Design Brief

Utilizing allicin as a means for Fusarium wilt resistance in Cavendish banana plants*

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Fusarium wilt is an infection caused by the fungus Fusarium oxysporum f. sp. cubense (FoC) that targets Cavendish banana plants. The fungus enters the plant through the roots and blocks the plant's ability to absorb necessary nutrients. The bananas' inability to ward off this infection results in plant death. Cavendish bananas' vulnerability to this disease has prompted billions of dollars in economic losses to countries that rely on banana production and exportation. While previous trials have attempted to eradicate the disease, an effective solution to eliminate FoC is yet to be discovered. Our proposed solution highlights the usage of allicin, a compound found in garlic that has demonstrated effectiveness in stopping the growth and proliferation of fungal infections. Our design is an allicin expression system for Enterobacter cloacae that works by inserting the gene encoding allicin into a bacterial endophyte (E. cloacae) and incorporating that endophyte into the soil. As a result, we hypothesize that Cavendish banana plants can gain resistance to FoC.

Keywords: Fusarium wilt, Cavendish banana, FoC, allicin, *Enterobacter cloacae*

Cavendish bananas are one of the top food crops in the world, and they are in grave danger due to a fungal infection. They rank as the second most popular fruit, with over 115 million metric tons harvested annually (Mala, 2020). In addition to their flavor, they contain high amounts of fiber, potassium, folate, vitamin C, and vitamin B6, contributing to their global popularity. As shown in Figure 1, bananas are primarily produced in Central America, South America, and Southeast Asia (Monfreda et al., 2008). While Cavendish bananas are native to Southeast Asia and the South Pacific (Figure 1), they are traded globally and grown in more than 120 countries in tropical and subtropical regions in a monoculture (Macedo-Raygoza et al., 2019; Monfreda et al., 2008).

Cavendish bananas are a subgroup of the banana cultivar group of *Musa acuminata,* which consists of three varieties: Grand Naine, Williams, and Valery (Porcher, 2011). They descend from a reproductive cross of two wild banana species: *M. acuminata and M. balbisiana* (Castle, 2009). They reproduce via cloning, where the stem of a mature bunch is planted, resulting in the growth of a new banana tree. Since this process bypasses sexual reproduction, the species lacks genetic diversity—making it more susceptible to fungal infections, such as *Fusarium oxysporum* f. sp. *cubense* (FoC).

FoC—primarily the Tropical Race 4 strand—is a soil-borne pathogen that infects Cavendish bananas and causes Fusarium

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Figure 1. Global Banana Production (in tons of bananas produced per hectare per harvest): bananas are primarily produced in Central America, South America, and Southeast Asia. This image was generated by ArcGIS, and the information used to make it was taken from Monfreda et al.'s 2008 paper.

wilt, a form of vascular wilt disease. FoC enters the plant through the roots and colonizes the xylem vessels (Mmadi et al., 2023). This colonization blocks the flow of water and nutrients, causing breakage in the pseudostem and the collapse of leaves at the petiole, resulting in plant death (Figure 2) (Dita et al., 2018). Infected plants show a reddish-brown internal discoloration of the vascular tissue (Figure 3) (Mmadi et al., 2023). FoC infection ruins substantial amounts of banana crops, destroying banana plantations, reducing total yield amounts, and

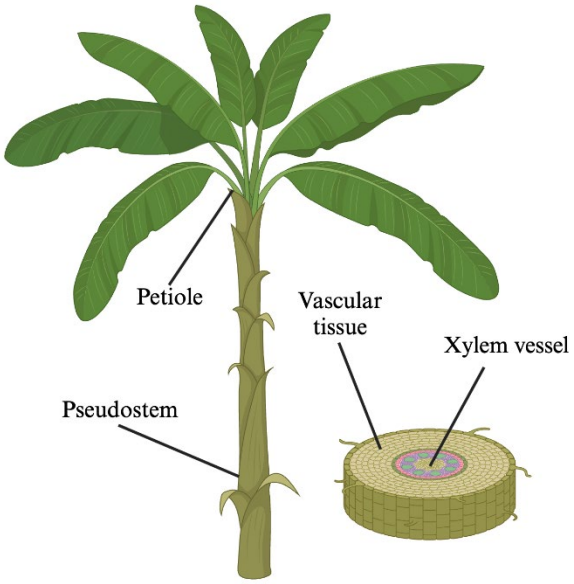

Figure 2. This image depits a Cavendish banana plant. It was created using BioRender, and the information was taken from Dita et. al.'s 2018 paper.

Figure 3. The reddish-brown discoloration of a banana plant infected with Fusarium Wilt is pictured here. This image was taken by S. Nelson on November 18, 2017, and they dedicated the image to the public domain by waiving all of their rights to the work worldwide under copyright law. The information was taken from Mmadi et. al.'s 2023 paper.

rendering economic loss.

Bananas are a major cash crop and economic staple for many countries across Asia, Africa, and South America. The over 150 million tons of Cavendish bananas produced annually create a $50 billion industry (Ploetz, 2021). Further, between 1998 and 2000, Cavendish bananas accounted for 47% of global banana production and 99% of global exports to developed countries (Arias et al., 2003). However, since its initial outbreak in the 1950s, FoC infection has dealt a staggering $2.7 billion in damages. For many "banana republics," or countries that heavily rely on the exportation of natural goods, FoC infection exacerbates social and economic stress, leaving many countries vulnerable (Mostert et al., 2017).

The persistence of FoC further intensifies economic stress. For example, once FoC infects a section of soil, it cannot be eradicated, making disease management difficult. In FoC-infested soil, resistant banana cultivars—or plants with desirable qualities selected for their fungal resistance—are the only consistently effective tools for managing the disease (Cannon et al., 2022). However, even though these resistant cultivars exist, ways to genetically engineer resistance to all races of Fusarium wilt (e.g., Race 1, Race 2, Race 3, and Race 4) have yet to be discovered, proving mass production unprofitable (Dita et al., 2018). Additionally, some chemical, cultural, and physical approaches to mitigate FoC proved both unsuccessful and cost-prohibitive (Ploetz, 2015). For example, the rice hull burning method (i.e. heat sterilization of the soil) and a land-flooding method aimed to revitalize the soil failed due to the disease's quick recolonization (Jamil et al., 2020). Due to these failures, there remains a critical need for resistant bananas.

Based on a banana plant's inability to fight off disease naturally, the biotechnology to create a method that does not harm the soil microbiome or change the fruit's taste proves necessary. Our solution incorporates a gene encoding the fungicidal compound allicin (diallylthiosulfinate), commonly found in garlic plants, into a bacterial endophyte. We selected allicin because of its active role in deterring and inhibiting the growth and proliferation of fungal infections, including FoC (Hayat et al., 2016). We plan to introduce the endophyte into the soil surrounding infected banana plants. Then, the endophytes will enter the plant through the roots, establishing a symbiotic relationship (Figure 4) (Borlinghaus et al., 2014).

A bacterial endophyte is a microorganism that inhabits a plant's rhizosphere (Figure 4). In many cases, the endophytes enter the roots because they are attracted to exudates (substances secreted by the roots). After colonizing the plant, endophytes release chemical signals to communicate with the plant (Afzal et al., 2019). One common endophyte function includes their ability to provide resistance to the host plant against both biotic and abiotic stressors via releasing antimicrobial compounds (Kandel et al., 2017). Previously, 150 bacterial endophytes of Cavendish bananas were compared and analyzed (Macedo-Raygoza et al., 2019). A later study then built upon that data by selecting the two endophytes that proved most promising: *Enterobacter cloacae* and *Klebsiella pneumoniae* (Macedo-Raygoza et al., 2019). Eventually, they found *E. cloacae* to be most effective since it establishes a nutrient-transfer symbiosis with Cavendish banana plants.

Our team plans to assist Cavendish banana plants in gaining resistance to FoC by inserting the genes encoding for each enzyme in the glutathione-to-allicin biosynthesis pathway into a plasmid. Next, we plan to pair it with a compatible promoter and transform the plasmid into *E. cloacae* for implementation into the soil. If successful, the proposed solution should aid Cavendish banana populations by bolstering them with another line of defense against Fusarium wilt. Overall, the vulnerability of the Cavendish banana plant creates a need for a solution to mitigate the effects of FoC.

Systems Level

As shown in Figure 4, our design includes the insertion of a bacterial endophyte that will live in the rhizosphere, the soil area directly surrounding the plant's roots. While there are many different kinds of endophytic bacteria, our design includes *E. cloacae* because of its

previous success against the Sigatoka disease caused by the fungus *Pseudocercospora fijiensis*. This disease infected banana plant leaves, ultimately reducing the plant's yield. Macedo-Raygoza et al.'s 2019 study provided ample evidence for *E. cloacae*'s ability to not only succeed in fighting this fungal disease but also in promoting growth in Cavendish banana plants (Macedo-Raygoza et al., 2019). In their research, the article discovered that, although all of the bacteria they tested provided some nutritional benefits to the banana plants, only *E. cloacae* set up a "sustainable symbiosis in the transference of nutrients to plants" (Macedo-Raygoza et al., 2019). The addition of a plasmid specifically designed to combat Fusarium wilt will prospectively yield positive results.

When choosing a plasmid to modify and insert into *E. cloacae*, we needed a plasmid suitable for cloning and expression within the endophyte we are using. The plasmid pUC19, a commonly used cloning vector, could be a viable option. pUC19 is commonly used for its foreign DNA compatibility and its multiple cloning site region (New England Biolabs, n.d.). Its demonstrated success in the insertion of foreign DNA will prove useful when inserting the gene encoding for allicin into the plasmid.

Allicin's demonstrated natural ability to stop the growth and proliferation of fungal infections makes it a promising compound for an effective solution. It has proved to have strong antibacterial properties as well as toxic effects on fungal cells (Hayat et al., 2016). Therefore, our primary mechanism will rely on allicin to stop the proliferation and mal effects of Fusarium wilt disease.

To understand how we could engineer *E. cloacae* to produce allicin, one first needs to understand how allicin is produced in garlic. Alliin is a sulfoxide that is present in the cell vacuoles of garlic plants. When the garlic is crushed and the structure is broken, alliin is released from the vacuoles. It comes into contact with alliinase, an enzyme stored in the cytoplasm of garlic cells. Alliinase then catalyzes the conversion of alliin into allicin,

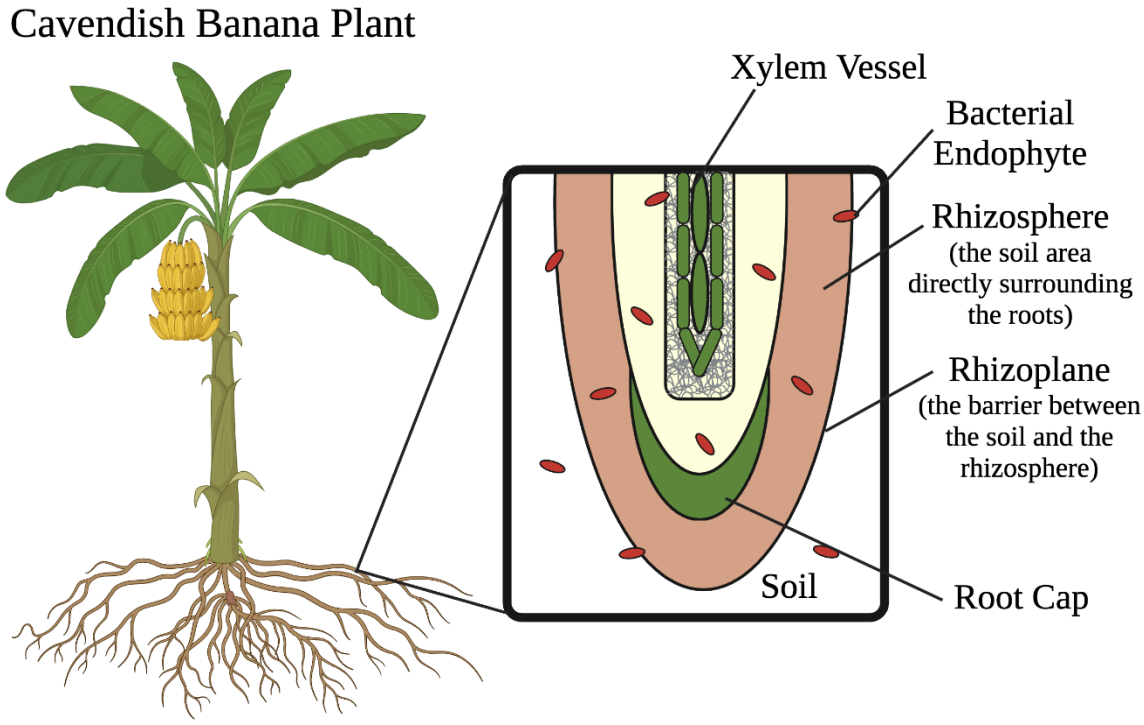

Figure 4. As shown above, endophytes colonize plant roots by first attaching themselves to the surface of the root (the rhizoplane) and then entering the plant through openings in the roots where root hairs (lateral roots) emerge. This image is a modified version of a rice plant image in Audipudi et. al.'s 2017 paper and was created using BioRender.

which is what exudes the sulfuric garlic odor (Bayan et al., 2014).

As shown in Figure 5 from Yamaguchi and Kumagai's 2019 paper, the allicin-optimized encoding gene pathway depicts two different biosynthetic routes to reach S-allyl-cysteine and then, allicin (Yamaguchi & Kumagai, 2019). The two potential substrates are serine and glutathione. Because the latter is already present in Gram-negative bacteria, we can begin our process with glutathione (Pophaly et al., 2012). The pathway for the biosynthesis of alliin progresses as follows. First, S-2-carboxypropyl glutathione is biosynthesized from glutathione with methacrylic acid that is produced from valine, an amino acid with a nucleophilic substitution at the a-carbon (Yamaguchi &

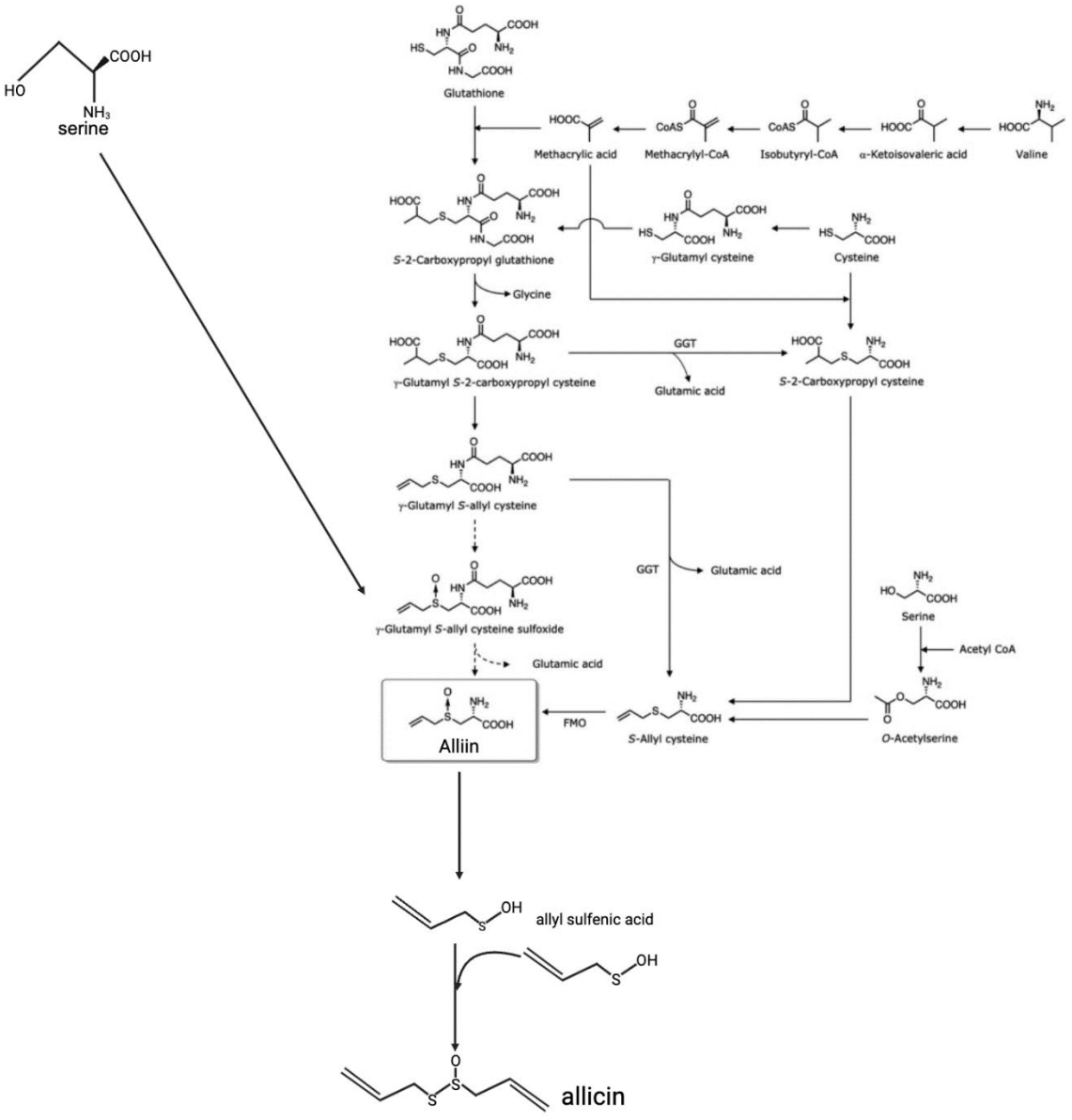

Figure 5. This diagram depicts the pathway for the biosynthesis of allicin. As shown above, there are two separate pathways by which allicin can be produced-one beginning from serine and the other from glutathione. This image was created in BioRender and adapted from a figure from Yamaguchi et. al.'s 2019 paper and a figure from Borlinghaus et al.'s 2014 paper.

Kumagai, 2019). We wanted to be sure that the valine would not alter or harm *E. cloacae*, but previous research has demonstrated that many *Escherichia coli* isolates and other bacteria secrete valine in biofilms, *E. cloacae* being one of them. Because of this, we can assume that valine will be harmless to *E. cloacae* (Valle et al., 2008). The article includes two possible pathways for forming S-2-carboxypropyl cysteine.

The first pathway uses γ-glutamyl S-2-carboxypropyl cysteine, which is derived from S-2-carboxypropyl glutathione with the elimination of glutathione using the enzyme γ-glutamyl transferase (Yamaguchi & Kumagai, 2019). The second uses merely generation from cysteine and methacrylic acid without glutathione. Both of these pathways can be compared to see which is most efficient. First, decarboxylation and oxidation of the carboxypropyl group produce an allyl group. Because of this, γ-glutamyl S-allyl cysteine can be produced from γ-glutamyl S-2-carboxypropyl cysteine using the enzyme γ-glutamyl transpeptidase, and S-allyl cysteine can be produced from S-2-carboxypropyl cysteine by flavin-containing monooxygenase (Yamaguchi & Kumagai, 2019). Then, The S-oxidation of γ-glutamyl S-allyl cysteine forms γ-glutamyl S-allyl cysteine sulfoxide, while that of S-allyl cysteine forms alliin. Finally, the enzyme alliinase converts alliin into allicin. A smell test can be used to test if the pathways and conversion have been successful because, if produced, allicin will produce a sulfuric garlic odor. The amount of allicin produced would be approximately 0.1 to 7 mg fresh weight due to the abundance of alliin in garlic.

Since we don't believe that allicin has previously been inserted into *E. cloacae*, the next steps in the process are less cemented. After purifying and obtaining allicin, it would then be inserted into our plasmid, pUC19. After designing the plasmid with one of the promoters and terminator sequences described in the following sections, the allicin would be cloned into the plasmid using polymerase chain reaction (PCR) to amplify the gene and subsequently insert it into the plasmid. Next, to transform *E. cloacae* with the plasmid, methods such as shock or electroporation could be adopted. Then, to confirm gene expression, a simple smell test will be used. Once we have selected the successfully expressed systems, we would inoculate them into the rhizosphere by mixing the culture directly into the soil.

Device Level

Efficient allicin output is essential to the overall design effectiveness. The genes that encode for the enzymes and the nitrate-driven *ZmNRT2.1* or *ZmNRT2.2* promoter will likely be inserted into our plasmid: pUC19 (Meng et al., 2020). However, further promoter testing of possible alternatives will be necessary since we have carried out no prior testing of the *ZmNRT2.1* and *ZmNRT2.2* promoters. So, in summary, the plasmid design will include one of the aforementioned promoters, the optimized allicin-encoding gene pathway, and likely the TTT GTA terminator sequence (Figure 6).

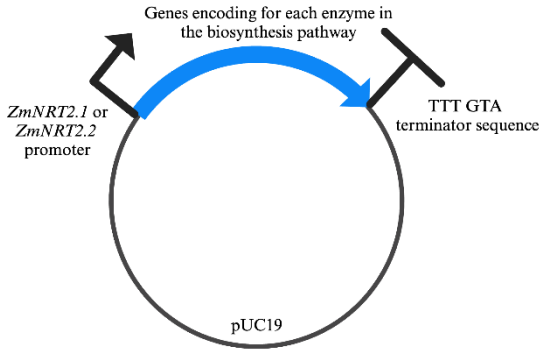

Figure 6. This image shows our plasmid design including our possible promoters and our terminator sequence. This image was created in BioRender.

Parts Level

As previously stated, when FoC infects Cavendish banana plants, it colonizes the xylem vessels, thereby inhibiting the flow of nutrients. Although reinstating any of the inhibited nutrients would not directly aid the banana plant in fighting FoC, they can be utilized to find a suitable promoter (Pegg et al., 2019). For example, since nitrogen is a key inhibited nutrient, we chose to investigate promoters that can detect a lack of

nitrogen. Because of that, *ZmNRT2.1* and *ZmNRT2.2* seem like plausible options. *ZmNRT2.1* and *ZmNRT2.2* are two gene sequences commonly found in corn that code for a nitrate transporter protein (Meng et al., 2020). The nitrate-responsive *cis*-elements of *ZmNRT2.1* and *ZmNRT2.2* can detect differences in soil nitrogen levels and, in turn, trigger the activation of specific gene sequences tailored to respond to the detected nitrogen levels (Meng et al., 2020).

However, if those promoters do not work with our expression system as planned, we are also looking into promoters that can detect changes in soil pH levels. Since a lack of nitrogen in the soil would decrease the overall pH, a promoter—such as a PASR promoter—that can detect that decrease, could also prove to be a possible solution (Sužiedėlienė et al., 1999).

Next, regarding allicin, or diallylthiosulfinate: it is an organosulfur compound derived from an amino acid (alliin) that is found in garlic. In fact, upon mechanical crushing, allicin makes up approximately 70% of the thiosulfinates present in garlic cloves. While allicin has proven to possess antimicrobial, anticancer, and hypolipidemic properties, we are focusing on its antifungal properties. These effects can be attributed to its potent SH-modifying capacity and antioxidant characteristics. Further, the active disulfide bond -S(O)-S- within allicin interacts with various thiol-containing compounds, including proteins containing SH groups (Miron et al., 2004).

Finally, as for our chosen terminator sequence, we selected TTT GTA. The terminator region is the part of the sequence that determines the detachment of RNA polymerase from the DNA strand, occurring at the end of the transcription process. The terminator gene sequence includes the following elements: coding sequence (CDS), cleavage element (CE), cleavage site (CS), downstream element (DSE), far upstream element (FUE), near upstream element (NUE), polyadenylation signal (PAS), and upstream element (USE) (de Felippes & Waterhouse, 2022). The three different terminator regions offered by de Felippes and Waterhouse are designed for mammals, yeast, and plants, respectively. Since allicin is ultimately derived from an enzyme found in garlic, our terminator region should use the code sequence designed for plants, hence the bottom sequence (Figure 7).

The terminator sequence for plants begins with the codon UUU GUA (corresponding to TTT GTA in DNA). Therefore, the 1,740 bp length alliinase DNA sequence will end with TTT GTA to determine the detachment of RNA polymerase (*Allium Cepa*, 1993). In conclusion, with the previously mentioned components, we will create a plasmid that

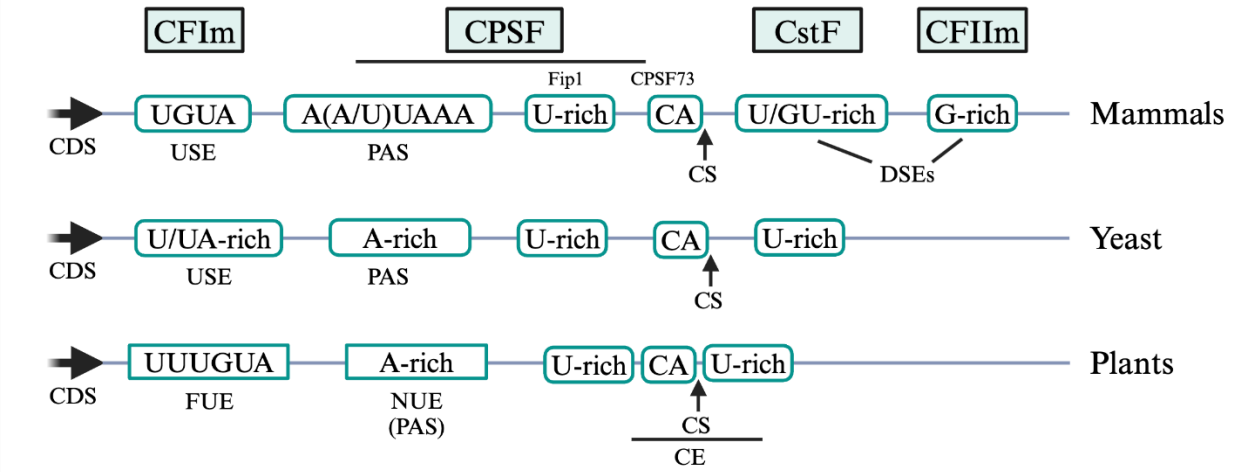

Figure 7. This is a schematic representation of the terminator regions of mammals, yeast, and plants. As pictured, mammals have a U/GU-rich and a G-rich section that plants and yeast lack. The image was created in BioRender and the information was taken from de Felippes, F. F., and Waterhouse, P. M.'s 2022 paper.

accommodates *E. cloacae*.

Safety

While *E. cloacae* colonies already live in banana plants' rhizospheres (Afzal, 2019), our planned modification of *E. cloacae* might yield other unintended consequences. Firstly, according to the Centers for Disease Control and Prevention, *E. cloacae* has a biosafety level of 2, meaning all necessary personal protective equipment should be worn while working with it. However, since *E. cloacae* already lives naturally among Cavendish banana plants, we do not anticipate it posing problems after insertion.

Another design concern is the introduction of an allergen, allicin, into the plant roots. Since allicin will prevent the growth of FoC, it will likely also inhibit the growth of other plant-pathogenic bacteria and fungi (Turacoz Healthcare Solutions, 2020). However, since our proposal was designed to be implemented only in isolated monoculture Cavendish banana plantations, this should not pose a threat. Additionally, allicin is a low molecular weight type IV contact allergen, meaning it is skin-permeable, and individuals with an allergy must have a genetic predisposition. We do not know if inserting allicin into the plant's roots will also introduce allicin into the fruit, barring individuals with allicin/garlic allergies from eating bananas. To create a specific test for fruit contamination, more research is needed, but enzyme-linked immunoassay (ELISA) and PCR tests are viable options.

Next, we also need to consider the regulations of genetically modified organisms (GMOs) and how we can ensure that our solution meets the necessary requirements for release into the ecosystem. Three federal agencies within the United States regulate GMOs: U.S. Food and Drug Administration (FDA), U.S. Environmental Protection Agency (EPA), and U.S. Department of Agriculture (USDA) (*How GMOs*, 2024). Therefore, we must examine each organization's policies to ensure our solution will be approved. Firstly, the FDA focuses on the consumption of genetically modified food. Although we will be modifying the bacterial endophyte and not the banana plant itself, we will still need to be certain that the quality of the banana fruit will not be compromised and that it will remain safe for human consumption (*How GMOs*, 2024).

Secondly, the EPA ensures that the environment remains unharmed by the release of GMOs. On its website, the EPA discloses the factors that they consider when approving GMOs, which include studies assessing the risks to human health, studies assessing risks to nontarget organisms and the environment, the potential for gene flow, and the need for insect resistance management plans (*EPA's Regulation*, 2024). Based on these factors, we will assess our design to ensure the environment is not compromised. However, as previously mentioned, this will likely not pose a problem because we only plan to incorporate our design into isolated Cavendish bananas operating as a monoculture system.

Lastly, we must look to the USDA, specifically their Animal and Plant Health Inspection Service (APHIS), for regulations surrounding the protection of agriculture from pests and diseases (*Regulation of Biotech*, n.d.). Our solution will need to be tested—by first undergoing controlled simulative tests in a laboratory setting—to guarantee that the allicin inserted into the soil does not impact the surrounding organisms and ecosystem. It is also important to note that these regulations are United States-specific and may not directly translate to the countries that would utilize our solution. In Central America, for example, as a part of the Central American Initiative on Biotechnology and Biosafety, genetically modified agricultural products must be labeled; however, they are not outlawed. Thus, our design should meet the necessary requirements for implementation (Rocha & Muñoz, 2013).

Furthermore, pesticides are currently the primary method for combatting Fusarium wilt. The pesticide residue has proven to have adverse effects on plants, soil, and even human health (El-Aswad et al., 2023). The residue may induce adverse health effects, including cancer and other negative effects on reproduction, immune, or nervous systems. Conversely, although our design has some risks, it seems a much safer option than

the harmful pesticides currently in use. So, in conclusion, by testing our design and working with federal and international agencies, we can ensure that it is safe for human consumption and the surrounding environment.

Discussion

We opted to use endophytes as a vehicle for expressing genes that combat fungi because, in their native environments, endophytes are nonpathogenic, symbiotic microorganisms dwelling among plant cells that are adept at enhancing both plant growth and immunity (Gouda et al., 2016). Specifically, we chose the bacterial endophyte *E. cloacae* as our chassis in which to insert the plasmid-containing genes since it has proven successful in a previous study because of its ability to establish a nutrient-transfer symbiosis with Cavendish banana plants (Macedo-Raygoza et al., 2019). Furthermore, since *E. cloacae* already exists within the soil and roots of Cavendish banana plants, it is naturally in a prime location to target FoC, since FoC is a soil-borne pathogen (Mmadi et al., 2023). Due to these reasons, we believe that incorporating *E. cloacae* in our proposed design will reduce the risk of Cavendish banana plants rejecting the insertion and optimize the effectiveness of plasmid expression. Additionally, we opted to utilize an endophyte instead of mass-producing allicin like a fungicide because it has been proven that allicin will enter the plant via the endophyte since *E. cloacae* lives naturally in and near Cavendish banana plants. Further, since allicin can only remain in the soil for a limited time on its own, the endophyte will allow the allicin to be produced continuously, thereby reducing the human effort needed to constantly add additional allicin. Thus, we chose to use biosensing promoters to regulate the amount of allicin produced.

Another significant benefit of this design lies in harnessing the unique attributes of endophytes. Endophytes, harmless bacteria that have the capacity for vertical transmission, can be transmitted between parent Cavendish banana plants and their offspring. By residing within and passing on desired genes to their offspring, our allicin expression system for *E. cloacae* has the ability to provide immunity to future generations by incorporating specific FoC-resistant genes. This proposal, when compared to traditional approaches such as fungicides, offers a more sustainable, long-lasting, and less labor-intensive means of safeguarding Cavendish banana plants (Gong et al., 2022).

There are several potential obstacles to consider when implementing and experimenting with endophyte design. The primary challenge lies in controlling other variables (e.g., soil type, insects, etc.) to ensure the collection of dependable data regarding the impacts of antifungal genes on Cavendish banana plants. Since some banana plants already possess antifungal properties and defense mechanisms, when testing, we must find a Cavensish banana strain that has not been previously exposed to FoC so that the strain lacks any natural defense against the infection (Behiry et al., 2019).

Next Steps

The following steps cover the testing and execution of our research. First, we will utilize the allyl-cysteine compound to extract the allicin compound. The Systems Level section expands upon the exact process undergone for the extraction of allicin. Second, we must purify the allicin to ensure that we are not harming the *E. cloacae* and that we are not inserting anything potentially harmful into the environment. After the purification of allicin, we will work to insert the genes encoding the enzymes for the pathway of allicin into pUC19. We will have to modify the plasmid by using nitrate-driven promoters and changing the terminator so the compound can directly sense the blockage of nutrients caused by FoC growth in the roots, which can begin to combat the infection. Third, for the successful expression of allicin in the soil, we must transform it into *E. cloacae*. Finally, we will need to confirm the gene expression through a smell test to ensure the compound will perform as hypothesized. Once we are sure our expression systems are successful and federal and international agencies approve the safety of our GMO, we will introduce our endophytes into the

surrounding soil of banana plantations.

Once our design proves successful in halting the growth and proliferation of Fusarium wilt in the Cavendish banana, we are prepared to expand our solution. FoC also impacts other plants because of a similar lack of biodiversity due to the importance of monoculture and high yield in modern agriculture. Thus, expanding our solution (with some slight modifications) can aid in combating other fungal infections besides solely FoC. We hope that the expansion of our solution will significantly aid developing countries whose economies heavily rely on crop exportation.

Author contributions

Evangeline Campbell is credited with the project idea. M.L., P.M., K.M., A.P., and H.W. contributed to the research and paper components. P.M. and K.M. created the images and video. M.L., P.M., K.M., A.P., and H.W. completed the citations.

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