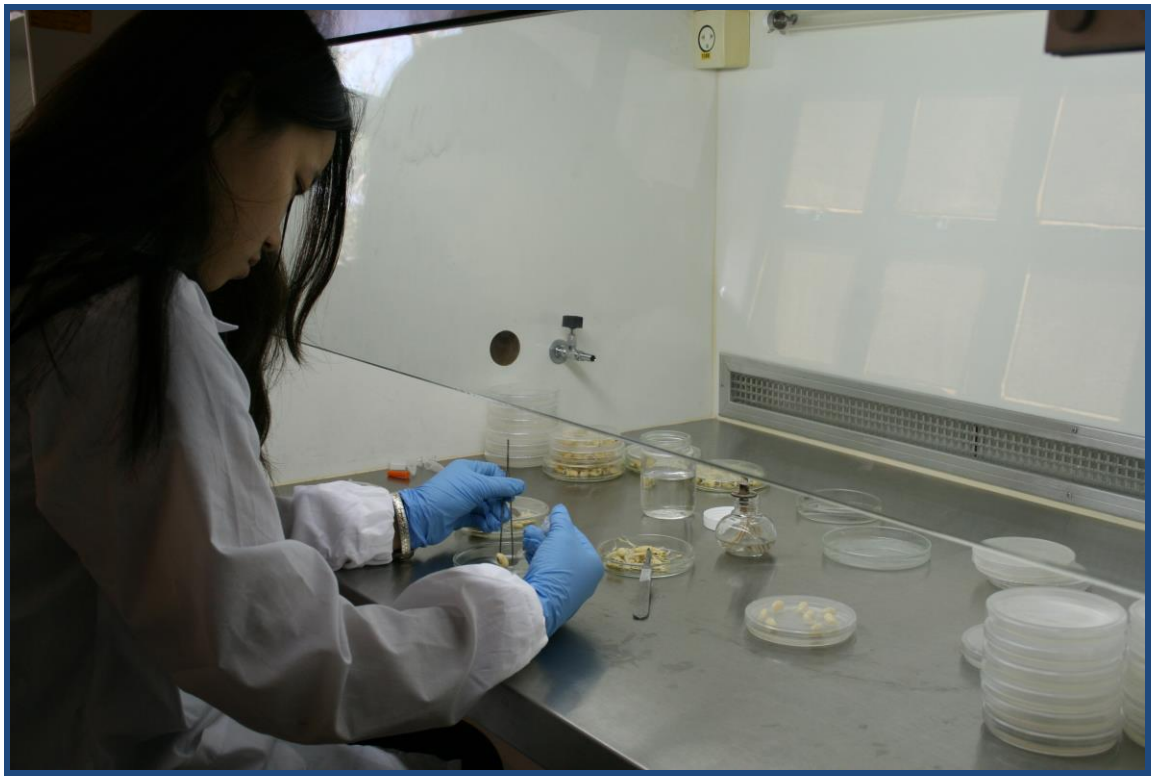

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Lastly, I thank God for always being there for me.

WEEKEND TRIP: FOZ DO IGUACU

I am in a dream, a trance.

I see, hear, and feel the formidable yet elegant rush of the water. It is the epitome of the amalgamation of tranquility and superiority. The billowing waves crashes against the mossy rocks that are ideally placed in the river and diverge the water perfectly. The varying shades and movement of the tenor of the water seems to convey a rhythm, a message. My eyes shift up to the falls. Although the sight is blurry from the rising mist, the sound and the feeling of drizzle on my cheeks and lips are limpid. The perpetual flood that rushes down is insurmountable. The cherry on top of the ice cream sundae is the perfect arch of the light rainbow; the hue is so pale as if it does not exist at all, but it is very much present – resting and floating in midair. The water, the greenery, the natural landscape and the sanguine blue sky complete the picture-perfect scene. I listen to the cadence of the surroundings for the consummate view. Smiling slightly, I close my eyes, trying to capture this moment for eternity. This glorious sight enamors and awes me and I fall in love with Brazil.

My weekend trip to Foz do Iguaçu was a memorable adventure. I was able to fully absorb the beauty of nature and the natural landscapes of this grand country. Brazil took me by surprise; the contrast of a city with European-like streets, loud traffic, swarms of people to a marvelous and breath-taking landmark. Brazil was a land of enigma and splendor. It was breathtaking.

BACKGROUND AND INVOLVEMENT

Childhood and Personal Background

I was born in a Huainan, a developing city of 2 million in China. Despite the dense and growing population, my city was not considered wealthy. Flashing back to my childhood, I can still see the rugged and cracked sidewalks, the vendors crowding either sides of the street, the overwhelming number of people and bicycles that traversed the crosswalks, the impatient cars honking. The litter that saturated the intersection of the walls and the ground consisted of wrinkled candy wrappers, sharp beer bottles, crushed aluminum cans, splintered kabobs, and oily eating utensils. A woman with graying hair and strips of cloth thrown over her body holds a metal can. Clink clink – there was only a penurious amount of coins in her piggy bank. Her well-like eyes implore pedestrians walk by, but they hardly notice that there is a person kneeled on the ground. You close your eyes to escape to a tranquil place, but the pungent scent of unsanitary food and trash creeps into your nostrils. Your eyes open and you can barely breathe. You look up to the sky but it is hazy. It's always hazy because of the pollution. These are my impressions of my hometown in hindsight. When I was growing up there however, I was habituated to the noise, the people, the rancid smells, and the lack of clear blue skies. In fact, I did not even notice. When I returned to my hometown a few years ago, new shopping malls and housing developments had been placed in the city. However, the vendors were still there along with the people, beggars, and turbid air. I now noticed.

My father grew up in a rural environment. He was the first in his family to graduate elementary school, and went on to graduate middle school, high school, college, and eventually, graduate school. He is one in a million. I would visit his hometown during Chinese New Year's and from what I can recall; it was not convenient or pleasant. His family depended on crops from the harvest and was much of an agricultural environment. The area was very underdeveloped; the only roads were cleared-out paths in the woods. When I returned to my dad's hometown in 2008 for a week, there was still no running water. Instead, we bathed with a small wooden tub and bucket, cleansed our hair in the lake, washed vegetables in the same lake, and had a gigantic outhouse that stored our feces. There, I was able to witness live the slaughtering of a chicken for our dinner. By the end of the week, I was sick, sweaty, exhausted, and infested with 87 mosquito bites.

I was able to "escape" this environment by moving to Ithaca, NY in the spring of 2003. My favorite thing about this town was the resemblance of nature and the fresh air that I could breathe. Ithaca is a small and educated town, surrounding Cornell University and Ithaca College. There, I rarely saw poverty with my own eyes, but I knew that there were people still hungry from volunteering at the local soup kitchen. I graduated from Ithaca High School in June 2012 and am attending Brown University. Although I don't know what I want to study yet, I know that I want to pursue something related to research through this internship.

My life experiences in two drastically different countries and my subsequent visits to my home country reinforced the reality of poverty and food insecurity in both places.

Getting Involved

Although I had an agricultural background, I never really considered agriculture as a modern field. However, I was proved wrong in the summer before my junior year of high school. I signed up for a 3-week program at the University of Maryland named Ag-Discovery sponsored by USDA-APHIS. There, I was able to take a college course in Agriculture and Natural Resources while taking field trips and hearing from guest speakers regarding the effects of plants, animals, and diseases on America's food supply, genetic engineering controversies, and controlling various populations. I left the 3-week training with a broader knowledge about agriculture and a passion for being involved with food insecurity.

The following summer, I applied to a plant sciences internship at the Boyce Thompson Institute at Cornell University. There, I was able to study transposable elements Ac and Ds in maize. In the same summer, I was accepted to the New Visions Program for Life Sciences at Cornell University. The program is directed towards students with an interest in the life sciences, ranging from veterinary studies to food insecurity to research. For our summer assignment, we were to write a paper regarding a developing country and a factor that was affecting poverty in the country. I read many sources both online and in print and presented my findings at the New York Youth Institute in September. I was pleasantly surprised to find later that day that I had been selected as one of four delegates to attend the Global Youth Institute in Des Moines, Iowa the following month.

During the week of the World Food Prize Laureate Awards Ceremony in 2011, I was able to absorb the speeches given by the laureates, Kufor and Lula de Silva, as well as other important people in the agricultural field. The speeches were very inspiring and really spoke to me. I remember when the director of the World Food Program concluded her speech with, "how do you end hunger? Be impatient, be unreasonable, and go for it." The room of teenagers, who are usually considered apathetic and lazy by society, cheered and gave her a standing ovation. At the Global Youth Institute a few days later, I was able to combine my ideas with the ideas of other youths around the world. It was then that I came to realize that the issue of food insecurity was much more than just education, genetic engineering, or policies such as giving food aid. It was all those factors combined and much more. We cannot categorize global hunger into one big category because the different parts of the world have different climates and their own issues. We cannot brainstorm one solution and distribute it to nations and groups all over the earth. With this in mind, I became more and more passionate in the topic of facing the challenge of global food insecurity and poverty.

I returned to my hometown wanting to make an impact, to make a change in the world. From the corner of my eyes, I saw the instructions sheet for applying to the Borlaug-Ruan summer internship. Immediately, I knew what action I needed to take. A cover letter, a resume, recommendation letters, and an essay later, I submitted my application just a few days before Christmas. When I received a letter from the WFP, I tore the envelope eagerly and rejoiced as I had passed the first stage and had obtained an interview. A few weeks later, I was informed that I had received the internship, but the location was still uncertain. After weeks of anticipation, I discovered that I was going to Brazil for the summer! I did a little research on the culture and attempted to learn some Portuguese. APs, finals, senior events and graduation occupied my mind

during the last few months of high school and my summer internship lingered at the back of my mind. Before I knew it, I stepped on the plane for my summer adventure. Final destination: Londrina, PR, Brazil.

First Impressions and Observations

Although Brazil doesn't look grand in size on the distorted version of world maps, the area of the country is almost the same as that of the U.S. Unequal income distribution and deforestation in the amazons are some of the problems that are prevalent in the country.

Londrina is located in the state of Paraná, located in Southern Brazil. Contrary to the beliefs of my friends, family, and even myself, it was not a rainforest in the Amazon filled with monkeys jumping from tree to tree and exotic plants. Instead, Londrina was founded by company owners from London. Therefore, Londrina is translated as "Little London."

I arrived in Londrina about 7 pm at night. After conversing with the airport staff regarding my missing luggage, I was welcomed into Londrina with open arms by my host mother and host father. I thought I would have a more difficult time adjusting to Brazil. Londrina was similar to a large city in the U.S. but with crazy traffic laws (or lack thereof), an abnormal amount of skyscrapers, and constant construction occurring. The grass was thicker, the soil redder in color, the days shorter, and the weather a bit cooler. We went to a restaurant where I was served parmesan beef, French fries, rice, and salad. Everything was what I expected it to be except for what looked like a chunk of cylindrical white plastic. I was hesitant to eat it at first, but realized that I wanted to experience new things including the culture of Brazil. I cut up the cylinder with a knife and placed a piece into my mouth. I would learn later that this was palmito, or palm tree trunk. I would also learn to love this dish and many other Brazilian dishes.

The apartment complex in which I lived reminded me of a luxurious hotel; complete with a lobby, a party room, a guard at the door, and one apartment per floor. I entered the elevator and pressed a few different digits as the password, and up we ascended to the 14th floor. My bedroom consisted of two twin-sized beds, a redolent bathroom, a TV, a magnificent view of Londrina, and a glorious sunset. Contrary to what I had expected, the apartment was not lacking in any "modern-day" accommodations.

Adjusting

There were some aspects that took some getting used to in Brazil. Coming from a small town of 100,000 people, I was not accustomed to the constant traffic and the overwhelming number of tall buildings. Fortunately, my childhood memories served me well as I knew the dangers of living in a large city. Another part was the time; I was to arrive at work by 8am each day. Because the drive took 30 to 40 minutes, my alarm went off at 6:50am each morning. Even when I had classes back in high school, I would wake up at 8am. Needless to say, waking up in the morning was truly difficult. My workday was 9 hours each day with about an hour of lunch break at midday, and plus time spent on the road, I would be out of the apartment for about 10.5 hours each day. The cultural differences included their form of greeting of exchanging hugs and kisses on the cheek, which I thought strange at first. Another experience was on the Fourth of

July. At about 7pm that night, I heard fireworks outside and rushed to the window to watch. *Oh well this is nice,* I thought to myself. It wasn't until later that night when another round of fireworks and parading outside when I realized that I was no longer in the U.S. but in Brazil and wondered if they actually celebrated the Day of Independence in the United States. My host parents finally explained to me that the Brazilian club soccer team, the Corinthians just won a title that they have not claimed in about a century. I enjoyed and endured the screams, cheers, fireworks, and music until about 2 am when I finally fell asleep.

Brazil was trying new things in a new environment with a hint of familiarity. I discovered that no matter where you are in the world and what language you speak, there will always be people sharing your interests. I met people who shared my love for chocolate, Harry Potter movies, and sense of fashion. They celebrated Father's day, birthdays, and soccer games. We all ceased to blink as our eyes fixated on the television during the final seconds of the Brazilian soccer game in the Olympics.

EMBRAPA AND ITS PEOPLE

Embrapa Facts

Embrapa, or otherwise known as Empresa Brasileira de Pesquisa Agropecuária (Brazilian Enterprise for Agricultural Research) is an agricultural company found in Brazil. It forms partnerships with other agricultural companies to conduct research on agriculture. With 45 decentralized research centers and over 8000 employees across the country, it is one of the main agricultural companies in the country. Embrapa Soybean, located on the countryside of Londrina, PR, is one of the Embrapa's research centers. Embrapa Soybean's mission is to "to provide feasible solutions for the sustainability of the soybean and sunflower productive chains, through research, development and innovation, for the benefit of Brazilian society." Embrapa soybean also shares their information internationally with organizations around the globe.

Researchers

Francismar C. Marcelino-Guimaraes, my primary research advisor completed her undergraduate degree at the Universidade Federal de Viçosa. She started working at Embrapa in December of 2005. She decided to work at Embrapa because she is a biologist and has always liked studying genetics and molecular biology. She also feels the necessity to make a difference in the world, helping decrease inequality and reducing people facing poverty. Dr. Francismar has been working with gene prospection in response to biotic stress factor affecting soybeans, such as nematodes and soybean rust and on the development of genetically modified soybean tolerant to drought. During my time at Embrapa, she has helped me choose my research project and guided me with her knowledge and various sources of readings.

My mentor, Carolina Rocha did her undergraduate degree at the Universidade Federal de Viçosa as well. Because of her love of working in the laboratory and researching, she started working at Embrapa Soja in August of 2011. She now works for a company called FuturaGene, as she was selected to perform research in the field of molecular biology.

PROJECT:

Evaluation of *Phakopsora pachyrhizi* genes required for biotrophic colonization in soybean

Abstract

Soybean is an economically important crop to many countries in the world, especially the U.S., Brazil, Argentina, and China. However, when pathogens strike, soybean growers suffer the consequences of the damage and destruction of soybean plants. Asian Soybean Rust, caused by the fungus, *Phakopsora pachyrhizi*, is one disease that has caused billions of dollars of loss in Brazil. Although the fungus functions the best in the warm climates of the southern hemisphere, it is also able to infect soybean plants in North America and is predicted to adapt to cooler climates. In order to better understand the fungus and various ways to combat it, we are evaluating the genes in *P. pachyrhizi* required for biotrophic colonization. The genes (chitin deacetylase [CD1], which hydrolyses the N-acetamido groups of N-acetyl-D-glucosamine residues in chitin; heterotrimeric G-protein α subunit [Gpα], which is a transmembrane receptor involved in signaling by activation of a cascade of reactions; mitogen-activated protein kinase [MAPK], which is a protein kinase that responds to extracellular stimuli and regulates various signaling pathways; amino acid transporter [AAT3], which is a membrane transport protein that transports amino acids; hexose transporter [HXT], which is a transmembrane protein that is responsible for carbohydrate transport; and aspartate aminotransferase [Asp-AT], which catalyses the reaction: (L-aspartate + 2-oxoglutarate = oxaloacetate + L-glutamate.) are chosen for our experiment because they are commonly involved in the rust pathogens and the biotrophic interactions. In the broader spectrum, this study deepens our understanding of fungi genetic factors that are involved in establishment and maintenance of the colonization in soybean.

Techniques include primer design and real time PCR for quantification of the expression levels of the genes during different stages of the infection cycle. It is expected that genes important to the establishment and maintenance of biotrophic interactions have expression activation in the beginning and during different stages of the infection, respectively. The main result that will be produced is the expression profile of the selected genes during pathogen infection. We will use SYBR green technology and relative quantification to compare the expression levels of the selected genes during the infection cycle with uninfected plants (controls).

The data will be analyzed by comparing the expression levels of the selected genes during the pathogen infection against uninfected plants (controls). We expected the expression profile to change during the infection cycle depending on whether or not the gene is important in each step of the infection. The comparisons of the expression levels will be verified using a statistical test. Based on Vieira *et al* 2012, these genes are important for closed pathogens (rust) in the infection in *Arabic coffee*. A high expression level of these genes during the infection is a good indication of their importance. Currently, I found that the highest levels of expression of RGT2 reached more than 6,000 times compared with the times and samples which haven't had contact with host tissues, such as spores and mock conditions. In the future, we expect that we will be able to identify at least 2-3 genes that are important for the initial interaction between soybean and *P. pachyrhizi*. Other experiments should be performed to validate our result.

If a gene is identified that shows high expression levels, we could confirm that the gene is important by annotating the gene and finding its specific location. We could then discover the function of the protein and confirm in vivo assays; for example, by silencing the gene in the fungus and checking its infection performance. By using biotechnological tools, important genes involved in fungus infection can be manipulated and used as alternative technology on the management of soybean rust disease.

Introduction

The soybean plant has had increased average yields and lower production costs in recent years. Soybean was developed first in Northeastern China in the 11 century B.C. and since has spread through the eastern hemisphere and all across the globe. Soybean grows optimally in temperate climates, but the crop could assist farmers if it was better adapted to tropical areas. Some of the problems with tropical areas include shorter days, poor storage for seed longevity, and lack of access to local markets. Today, production areas are concentrated in the U.S., Brazil, Argentina, and mainland China. There is a high demand for soybean as it is a valuable and profitable crop. About two-thirds of all soybean crops are used for the consumption of humans or animals, and the other one-third is dedicated to oil production. Soybeans are very nutritious foods: consisting of proteins, carbohydrates, fibers, and healthy fats. In addition, soybeans also include various types of vitamins and minerals. Soybean oil is the most important vegetable oil, making up about 20% of all vegetable oil production. In genetic engineering and crop modification, soybean is a very useful crop. In short, the soybean plant is a very important crop all over the world for various purposes, but when pathogens strike, the loss can be devastating. One disease that infested Brazil and other parts of the world was the Asian Soybean Rust.

Asian Soybean Rust is the caused by both pathogens, *Phakospora pachyrhizi* and *Phakospora meibomide.* Originally, they were thought to be the same, but later studies only found 80% nucleotide similarity within the ribosomal internal transcribed region. *Phakospora pachyrhizi* infects the leaf tissue at a broad range of 31 species across 17 genera of legumes. 60 species in other genera are also able to be infected under laboratory conditions. The fungus first causes small tan-colored lesions on the leaves of plants; after about a week, the lesions enlarge and rust pustules become visible. It eventually defoliates the soybean and leads to plant death and complete crop failure.

Phakospora pachyrhizi originated in the Asia-Australia region, with its first sighting in Japan in the year 1902. This fungus first appeared in South America in 2001, and has been virulent in spreading to major soybean regions of Brazil. In 2003, the fungus caused a loss of $2 billion in USD. In November 2004, the fungus was noted in the continental USA. However, U.S. soybean growers did not experience the same magnitude of loss probably because of unfavorable weather conditions and the lack of ability of the adaptation of the pathogen. Because *Phakospora pachyrhizi* is not frost tolerant, the fungus attacks the legume kuzdu in the winter months and attempts to spread to other plants under warm conditions. However, it is not always able to spread to the soybean plant. Even though the pathogen is not very detrimental to U.S. soybean plants at the moment, it is expected to adapt, so researchers need to continue studying the fungus and discovering new ways to combat it. (3)

It is important to identify resistance genes and endogenous up-regulation of genes in response to disease in order to find or engineer ASR resistance soybean cultivar. The real –time PCR technology has been useful in identifying the resistance genes for fungal diseases in plants (Luo *et al.* 2005).Transcriptional expression levels play an important role during the plant defense process. The corresponding up-regulation genes lead to defense responses such as toxin production, cell wall structure changes, programmed cell death, nutrition regulation and so on. To study differentially expressed genes of soybean in response to *P. pachyrhizi* should help us to understand the mechanisms of rust infection and engineer ASR resistant soybean varieties. The main objective of this study was touse real-time PCR to perform a transcriptional expression level of selected genes of *P. pachyrhizi*-exposed young soybean plants (V2 growth stage). A few molecular and biological analyses have been done on *P. pachyrhizi* and on the interaction between *P. pachyrhizi* and its soybean host. Fourhundred eighty-eight unique expressed sequence tags (ESTs) were generated within *P. pachyrhizi* germinating spores (Posada-Buitrago and Frederick, 2005). Panthee *et al.* (2007) also found that forty-six of these genes were up-regulated and 66 were down-regulated in soybean plants inoculated with *P. pachyrhizi* 72 h after infection (hai).
In order to better understand the fungus and various ways to combat it, we are using real-time QPCR to evaluate the genes in *P. pachyrhizi* required for biotrophic colonization.

Methods

Growth and Collection
Soybean leaves infected with *P. pachyrhizi* were collected in different hours after infection: 0 hours, 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, 96 hours, 192 hours, and 10 days. The urediniospores of *P. pachyrhizi* were collected and re-suspended in 0.05% Tween20 (v/v) solution to a final concentration of $13x10^4$ spores/mL. This suspension was pulverized over soybean susceptible plants at V2 (second trifoliolate) growth stage (Fehr and Caviness 1977). The same solution minus spores was used to mock inoculations as a control of the infection process. Plants were recovered with humid plastic bags immediately after inoculation for two days long to optimize fungal infection and avoid cross contamination of mock-inoculated plants. After ten days post inoculation (10dpi) we could see Tan lesions on the leaf abaxial surface in the inoculated plants but not in the mock ones (Figures 1&2).

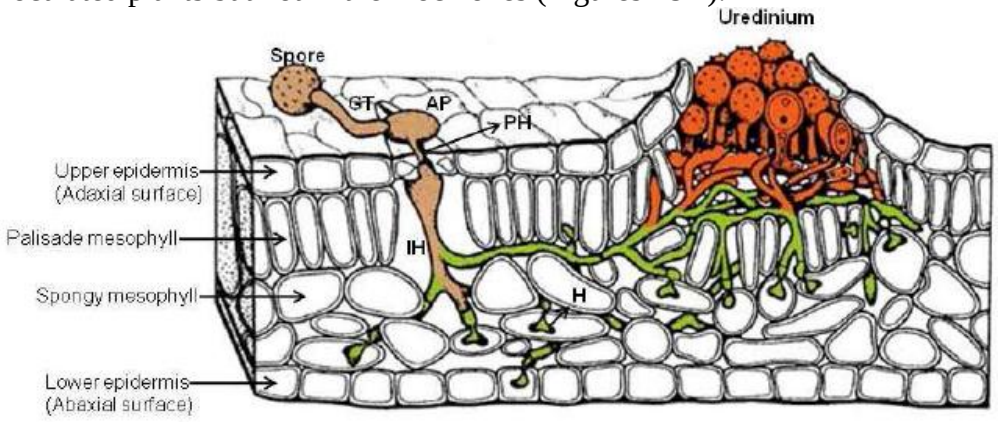

Figure 1. Internal structure of a typical dicotyledon leaf showing the different cell layers and infection by a rust fungus. GT, germ tube; AP, appressorium; PH, penetration hyphae; IH, infection hyphae; H, haustorium. Schema was taken from Hahn (2000).

Once germination occurs, a single germ tube (GT) was produced by the uredospore that grows across the leaf surface until it reaches an appropriate surface where an appressorium (AP) forms. This penetration occurs between 7-12 hours after the spore lands on the leaf adaxial surface. Approximately 20 hours after the spore landing, the *penetration hyphae* (PH), stemming from the appressorium cone, pass through the cuticle to emerge in the intercellular space where a septum is formed to produce the *primary infection hypha* (IH). This IH grows between palisade cells to reach the spongy mesophyll cells where it forms the haustorium (H) (Tremblay et al., 2010). Once this first stage has been reached, additional hyphae emerge and spread through the apoplast where many other haustoria are formed. Yellow mosaic discoloration observed at 7 dai (Figure 2A) and Tan lesions observed at 21 dai (Figure 2B).

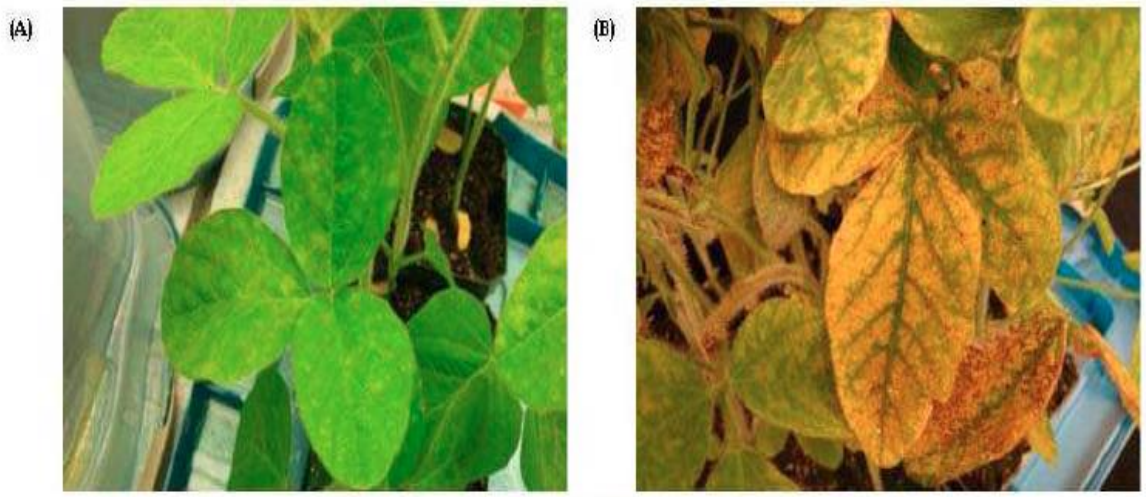

Figure 2. A) Tan lesions forming on a soybean leaf 7 days after infection. B) Tan lesions saturating the soybean leaf 21 days after infection.

These times were chosen because of an experiment was performed under greenhouse conditions at EMBRAPA soybean and are related with the different phases of the disease. RNA samples were then extracted and immediately frozen in liquid nitrogen and then placed at -80℃ used for cDNA preparations. The samples from February were used in my experiments in a quantitative PCR for gene quantification.

Selection of Genes

In this experiment, six genes were chosen (CD1, Gpα, MAPK, AAT3, HXT, and Asp-AT). These genes are important in our experiment because they are commonly involved in the rust pathogens and the biotrophic interactions. In another similar experiment involving Arabic coffee and the pathogen *Hemileia vastatrix*, these same genes are analyzed for its involvement in the success of the pathogen in infecting and colonizing the host (6). These genes were important in different steps during pathogen infection, such as recognition, signaling, penetration and the up taking of nutrients. We are only focusing on two of these six genes because of issues dealing with the delivery of primers. One of the two genes was mitogen-activated protein kinase [MAPK], a protein kinase that responds to extracellular stimuli and regulates various signaling pathways; MAPK is also highly conserved between species and would therefore be able to signal host infection. The other gene, hexose transporter [HTX], which is a transmembrane protein responsible for carbohydrate transport and is involved with nutrient acquisition.

Primer Design and Test for RT-qPCR

For PCR amplification, it is necessary to design primers that are specific to the genes. First, the accession number is typed into the BLAST function on the NCBI website (an international database of DNA sequences) in order to obtain the sequence for the gene. The sequence is then put into local blast (Database of Embrapa Soja containing *Phakospora pachyrhizi* genes expressed during infection in soybean) to check for specificity. If the gene is specific, it is put into an application that will help us find primers within the stated parameters (melting temperature 60°C, primer lengths 18-24 bp, 50-60% GC content.) for use in quantitative PCR. Originally, 6 primers were designed, but because of the enormous amount of time that it took for the primers to arrive, I only had enough time to perform experiments on two primers (Table 1).

In order to test the efficiency of the primers, the two primers, named "23236" and "3443" were then diluted to 5 µM by adding 5 µL of primer and 95 µL of H_2O. The RNA samples from 10 days-post-innoculation were diluted leaving a result of five different concentrations consisting of the original concentration, 1/10, 1/100, 1/1000, and 1/10000. A PCR plate was then designed incorporating the 2 primers and different concentrations of the RNA. The Ct value of each dilution was used to prepare a standard curve. To obtain the efficiency value for each primers, the slope of each standard curve prepared with the dilutions and Ct values was used in the formula $E=10^{(-1/slope)}-1$.

Table 1 Contigs, their respective primers, and the predicted functions of the genes.

Contigs	Primers	Function
1482-F	CAAGTCCATGAGCTATCACGAG	Secretoma
1482-R	TATTCCACGAGCTGCTTAGG	
4692-F	GGAACGCAAGAAGTGGATTC	G-protein alpha subunit
4692-R	GCTTCAGCCATTCGATTCAC	
3443-F	TTCTGCAGCGGTACTTCATC	**putative MAP Kinase**
3443-R	AATCCTGTTGCCTCCTGTTC	
3392-F	CTGCTGTGTTCCAATGATCG	histidine permease
3392-R	CAACGTATGCGCTGGTTATG	
23236-F	AAACAAGAGGCAGTGCCAAC	**high-affinity glucose transporter RGT2**
23236-R	TGGCCGAAGAATCGGTATAG	
389-F	ACCTGACCCAATTCTTGGTG	
389-R	AACTGAGGGAAGCACAAACG	

Preparation of RNA for RT-qPCR.

Using the RNA from plant samples that were collected and extracted in February 2012 and subsequently stored in the -80°C fridge, I used the Deoxyribonuclease I, Amplification Grade kit to prepare RNA samples to RT-qPCR analysis. The RNA samples need to be treated with DNase to clean the RNA of any DNA contamination.

The following mixture was prepared in a test tube:

1 µg of RNA (see table 2 below for dilution)

1µL of 10X

DNase I Reaction Buffer

1µL of DNase I, Amp Grade, 1U/µL

DEPC-treated water to 10µL (see table below for dilution)

Table 2 Dilution of RNA to 1µg.

Time after inoculation	Amount of RNA (in µL)	Amount of H_2O (in µL)
0 hours	1.54	6.46
6 hours	1.08	6.92
12 hours	0.73	7.27
24 hours	0.84	7.16
36 hours	1.46	6.54
48 hours	1.17	6.83
72 hours	1.19	6.81
96 hours	0.91	7.09
192 hours	1.14	6.86
10 days	1.18	6.82

The tubes were then incubated at room temperature in order for the DNase to start a reaction within the test tube. After 15 minutes, 1µL of 25mM EDTA solution was added to each tube to cease the reaction. The samples were then incubated for 10 minutes at 65°C.

First-Strand cDNA Synthesis
Mixing these components together in a separate test tube:
RNA (from previous step)
1 µL of Primer 50µM oligo $(dT)_{20}$
1 µL of 10mM dNTP mix
Up to10 µL of DEPC-treated water

The samples were incubated at 65°C for 5 minutes and immediately place on ice for 1 minute.
10 µL of cDNA synthesis mix was added to each tube and then incubated in a PCR machine at 50°C for 50 minutes followed by 85°C for 5 minutes and then chill on ice. Finally, 1 µL of RNase H to each tube and incubate for 20 minutes at 37°C.

Table 3 cDNA Synthesis Mix

Component	x 1 reaction	Master Mix (x 11 reactions)
10X RT buffer	2 µL	22 µL
25mM $MgCl_2$	4 µL	44 µL
0.1 M DTT	2 µL	22 µL
RNaseOUT (40 U/ µL)	1 µL	11 µL
SuperScript III RT (200 U/ µL)	1 µL	11 µL

RT-qPCR
Table 4 preparation of the PCR component

Component	x 1 reaction	Master Mix (x117 reactions)
SYBR	6.5 µL	760.5 µL
Primer F	0.5 µL	58.5 µL
Primer R	0.5 µL	58.5 µL
H_2O	3.0 µL	351.0 µL

cDNA	2.0 µL	--
Total:	12.5 µL	

Using a 96-well PCR plate and put 6 µL of cDNA + 31.5 µL Master Mix in the wells in column 1, 4, 7, and 10. After mixing the cDNA with the Master Mix, 12.5 µL of the mixture was distributed to the adjacent cells to ensure uniform results.

Real-time reverse-transcription was performed on a 7300 RT-qPCR thermocycler (Applied Biosystems), following the manufacturer's instructions. After the initial steps at 50°C for 2 min (UNG activity) and at 95°C for 10 min (activation of the AmpliTaq Gold polymerase), a two-step program of 95°C for 15 s and 62°C for 1 min was conducted for 40 cycles. The dissociation curves were obtained to guarantee the absence of unspecific amplifications. The data were collected in the log phase, and the results were analyzed by the Sequence Detection program (Perkin Elmer, Massachusetts, CA, USA). A final relative quantification of each gene compared with the control conditions was estimated considering the RQ (Relative quantification) obtained in each biological replicate, represented by each independent experiment, each with three replicates. The expression levels were determined using the formula RQ= $2^{-\Delta\,\Delta ct}$ after efficiency adjustments (Livak, 2008).

Table 5 - Samples laid out in the 96-well plates

	Placa1 = 23236											
	1	2	3	4	5	6	7	8	9	10	11	12
A	Esporo1	Esporo1	Esporo1	EspGerm1	EspGerm1	EspGerm1	Mock1	Mock1	Mock1	Mock2	Mock2	Mock2
B	Mock3	Mock3	Mock3	0hpiRep1	0hpiRep1	0hpiRep1	0hpiRep2	0hpiRep2	0hpiRep2	0hpiRep3	0hpiRep3	0hpiRep3
C	6hpiRep1	6hpiRep1	6hpiRep1	6hpiRep2	6hpiRep2	6hpiRep2	6hpiRep3	6hpiRep3	6hpiRep3	12hpiRep1	12hpiRep1	12hpiRep1
D	12hpiRep2	12hpiRep2	12hpiRep2	12hpiRep3	12hpiRep3	12hpiRep3	24hpiRep1	24hpiRep1	24hpiRep1	24hpiRep2	24hpiRep2	24hpiRep2
E	24hpiRep3	24hpiRep3	24hpiRep3	36hpiRep1	36hpiRep1	36hpiRep1	36hpiRep2	36hpiRep2	36hpiRep2	36hpiRep3	36hpiRep3	36hpiRep3
F	48hpiRep1	48hpiRep1	48hpiRep1	48hpiRep2	48hpiRep2	48hpiRep2	48hpiRep3	48hpiRep3	48hpiRep3	72hpiRep1	72hpiRep1	72hpiRep1
G	72hpiRep2	72hpiRep2	72hpiRep2	72hpiRep3	72hpiRep3	72hpiRep3	92hpiRep1	92hpiRep1	92hpiRep1	92hpiRep2	92hpiRep2	92hpiRep2
H	92hpiRep3	92hpiRep3	92hpiRep3	196hpiRep1	196hpiRep1	196hpiRep1	196hpiRep2	196hpiRep2	196hpiRep2	196hpiRep3	196hpiRep3	196hpiRep3

RT-qPCR is a preferable method in studying gene expression because it is sensitive and accurate.

Results

Ct values were evaluated to test the expression levels. Ct values, otherwise known as the cycle threshold, is the PCR cycle where the target amplification is first detected. It is where the fluorescence intensity is greater than the background fluorescence. "Consequently, the greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower Ct."

Table 6 - Efficiency Reaction for "3443" primer.

Detector	Reporter	Start
3443	SYBR	3
Well	SampleName	Ct
13	3443 (1)	30.8382
14	3443 (1)	30.6622
15	3443 (-1)	28.2744

16	3443 (-1)	32.1935
17	3443 (-2)	34.4391
18	3443 (-2)	38.29
19	3443 (-3)	
20	3443 (-3)	
21	3443 (-4)	
22	3443 (-4)	
23	blank	
24	blank	

The first primer, "3443", did not function; Ct values for wells 19-22 are blank. I repeated the PCR for "3443" three times but received similar results to the table shown above. We assume that the company that manufactured the primers made a mistake as this often happens in Brazil.

Table 7 - Efficiency Reaction for "23236" primer.

Detector	Reporter	Start	End	Threshold
23236	SYBR	3	15	0.2
Well	SampleName	Ct	Average	
1	(23236) 10^0	15.6293	15.6	0
2	(23236)10^0	15.5778		
3	(23236)10^-1	18.1822	18.2	-1
4	(23236)10^-1	18.2426		
5	(23236) 10^-2	21.6028	21.7	-2
6	(23236) 10^-2	21.7271		
7	(23236) 10^-3	23.86	24.7	-3
8	(23236) 10^-3	25.4432		
9	(23236)10^-4	29.0355	29.0	-4
10	(23236) 10^-4	29.0193		
11	blank			
12	blank			

The Ct values for "23236" looks more consistent and showed expected results, unlike "3443". Within the same sample, the Ct values are close to each other in value.

Plotting the graph of efficiency versus the dilution, we obtain the following graph. Plotting the equation of best fit, which is also the equation for the efficiency, we obtain an extremely high efficiency of 99%. Because the efficiency is so high, we know that the primer "23236" functions properly and we are able to use it in our experiments.

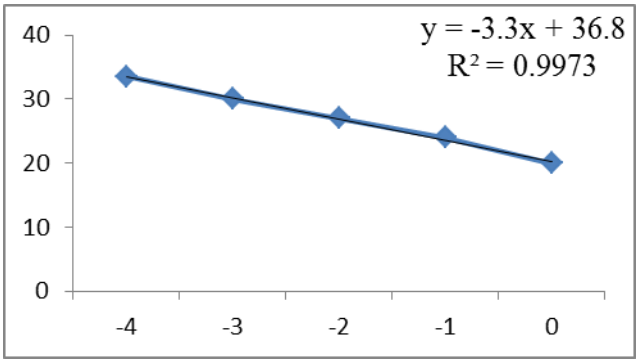

Figure 3 - The efficiency of "23263"

RT-qPCR Output:

Table 8 - Average Ct values

	Tubulina (fungus endogenous reference)			23236 (Target Gene)		
SampleName	Ct_Rep1	Ct_Rep1	Ct_Rep3	Ct_Rep1	Ct_Rep1	Ct_Rep3
Esporo	21.46567	21.46567	21.46567	15.58387	15.58387	15.58387
Esp.Germ.	23.19487	23.19487	23.19487	18.5196	18.5196	18.5196
Mock	38.31677	37.2497	38.1855	31.4241	31.16873	31.61693
0hpiRI	36.40123	36.7478	37.20467	24.33787	29.06843	23.96333
6hpiR1	37.2045	36.5283	36.3955	23.79673	22.92243	23.16347
12hpiR1	37.00557	36.96577	37.4865	26.02227	25.5119	26.13063
24hpiR1	37.36587	36.96977	36.8466	31.45443	30.757	29.7416
36hpiR1	36.89917	36.68893	37.1709	32.34273	32.76527	32.7038
48hpiR1	36.63127	36.97267	35.60513	34.18727	30.19823	30.41007
72hpiR1	33.622	35.50263	34.73293	31.19737	30.22147	34.526
96hpiR1	32.47837	33.4537	33.4537	33.49033	35.81997	35.81997
192hpiR1	28.59607	29.02927	28.22753	25.50823	26.02193	25.42623
10dpiR1	27.20743	27.20743	27.6402	17.2091	22.43147	19.06587
Branco						

Table 8 - Delta Ct

	23236 (Target Gene)		
SampleName	ΔCt1	ΔCt2	ΔCt3
Esporo	-5.8818	-5.8818	-5.8818
Esp.Germ.	-4.67527	-4.67527	-4.67527
Mock	-6.89267	-6.08097	-6.56857
0hpiRI	-12.0634	-7.67937	-13.2413
6hpiR1	-13.4078	-13.6059	-13.232
12hpiR1	-10.9833	-11.4539	-11.3559

24hpiR1	-5.91143	-6.21277	-7.105
36hpiR1	-4.55643	-3.92367	-4.4671
48hpiR1	-2.444	-6.77443	-5.19507
72hpiR1	-2.42463	-5.28117	-0.20693
96hpiR1	1.011967	2.366267	2.366267
192hpiR1	-3.08783	-3.00733	-2.8013
10dpiR1	-9.99833	-4.77597	-8.57433
Blank			

Table 9 - Expression Levels

	23236 (Target Gene)		
SampleName	0.9038	0.9038	0.9038
Esporo	47.22356	47.22356	47.22356
Esp.Germ.	21.41601	21.41601	21.41601
Mock	14.50097	10.58346	12.78754
0hpiRI	2714.195	153.3934	5873.949
6hpiR1	6550.926	7459.138	5838.255
12hpiR1	1337.266	1820.358	1707.115
24hpiR1	48.14967	58.66282	105.2744
36hpiR1	19.81138	13.08603	18.68475
48hpiR1	4.961864	84.76806	30.10865
72hpiR1	4.899282	31.85651	1.14525
96hpiR1	0.515181	0.212071	0.212071
192hpiR1	7.566613	7.177753	6.271111
10dpiR1	701.234	22.87711	275.7684
Blank			

Table 10 - Average Expression Levels

	23236 (Target Gene)
SampleName	
Esporo	47.22355652
Esp.Germ.	21.41601217
Mock	12.62398801
0hpiRI	2913.845757
6hpiR1	6616.106084
12hpiR1	1621.579499
24hpiR1	70.69562421
36hpiR1	17.19405246

48hpiR1	39.94619018
72hpiR1	12.6336803
96hpiR1	0.313107595
192hpiR1	7.005159009
10dpiR1	333.2931601
Branco	

Ct = Cycle threshold
Target gene ct – endogenous gene ct = Δ ct
$\Delta\Delta$ ct = Δ ct_{sample} - Δ $ct_{calibrated}$
Expression level = $(1 + E)^{-\Delta\ \Delta ct}$

From table 10 above, we know that the average expression levels of RGT2 were from 1621 to 6616 times compared with the control in the initial steps of the infection. The expression levels of RGT2 reached the highest level at 6 hours after inoculation. Then the expression levels decreased after 6 hai. But the expression level increased to 333 times at 10 days after inoculation.

Conclusions and Discussions

Based on the expression levels results determined by RT-qPCR the gene RGT2, a potential glucose transporter has an important role on the initial steps of the *P. pachyrhizi* infection in soybean. An invariant endogenous control (reference gene) changes very little, or not at all, in expression across your experiment so any change in the signal generated by these genes is due to things like differences in the amount of cDNA loaded in each reaction. By normalizing the signal generated by your genes of interest to the signal generated by a reference gene (tubuline) then you can infer if your genes are up or down regulated. The expression levels on the initial time points tested in this experiment showed an up regulation since the first contact of the pathogen with the host tissues. The highest levels of expression reached more than 6,000 times compared with the times and samples which haven't had contact with host tissues, such as spores and mock conditions.

RGT2 are plasma membrane proteins, with 12 transmembrane domains, highly similar to the HXT glucose transporters. RGT2 would be a sensor for high levels of glucose, as it is required for maximal induction of HXT1 by high glucose (O¨ zcan et al., 1996). There is strong experimental evidence that RGT2 interacts with the membrane-bound, type I casein kinases Yck1 and Yck2 and, upon binding glucose, activates them (Moriya & Johnston, 2004).

Rgt2 influences several HXT-transporters which are responsible for the glucose uptake. Low extracellular glucose concentrations are sensed by the Snf3 protein which probably leads to the expression of HXT2-Genes for high affinity glucose transporters, while RGT2 senses high glucose concentrations and leads to the expression of low affinity glucose transporters, like HXT1 Although the downstream pathway is poorly understood it seems that Snf3 and Rgt2 transmit a signal directly or indirectly to Grr1, the DNA binding protein RGT1, and the two cofactors Ssn6 and Tup1.

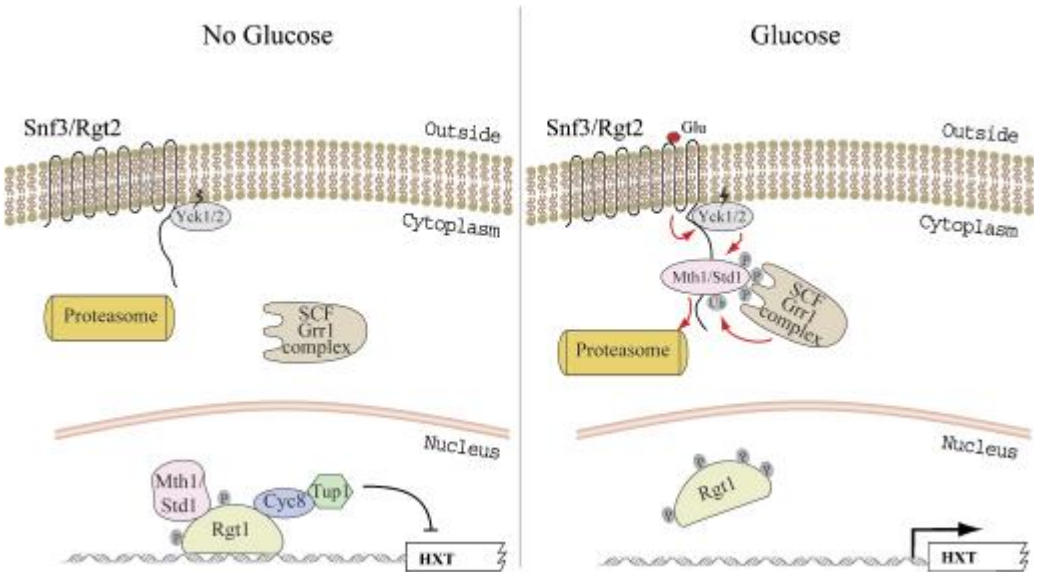

Figure 4: Snf3/Rgt2-signalling pathway. In the absence of glucose a repressing complex, including Rgt1, Mth1/Std1, Cyc8 and Tup1, binds to the promoters of the HXT genes, and blocks their transcription. When glucose (Glu) is present, it binds to the Snf3/Rgt2 sensors, thus activating the membrane bound casein kinase I (Yck1/2). Activated Yck1/2 phosphorylates Mth1/Std1, bound to the C-terminal tails of Snf3 and Rgt2. Phosphorylated Mth1/Std1 are recognized by the SCFGrr1 complex, which tags them, through ubiquitination, to be degraded by the proteasome. Removal of Mth1/Std1 allows the phosphorylation of Rgt1, which dissociates from the promoter, allowing derepression of the HXT genes.

Fungi possess sensitive gene regulatory mechanisms for responding to nutrient fluctuations in the environment, but until recently little was known about these systems in pathogens. Within the host cell, the fungus spreads undetected from cell to cell in a biotrophic growth phase, extracting nutrients from the host in a manner that does not immediately kill the plant cell (Jessie, 2012) . In planta growth is rigorously controlled and choreographed during the early stages of infection, with the fungus residing in one cell for 8–12 hr before moving to the next in a biotrophic and symptomless manner (Jessie, 2012) From our data it is likely RGT2 up-regulate the glucose uptake of the *P. pachyrhizi* after inoculation.

Final Remarks

This experience was eye-opening and really changed my perspective on global poverty and food insecurity. I learned about the culture of another country, Brazil, and experienced living in another country. As a result of this internship, I am more aware of the whole global community and know that I want to definitely pursue a career related to scientific research.

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