Knockout on *Fusarium oxysporum* f. sp. cubense

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Cavendish bananas are in danger of being commercially wiped out because of a fungus called *Fusarium oxysporum* f.sp. cubense (Tropical Race 4), more commonly known as the fungus that causes Panama Disease in bananas. The *F. oxysporum* fungus has had multiple strains such as *F. oxysporum* f.sp. lycopersici Tropical Race 1, which commercially wiped out the Gros Michel bananas in the 1960s. The current strain of *F. oxysporum* is uncontrollable and is devastating the economies of countries that depended heavily on exporting bananas. This strain could commercially eradicate Cavendish bananas in the next 10 years if methods to kill the fungus are not detected and carried out. Foc. TR4 has fourteen genes called Secreted in Xylem genes, abbreviated as SIX genes, which control the pathogenicity and virulence of the fungus. Hence the name, these genes are secreted into the xylem, or vascular system, of the banana plant that the fungus infects. While the SIX genes present one way to approach the pathogenicity of Foc., they are not well understood. Here, we propose to develop CRISPR Cas9 tools in *E. coli*, that will be later used to investigate *F. oxysporum* SIX genes. Of the 14 SIX genes, genes one and eight are suspected to be especially essential to the fungus's virulence. In this project, the goal is to use CRISPR Cas9 to perform a proof of concept using green fluorescent protein (GFP) in *E. coli* strain K12. The GFP will be inserted into the cell using CRISPR Cas9 technologies.

Keywords: fusarium wilt, panama disease, *Fusarium oxysporum*, CRISPR Cas9, bananas, SIX genes

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Background

In the 1940s and 50s, *F. oxysporum* infected the Gros Michel banana population and resulted in a wipeout of the commercial production of those bananas. This strain of the fungus was called *F. oxysporum* f.sp. In 1947, before they were commercially eradicated by the disease, a lab in the United Kingdom developed a new strain of bananas called Cavendish (Kramer, 2016). Currently, Cavendish are the most common form of banana sold and consumed, making up 96% of all bananas sold worldwide and contributing $12.7 billion yearly to worldwide GDP (CNN, 2016). In 1990, a new strain of *F. oxysporum* f.sp. cubense Tropical Race 4—known

as Foc. TR4—was found in soil samples from banana plantations in Taiwan (Vézina, n.d.). This new outbreak of Foc. TR4 soon travelled to South America and now affects Cavendish Banana fields all over the world. Since Cavendish bananas were developed in a lab, and as they asexually reproduce, they are all genetically similar. Due to their lack of genetic diversity, they have difficulty combatting this disease. Once the bananas are infected, they begin to exhibit symptoms of Foc. TR4 and die. According to Kramer (2016), author at Business Insider, the world's banana population could be wiped out in the next 40 years, and the banana industry in the next 10, if this fungus is not abated.

Latin American and African local and global economies are heavily dependent on banana production. In fact, up to 70% of bananas sold worldwide are grown in Latin America, and bananas grown in Latin America contribute $8.9 billion to the world economy (Guilford, 2014). Additionally, the East African Highlands are one region where the loss of bananas could devastate the region. A third of the world's bananas are grown in Africa. Of this third, 40% are grown in East Africa. Banana production in these high producing regions are not just a massive source of income, they are also a dominant component to peoples' diets as bananas are usually eaten multiple times per day. Bananas are a highly nutritious food staple as they are rich in potassium, vitamin C, and fiber. Yearly banana consumption can reach up to 400 lbs. per capita in the East African Highlands (Baggaley, 2017). Since the bananas provide nutrition for individuals throughout the world and are a main source of income, it is important to protect the fruit.

Banana production is not only critical to the world economy, but many small-scale farmers and local communities are dependent on the industry for income stability and nutrition. The majority of banana crops are grown by small farmers in East Africa's Southern Nations and Nationalities Peoples' Regional State (Alemu, 2017) . In 2018, one of the region's most prolific countries for banana production, Ethiopia, had their banana exports reach 86%—478,251.04 tones—of its total fruit exports and create job opportunities for many farmers in the country. The cash crop is cited by small farmers as their number one source of income. Farmers from this region report higher income, better employment opportunities, and increased local and regional economic developments due to their banana crops (Alemu, 2017). The banana crop has become an essential pillar of the farming economy and is significant for the life of the farmers. If these banana farmers were out of work, it would cause a severe blow to Ethiopian economy. This crisis would cause economic turmoil as important jobs would be lost, and an entire integral aspect of the Ethiopian economy would disappear. If the global banana population were to diminish, farmers around the world, as in Ethiopia, would lose their jobs, livelihoods, and the ability to feed their families. If the fungus Foc. TR4 continues to destroy banana plants at its current rate, this would not just cause economic consequences as a cash crop, but its loss in dependent regions could also promote food instability.

Foc. TR4 is spread by direct contact between fields. The United Fruit Company, which had a monopoly on the banana industry in Latin America in the 1900s tried to put off the spread of the disease by uprooting healthy plants and replanting them in fresh dirt, miles away from infected fields. Eventually, they ran out of virgin land to plant their bananas, so they resorted to flooding their fields, in hopes that they would finally drown out the fungus. However, drowning the fields did not help prevent the fungal spread due to the persistence of the fungal spores, and the problem remained to infect the non-infected trees (Scharping, 2017). Other strains of Foc. that attack different types of plants can be burned or pulled up by the root to stop the fungus from spreading. However, these methods do not work for Fusarium Oxysporum f. Sp. Cubense. In order to combat the economic, social, and human health implications from the destruction of banana crops due to Foc. TR4, elimination of Foc. TR4 is crucial.

Literature Review

Life Cycle and Spread of *F. oxysporum* cubense

The Fusarium fungi can be split up into multiple sub species of fungi. These include the sub species, fusarium graminearum, fusarium verticillioides, and fusarium oxysporum. *F. oxysporum* f.sp. cubense Tropical Race 4 (abbreviated Foc. TR4), the species that is currently devastating world banana populations, is resistant to all known fungicides. It can live up to 150 cm underground, is capable of withstanding both dry spells and flooding, and can stay in the soil for up to 40 years in spore form. (Garden, 2018). The disease is spread by its spores that target the roots of the plant and enter through broken sections or natural openings on the root. Chlamydospores—Fusarium spores—are responsible for Foc. TR4's ability to spread rapidly. In a single inch of stalk where Foc. TR4 symptoms have developed, over 20 million individual spores may be present (Garden, 2018). The spores move from an infected field to a non-infected one via dust and wind, and have a 2 to 13 week incubation period, allowing them to avoid detection. They may also be transmitted by insects and those handling the plants. The Chlamydospores can survive up to one year without infecting a new plant if they are in water. Additionally, the spores are highly virulent. A plant in a one-litre plot will be infected and killed if as little as 50,000 spores are present (Garden 2018).

Plant and Pathogen Interaction

A plant's ability to recognize pathogens is a critical component to its survival and resistance against pathogens. Plant pathogens produce effectors, small proteins that play a key role in the infection process and allow the pathogen to infect the host. Pathogen effectors appear to facilitate infection by manipulating the structure and function of the plant host cells. These pathogen effector proteins are referred to as avirulence proteins controlled by avirulence genes (AVR genes) within the pathogen. In response to this pathogen threat, plants have evolved a

defense response that includes resistance protein action, R genes. Plants secrete a specific protein product of R genes when a specific effector protein of a pathogen is detected. When a plant develops a specific R protein that matches a specific AVR protein of the pathogen, the plant can create an immune response against that particular pathogen in the future (Rouxel & Balesdent, 2010). However, pathogen effector proteins (AVR proteins) continually evolve to counter the resistance proteins (AVR proteins) of plants. Thus, the genes of plant and pathogen continually evolve, as pathogens develop virulence against plants that are ultimately countered by resistance proteins in plants.

Currently, there are seven known species of fungi that contain avirulence genes, Foc. TR4 being one of the seven. The effector proteins in Foc. TR4 are controlled by genes known as secreted in xylem (SIX) genes (Di, 2017). These effectors have been termed "secreted in xylem" genes because they release AVR proteins into xylem tissue, or the vascular system of plants. This vascular system transports water and nutrients from the roots of the plant upward throughout the entirety of the plant and is fundamentally important for plant survival (Petruzzello, 2016).

Foc. TR4 has fourteen SIX genes. Of the fourteen SIX genes, some are found to be essential to virulence, while others have unknown functions. The exact mechanism of each is unknown; however, SIX 1 and SIX 3 are found to secrete small AVR proteins linked to virulence (Czislowski, 2017).

Researchers think that fusarium wilt occurs due to water blockage in xylem tissue. After a fungus such as Foc. TR4 enters a plant through openings in the roots, blockages develop in xylem and prevent the flow of water and nutrients. These blockages are believed to form as a result of the plant response to the fungal pathogen. This blockage then causes symptoms such as discoloration and eventual death of the host (Deacon, n.d.). It takes one to two months from the first sign of discoloration in leaves until the leaves complete their wilt and cover the trunk in wilted leaves. Within a few years, the entire aboveground portions of the plant will wilt and die while Foc. continues to thrive in the soil ("Pamana disease"). Knocking out SIX genes may be one of the most promising methods in order to combat fusarium wilt.

Question

We hypothesize that knocking out SIX 1 and SIX 3 in Foc. Tr4 using CRISPR-cas9 technologies would knockout the virulence and pathogenic abilities of the fungus and remove the risk of the disease. If the genes are knocked out, when Foc. TR4 infects the host, as SIX 3 and SIX 1 will not be synthesized, the plant would react less to the AVR proteins synthesized by remaining genes, and the host would not be killed. We believe that the disease's ability to spread rapidly, and be highly virulent is best combated by lowering or disabling Foc. TR4's virulence and causing the plant to minimize its immune reaction, and not be killed or maimed by the fungus.

Hypothesis

If SIX 1 and SIX 3 are knocked out, Foc. TR4 will no longer be able produce AVR proteins that will trigger an immune response in the plant, thus blocking the flow of nutrients in xylem tissue and, therefore, the knockout will eliminate the effects of fusarium wilt on Cavendish bananas.

Systems Level

We are uncertain about the delivery method of the CRISPR treatment into the fields. We hypothesize that the most effective method would be to create a spray treatment for the fields, administered before or after planting. The spray would work on fields infected by Foc TR4 and would contain the programmed CRISPR molecules primed to migrate into the cells of the fungus. Spray droplets would land on the plants and the ground and allow any fungus which contacts the large surface area of the spray to be infected by CRISPR. The farmers would have to treat their fields more than once to account for spores moving from their spore phase into an active stage. After treatment, farmers would see a decrease in fusarium wilt, directly reflected by the lowered number of pathogenic Foc. Tr4 molecules. Once Foc. Tr4 pathogenic populations are lowered, farmers would see increased banana crops.

As the treatment is specifically targeting one organism, the effects on the ecosystem as a whole would be negligible. A lowered fungal presence on the floor of the fields would allow small species of insects that consume dead plant matter to increase in number as well as allowing other fungi to take the place of fusarium without having a negative impact on the ecosystem. There are many fields not infected by Foc. TR4 in which an opportunistic fungus could thrive without Foc. TR4 competition, yet these uninfected fields are not infected by an opportunistic fungus. Because there is no evidence of such opportunistic fungus, it is reasonably inferred that in the absence of Foc. Tr4, another fungus would not emerge to take its place. Therefore, the removal of Foc. Tr4 would not have a negative impact on the ecosystem. The introduction of the treatment, that would remove Foc TR4 in its pathogenic form, would not allow rival species detrimental to the Cavendish banana to overtake fields in the absence of competition. Insects in areas affected by Foc. TR4 would likely be similarly affected and non-conse-

quential to the ecosystem if Foc. TR4 was removed. Uninfected fields face only treatable fungi and insects that do not currently negatively affect the ecosystem. Likely, if Foc. TR4 was removed from the ecosystem or removed in its pathogenic form, there would be no consequential changes in regard to insect populations in the area.

The possibility of a random CRISPR mutation in Foc. Tr4 is a possibility, however low, that needs to be mentioned. Our team does not have the technology to test for possible mutations in CRISPR, so we are uncertain at this time how or even if it could mutate to interact with a different fungus. If the CRISPR molecules do not mutate, they would be useless if they were to interact with any organic organisms that are not genetically identical to Foc. Tr4. With extensive testing, we could discover the success rate of the treatment and calculate the probability of success in the field. If the treatment were to not be successful, the farmer would continue to see necrosis and wilt in their banana plants.

Device Level

The overarching goal of this project is to determine a way to knockout SIX 1 and SIX 3 using CRISPR Cas9 technology and demonstrate this process in a proof of concept. CRISPR Cas9 is an efficient and effective gene-editing technology. In her book, A Crack in Creation, Jennifer Doudna, who originally developed CRISPR for prokaryotic use, describes the uses and function of CRISPR Cas9. CRISPR Cas9 technology has the ability to prevent a cell from producing a protein by altering the very DNA that the chosen protein is produced from. The CRISPR Cas9 process that prevents a section of DNA that codes for a certain protein to be expressed is known as a gene knockout procedure (Doudna, 2017, p.103). This process will be adapted to knockout the SIX 1 and SIX 3 genes in Fol TR4 so that the fungus is unable to produce the AVR proteins that SIX genes code for.

Figure 1 shows the Cas9 nuclease being guided to the SIX gene and attaching upstream of the SIX gene. Next, the Cas9 nuclease cuts the SIX genes. The fungal DNA will attempt to repair itself, however, in repair without a guide molecule showing the cell how to repair, the pieces of the SIX gene will either be missing, jumbled, or rearranged in a fashion so that the stop codon appears earlier than expected or the codons are so misaranged so that they cannot be read. Thus, the DNA section will be unable to produce a functional mRNA that could be expressed as a protein. In the case of Foc TR4, the AVR proteins will likely not be produced.

Before the knockout procedure is performed with Foc TR4, a proof of concept will be performed with *E. coli* and a GFP plasmid. First, *E. coli* that expresses green fluorescent protein (GFP) will be acquired. Next, guide RNA with a corresponding amino acid sequence to that of the GFP plasmid will be acquired. Cas9 nucleases will also be acquired. The guide RNA and the Cas9 protein will act together in a CRISPR system to knockout GFP from the E. coli genome and, thus prove the concept of knocking out SIX genes from Foc TR4.

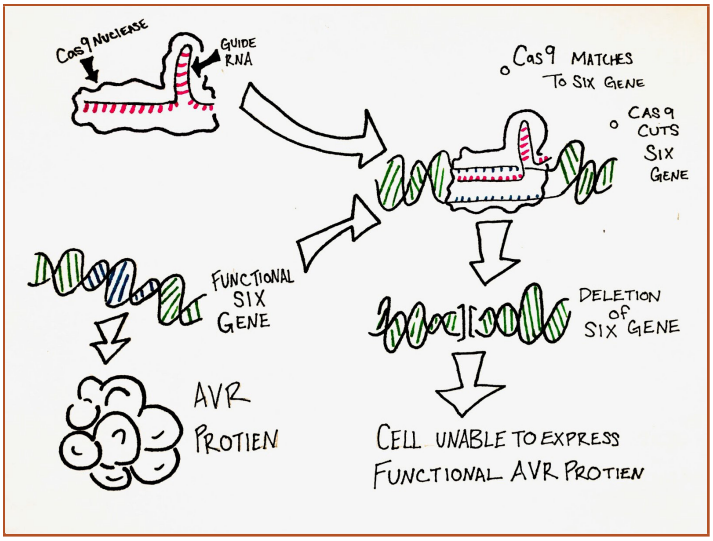

Figure 1. *CRISPR targeting SIX genes.*

Despite having a clear plan for the proof of concept, there are still roadblocks to overcome before the procedure is used on Foc TR4. First, the exact sequence of Foc TR4 DNA of SIX 1 and SIX 3 is unknown. We are currently uncertain of the exact sequence of SIX 1 and SIX 3 on the Foc TR4 chromosome. This information is necessary in order to properly program the CRISPR RNA to tell the Cas9 enzyme where to target in order to properly knock out SIX genes.

Secondly, a method of delivery must be developed in order to properly ensure that the programmed CRISPR Cas9 system would make its way into the Foc TR4 cells. Currently, we are unsure of how the CRISPR cas9 programed to target SIX genes would be delivered into the fungal cells. One possible route would be to spray infected fields with a mix that contains Cas9 enzymes with CRISPR guide RNA programed to match SIX genes and knock out the genes from Foc TR4.

Parts Level

In order to complete the experiment, all parts of the CRISPR cas9 system must be acquired. CRISPR cas9 is comprised of two main parts. The first is the cas9 protein. Cas9 is a protein that is able to bind to the DNA that it is directed to by the guide RNA and to cleave that matching DNA. Within the Cas9 enzyme, there are two nucleases capable of cutting nucleic acids. These two

modules are responsible for cutting the nucleic acids that parallel that of the corresponding guide RNA (Doudna, 2017, p. 77).

Guide RNA, also known as CRISPR RNA, is the sequence of nucleic acids that corresponds with base pairs in the locus that is to be edited, allowing the Cas9 enzyme to go to the exact location of the targeted gene, and edit out or add a specific sequence.The guide RNA also includes nucleic acids that correspond to the gene that the Cas9 enzyme will target (Doudna, 2017, p.81).

Safety

There is no evidence to suggest that this knockout could affect other organisms or the ecosystem. The knockout targets only one specific organism, and CRISPR will not edit other organisms genomes. The genes that are being targeted in this knockout are specific to *F. oxysporum*, so the knockout would not affect other species. The CRISPR that performs the knockout will be concentrated on banana fields; therefore, it would not be widespread throughout the environment. The death of this fungus would possibly allow for other fungi to take the place of it in the ecosystem, which could possibly affect the ecosystem. By killing a fungus that is prevalent within the ecosystem, there could be an opportunity for another fungus to thrive in its place. he resources being used by *F. oxysporum* f.sp. cubense would be available for other fungi to take advantage of *F. oxysporum* that could be out-competing these other fungi in the ecosystem. Without this competition, the fungi could increase in quantity.

Discussion

Given that this disease is a fungus, one of our first thoughts was that it could be useful to mimic the method of operation of an antifungal medication once inside the fungal cells of the Foc. TR4 disease. A majority of antifungal medications lyse the fungal cell walls and cause the contents to leak out and the cell to die or prevent new growth. The fungal cells may believe that they are "under attack" and commit apoptosis in an attempt to prevent further spread of the supposed attack. Unlike other plants, fungi cell walls are not composed of cellulose, they are composed of chitin, which is a strong polysaccharide that forms a strong barrier between the inside of the cell and its surroundings (Antifungal Medications, n.d.).

Ultimately, it was decided that this was not the best idea for combatting this disease. We eventually decided on researching the mapped genome of Foc. TR4 and began to do research on what makes the fungus pathogenic. We discovered that there are fourteen genes in the fungus's DNA called the Secreted in xylem genes, or SIX genes, which are essential to the pathogenicity of the fungus. We decided to figure out a method of knocking out two of the fourteen genes in an attempt to restrict the pathogenicity of the fungus.

A lot had to be considered in order for this idea to be fully fleshed out and ready for a team to come up with a solution on how to deal with this disease. The economic, international, social, and societal issues surrounding this problem had to be addressed in order to properly deal with the conundrum of Fusarium wilt. How would this affect international trade? How would this affect the local industries of small banana farmers who only sell locally and get their entire income from selling their bananas? What would ensue in Latin America, South America, East Asia, and Africa if this fungus isn't dealt with? Bananas are a huge part of the economy in many parts of the world that are already impoverished and not developed. If their main source of income were to be smothered by this disease, they would have to find another job, which would put millions of people out of work and would put an enormous strain on the local and global economy.

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