PPB Engineering Success

Overview

1) **Design**: Designed two toehold biosensors: Fusarium pair 1 (BBa_K3725020) and Phytophthora (BBa_K3725010) and complementary triggers.
2) **Build**: Performed dual plasmid transformation on both Fusarium pair 1 (BBa_K3725020) and Phytophthora (BBa_K3725010).
3) **Test**: Quantified fluorescence and optical density using the plate reader - results showed that LB had more fluorescence than the dual plasmid cells.
4) **Learn**: Deduced that background GFP expression in LB was skewing fluorescence data and found a better basis for comparison, the lack of IPTG to induce the T7 promoter could explain the lack of dual plasmid fluorescence, and there were potential compatibility issues with Fusarium pair 1.
5) **Design**: Designed Fusarium pair 2 (BBa_K3725022) to account for compatibility issues in Fusarium pair 1.
6) **Build**: Performed dual plasmid transformation on both Fusarium pair 2 (BBa_K3725022) and Phytophthora (BBa_K3725010).
7) **Test**: Quantified fluorescence and optical density using the plate reader - results showed that dual plasmid cells had more fluorescence than pUC19 and toehold cells.
8) **Learn**: Concluded that Fusarium pair 2 (BBa_K3725022) and Phytophthora (BBa_K3725010) were compatible and functioning toehold biosensors.
9) **Future**: Planned on implementing toehold biosensors in a safe cell-free system and developing more biosensors as part of an affordable plant pathogen diagnostic kit.

Design

Our team designed two novel biosensors, parts BBa_K3725020 and BBa_K3725010, to detect *Fusarium oxysporum f. Sp. lycopersici* and *Phytophthora cryptogea* respectively. Both biosensors followed a similar construct, but with different switch and trigger sequences. The toehold and trigger constructs contained eight random base pairs at the beginning and end of the sequence to prevent our prefix and suffix from getting compromised due to degradation. The presence of the trigger sequence will cause it to bind to the toehold switch, unraveling the hairpin loop and exposing the ribosomal binding site. A ribosome will then attach and GFP will be expressed [1]. Because the trigger sequence is derived from a gene specific to the pathogen of interest, GFP expression allows us to confirm that the specific pathogen is present. For the detection of *Phytophthora cryptogea* and *Fusarium oxysporum f. sp. lycopersici*, Lambert iGEM focused on the X24 and FRP1 genes, respectively. We selected these genes because they were

required for pathogenicity in their respective host organisms and were unique to the species of interest. We obtained these sequences via UniProt, an online database of protein sequences.

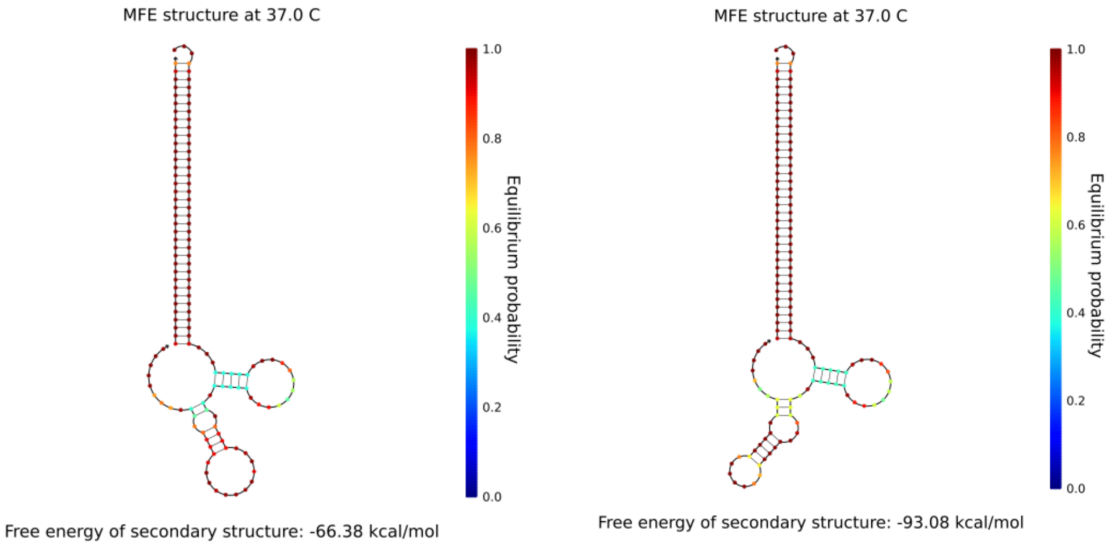

Figure 1. *NUPACK's calculated optimal toehold switch structure designs for the Fusarium (left) and Phytophthora (right) toeholds.*

The *Fusarium* Toehold w/ GFP Reporter (BBa_K3725020) is composed of four basic parts: the T7 promoter (BBa_J64997), the switch sequence (BBa_K3725050), a GFP reporter (BBa_E0040), and the T7 terminator (BBa_K731721).

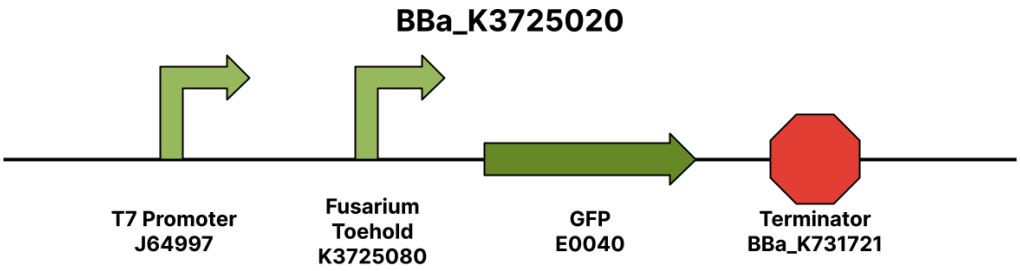

Figure 2. *Diagram of the BBa_K3725020 construct*

The T7 *Fusarium* Trigger (BBa_K3725070) is composed of eight random base pairs, the biobrick prefix, the T7 promoter (BBa_J64997), the trigger sequence (BBa_K3725060), and the T7 terminator (BBa_K731721).

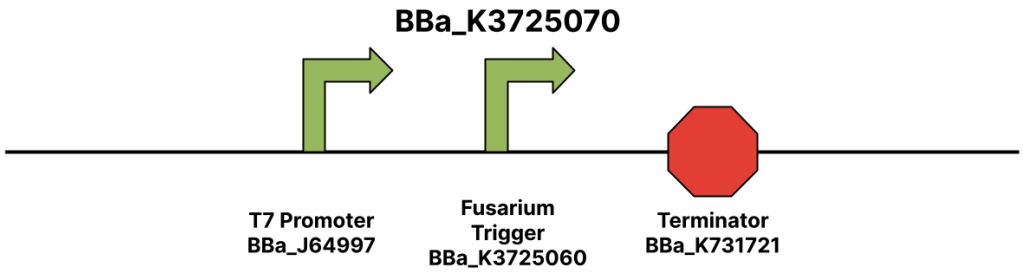

Figure 3. *Diagram of the BBa_K3725070 construct.*

The *Phytophthora* Toehold w/ GFP Reporter (BBa_K3725010) is composed of four basic parts: the T7 promoter (BBa_J64997), the switch sequence (BBa_K3725050), a GFP reporter (BBa_E0040), and the T7 terminator (BBa_K731721).

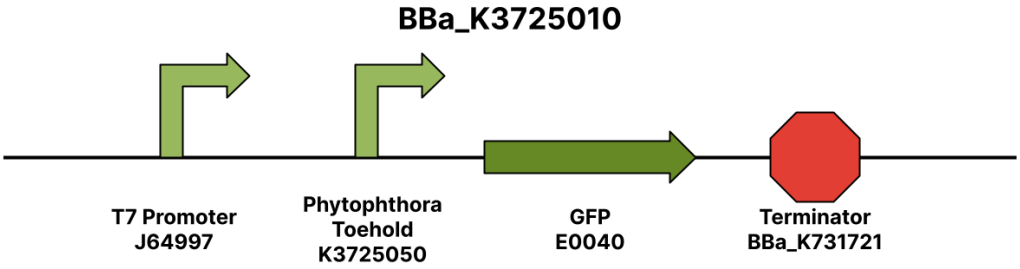

Figure 4. *Diagram of the BBa_K3725010 construct.*

The T7 Phytophthora Trigger (BBa_K3725040) is composed of four basic parts: the T7 promoter (BBa_J64997), the trigger sequence (BBa_K3725030), and the T7 terminator (BBa_K731721).

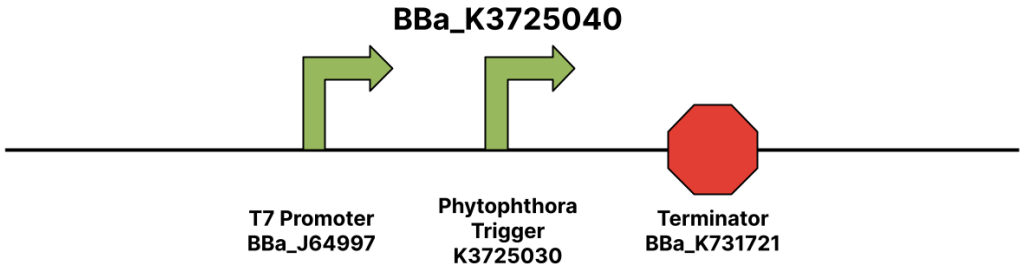

Figure 5. *Diagram of the BBa_K3725040 construct.*

Build

After ordering the toehold sequence assembled into the puCIDT Amp GoldenGate plasmid and the trigger sequence assembled into the puCIDT Kan plasmid from Integrated DNA Technologies (IDT), we attempted dual plasmid transformation. We hydrated both parts with 20µL of sterile Milli-Q water to make 20µL of 200 µg/µL of storing stock. For dual plasmid purposes, we diluted the storing stock to 30µL concentration working stocks. We performed electrocompetent transformation with a voltage of 1.8kv using a micropulser, grew the electrocompetent cells in SOC, and plated on kanamycin-carbenicillin plates. Cells grew within 1-2 days, and after performing a miniprep and restriction digest, we confirmed the uptake of both plasmids by running a gel and seeing two clear bands at the correct lengths for both parts.

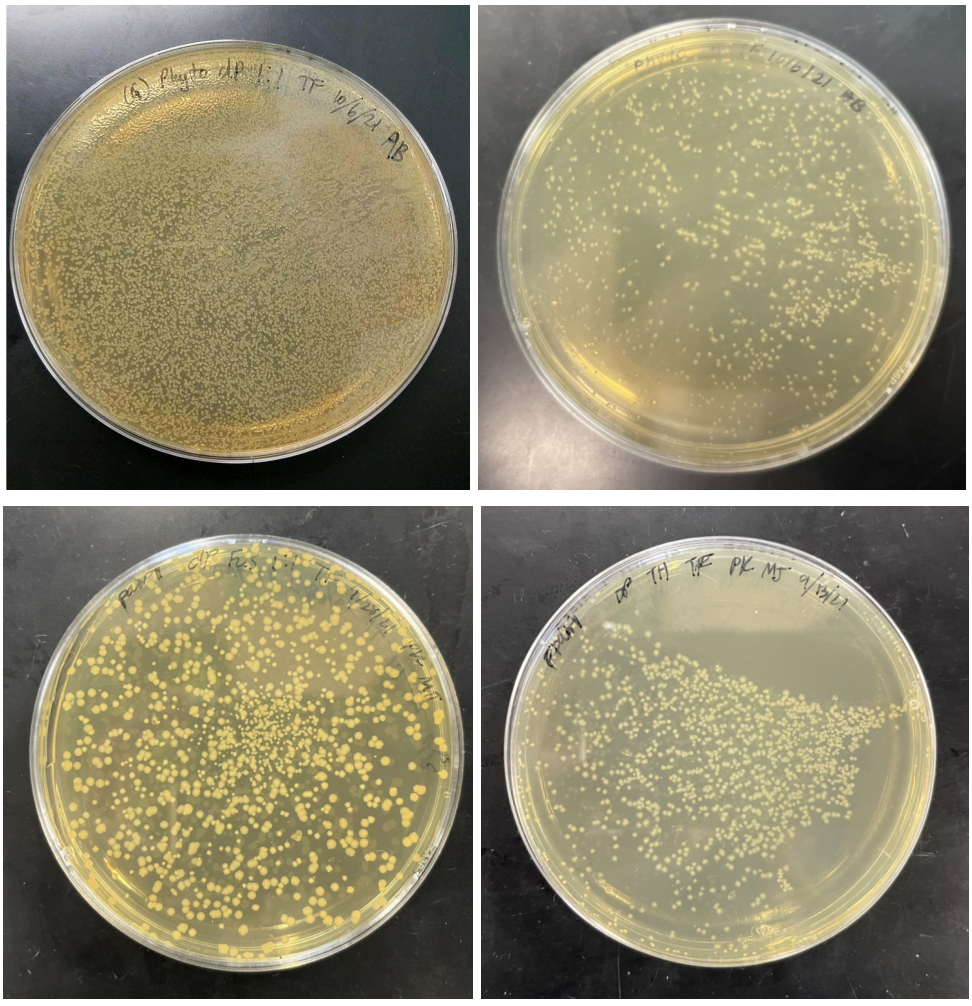

Figure 6. *Dual plasmid transformation on Fusarium (bottom left) compared to transformation of Fusarium toehold (bottom right) after 2 days of growth. Phytophthora dual plasmid transformation (top left) compared to Phytophthora toehold (top right) is displayed after 2 days of growth.*

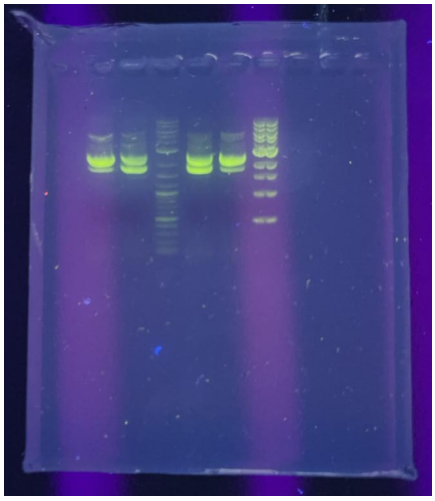

Figure 7. *Gel confirming successful dual plasmid transformation of Phytophthora toehold switch and trigger. Wells 1, 2, 3, and 4 contain the Phytophthora toehold and trigger at 2,800bp and 2,000bp, respectively.*

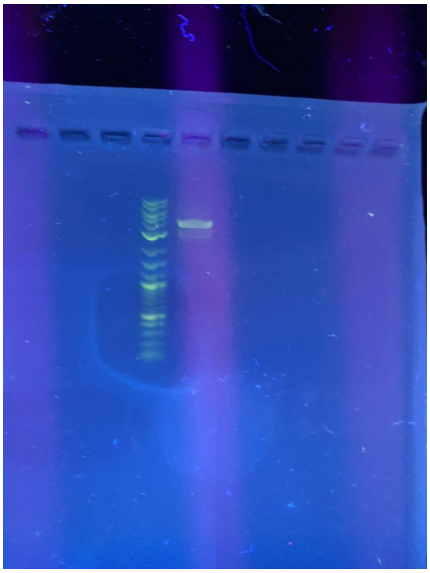

Figure 8. *Gel showing unsuccessful dual plasmid transformation of Fusarium toehold switch and trigger. Despite seeing two bands, the sizes of both are above our desired lengths of 2,800bp and 2,000 bp for toehold and trigger. Well 5 contains bands at 4,000bp and 3,000bp, respectively.*

Test

Experimental Design

To test whether our dual plasmid transformations were successful, we measured and compared the fluorescence and optical density (OD) of our dual plasmid transformations, both toeholds, and plain LB. In order for our dual plasmid transformations to be deemed successful, the fluorescence/OD of the dual plasmid cells should have a significant difference compared to that of the toehold and plain LB.

The dual plasmid cells were grown in culture tubes containing 5mL of kanamycin-carbenicillin LB and the toehold cells were grown in culture tubes containing 5mL of carbenicillin LB. After overnight incubation, cell growth and fluorescence were measured using a plate reader.

We first centrifuged the culture tubes containing our dual plasmid and toehold cells, then they were vortexed to ensure that the cells were evenly distributed. The resultant liquid was divided into 150μL increments and pipetted into a well plate, along with 150μL increments of plain LB. The fluorescence and OD values were quantified using a plate reader.

Results

We ran multiple trials and created a graph displaying mean fluorescence/OD and SEM error bars using the data from the plate reader. The SEM bars for the *Fusarium* dual plasmid transformation compared to LB and toehold overlapped, showing no significant difference in fluorescence/OD. However, the fluorescence/OD data of the *Phytophthora* dual plasmid transformation showed that plain LB had more fluorescence than the dual plasmid cells.

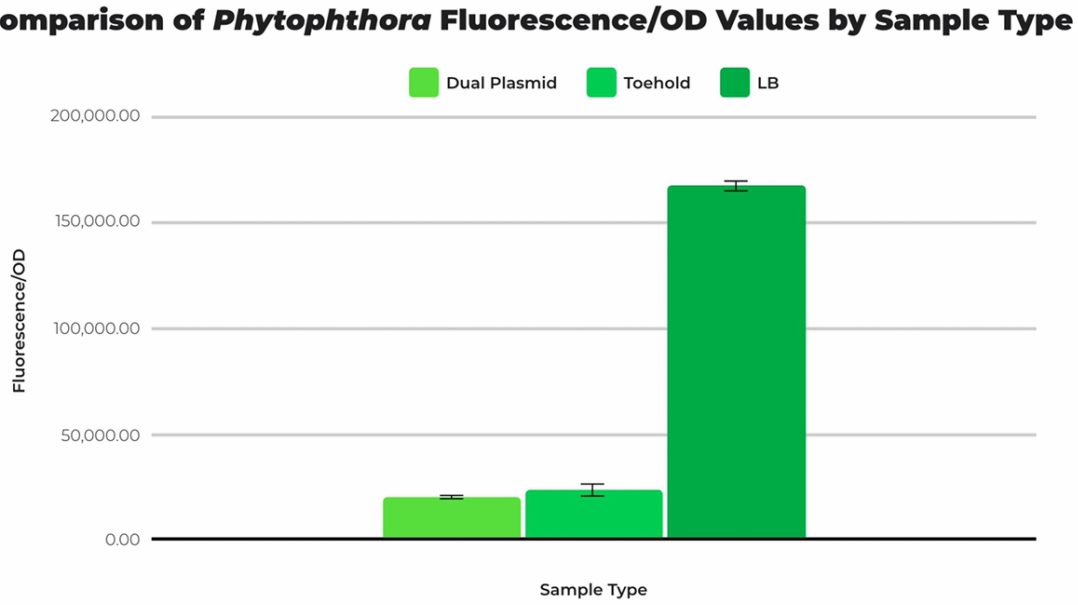

Figure 9. *Mean fluorescence/OD of Phytophthora dual plasmid transformation compared to plain LB with SEM error bars. DP stands for dual plasmid. Ran at gain of 70.*

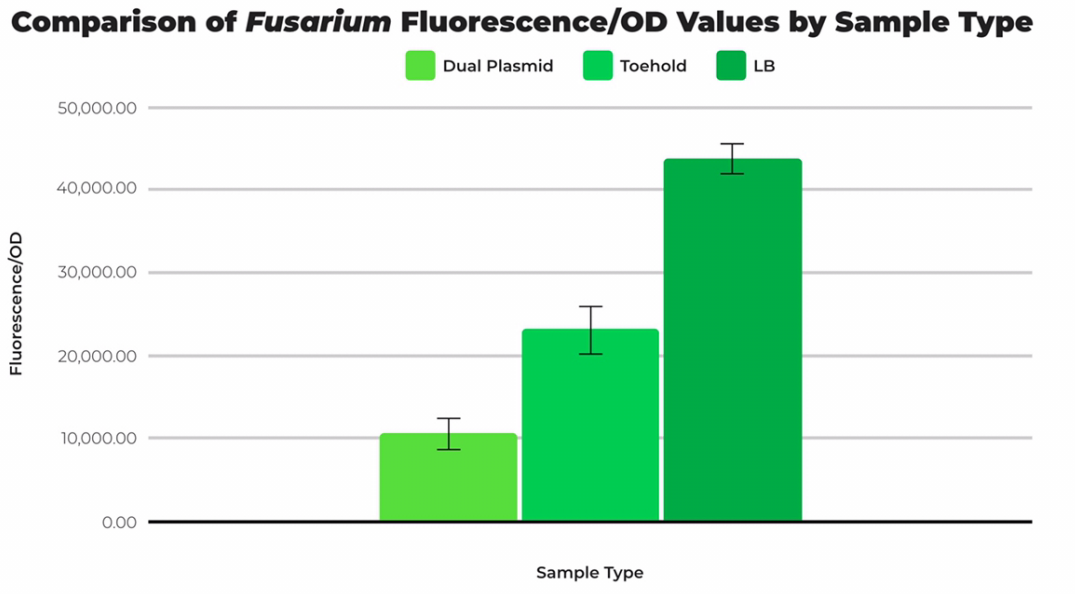

Figure 10. *Mean fluorescence/OD of Fusarium dual plasmid transformation compared to toehold and plain LB with SEM error bars. DP stands for dual plasmid, TH stands for toehold only. Ran at a gain of 60.*

Learn

Potential Problem	Solution
Background expression of GFP in LB	We did not take into account the potential of LB background fluorescence skewing our data. Because of background expression in GFP inflating our fluorescence values for LB, we decided to grow our cells in LB and resuspend them in water. Since water is clear, the possibility of background fluorescence interfering with our plate reader results would be eliminated..
Expression was not induced	Because we used the T7 promoter, IPTG is needed to induce expression of GFP. IPTG is not present in the NEB 10 beta electrocompetent cells used in our transformation, so our low fluorescence may be attributed to a lack of IPTG. As a result, we added 30μL of IPTG into each liquid culture before quantifying fluorescence and OD using the plate reader.
Did not have a reliable basis for comparison	LB served as a poor basis of comparison since it contains background fluorescence and no cells, so the fluorescence values were inflated while the OD values were very low, resulting in a high value for fluorescence/OD. As a better basis for comparison, we decided to compare our dual plasmid cells to pUC19 plain cells that would yield more similar OD values. We continued to compare our dual plasmid cells to the toehold cells to ensure that GFP expression was not constitutively expressed in the absence of the trigger.
Compatibility issue between toehold and trigger	After analyzing the gel results for Fusarium pair 1, we thought the bands were at the correct base lengths, but our fluorescence data was statistically insignificant, so we concluded that we had a compatibility issue. To design Fusarium pair 2, we selected the pair with the second-lowest normalized ensemble defect. We retried dual plasmid transformation, but after revisiting our Fusarium pair 1 data to revise our protocols, we realized that the base lengths on the gel were actually incorrect. Because of time constraints, we decided to continue our dual plasmid transformation with Fusarium pair 2 and followed the changes applied to the problem listed above.

Design

BBa_K3725022 Design

The Redesigned *Fusarium* Toehold w/ GFP Reporter part BBa_K3725022 is designed to be used in conjunction with the T7 F. oxysporum f.sp. lycopersici trigger to express GFP as a part of the engineered toehold switch system. After inputting the FRP1 gene sequence into the NUPACK software, pairs of trigger sequences and switch sequences were outputted. The pairs were ordered by normalized ensemble defect, which is the average percentage of nucleotides incorrectly paired relative to the specified secondary structure [2]. Part BBa_K3725022 was the generated pair with the second-lowest normalized ensemble defect. The sequence was then ordered as an insert in a pUCIDT (Amp) Golden Gate vector provided by IDT.

The Redesigned *Fusarium* Toehold w/ GFP Reporter (BBa_K3725022) is composed of four basic parts: the T7 promoter (BBa_J23100), the switch sequence (BBa_K3725080), a GFP reporter (BBa_E0040), and the T7 terminator (BBa_K731721).

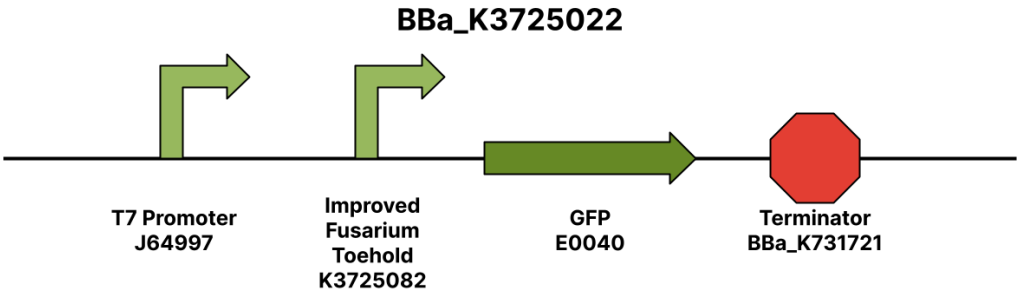

Figure 11. *Diagram of the BBa_K3725022 construct, the redesigned Fusarium Construct*

Build

We ordered the DNA for part BBa_K3725022 and hydrated following the same method as the first cycle. We performed electrocompetent transformation with a voltage of 1.8kv using a micropulser, grew the electrocompetent cells in SOC, and plated on kanamycin-carbenicillin plates. Cells grew within 1-2 days, and after performing a miniprep and restriction digest, we confirmed the uptake of both plasmids by running a gel and seeing two clear bands at the correct lengths for both parts.

Figure 12. *Dual plasmid transformation on redesigned Fusarium toehold and trigger pair*

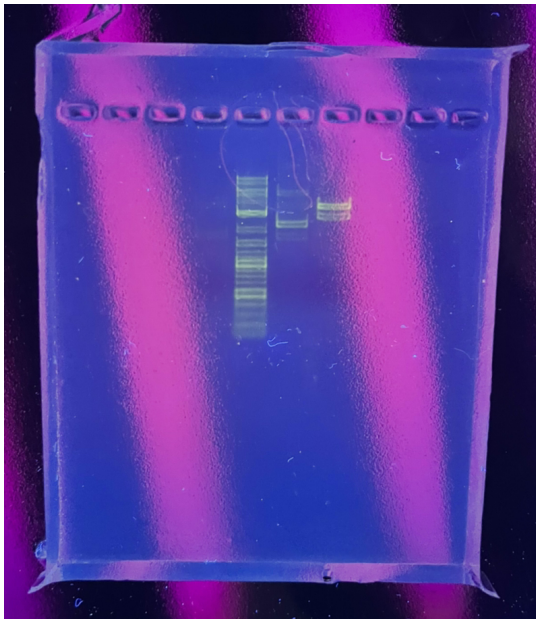

Figure 13. *Gel confirming successful dual plasmid transformation of Fusarium toehold switch and trigger. Well 6 contains the correct Fusarium toehold and trigger at approximately 2,800 bp and 2,000bp, respectively.*

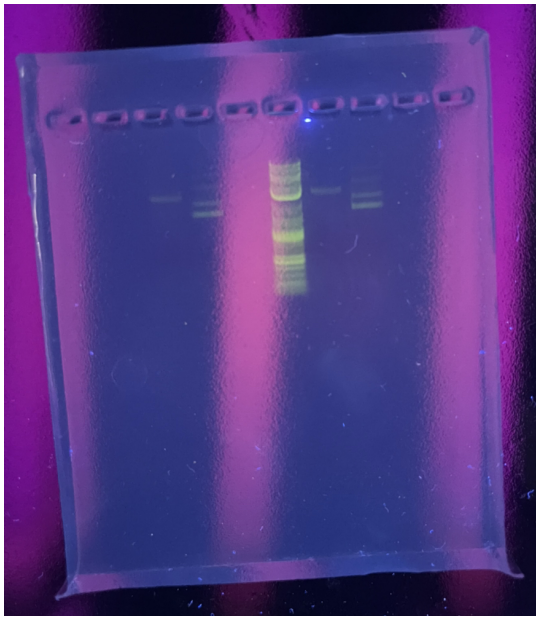

Figure 14. *Gel confirming successful dual plasmid transformation of Phytophthora toehold switch and trigger. Wells contain the Phytophthora toehold and trigger at 2,800bp and 2,000bp, respectively.*

Test

Experimental Design

To test whether our second dual plasmid transformations were successful, we measured and compared the fluorescence and optical density (OD) of the dual plasmid transformation, toehold, and pUC19 cells. In order for the dual plasmid transformation to be deemed successful, the fluorescence/OD of the dual plasmid cells should have a significant difference compared to that of the toehold, trigger, and pUC19 cells.

The dual plasmid cells were grown in culture tubes containing 5mL of kanamycin-carbenicillin LB, the toehold cells were grown in culture tubes containing 5mL of carbenicillin LB, and the trigger cells were grown in culture tubes containing 5mL of kanamycin LB. After overnight incubation, cell growth and fluorescence were measured using a plate reader.

We first transferred the 5mL liquid cultures into 250mL Erlenmeyer flasks, adding LB to each flask to a total volume of 30mL. After growing the liquid cultures in the shaking incubator for two hours, we measured the OD of each culture using a spectrophotometer. Because the active growing range of *E.coli* cells is at an OD value between 0.4 and 0.8, we added 30μL of IPTG once the OD was at 0.5. After adding IPTG, each liquid culture was allowed to grow in the shaking incubator for 40 more minutes.

After the incubation period, the liquid cultures were transferred into a falcon tube, centrifuged down, and the LB supernatant was discarded. Because of background GFP expression in LB potentially interfering with our fluorescence readings, we washed each liquid culture twice by first resuspending the cells in 6mL of water, centrifuging it down, and discarding the supernatant. After the second cycle, the liquid cultures were vortexed to ensure that the cells were evenly distributed. In a well plate, each liquid culture was divided into 150μL increments and the fluorescence and OD values were then quantified using a plate reader.

Results

We ran multiple trials and created a graph displaying mean fluorescence/OD and SEM error bars using the data from the plate reader. The SEM bars for the dual plasmid transformations for both *Fusarium* and *Phytophthora* compared to their respective toeholds and pUC19 yielded statistically significant results since the SEM bars did not overlap.

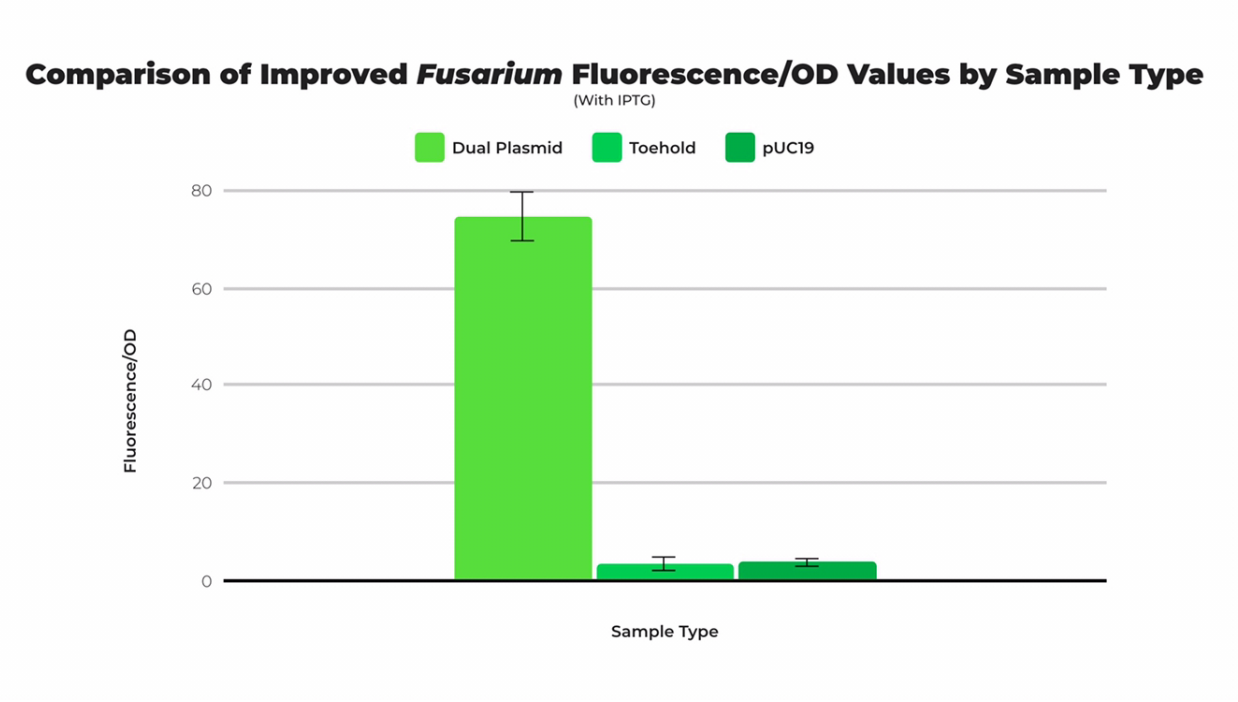

Figure 15. *Mean fluorescence/OD of IPTG-induced Fusarium pair 2 dual plasmid transformation compared to toehold and pUC19 with SEM error bars. Ran at gain of 40*

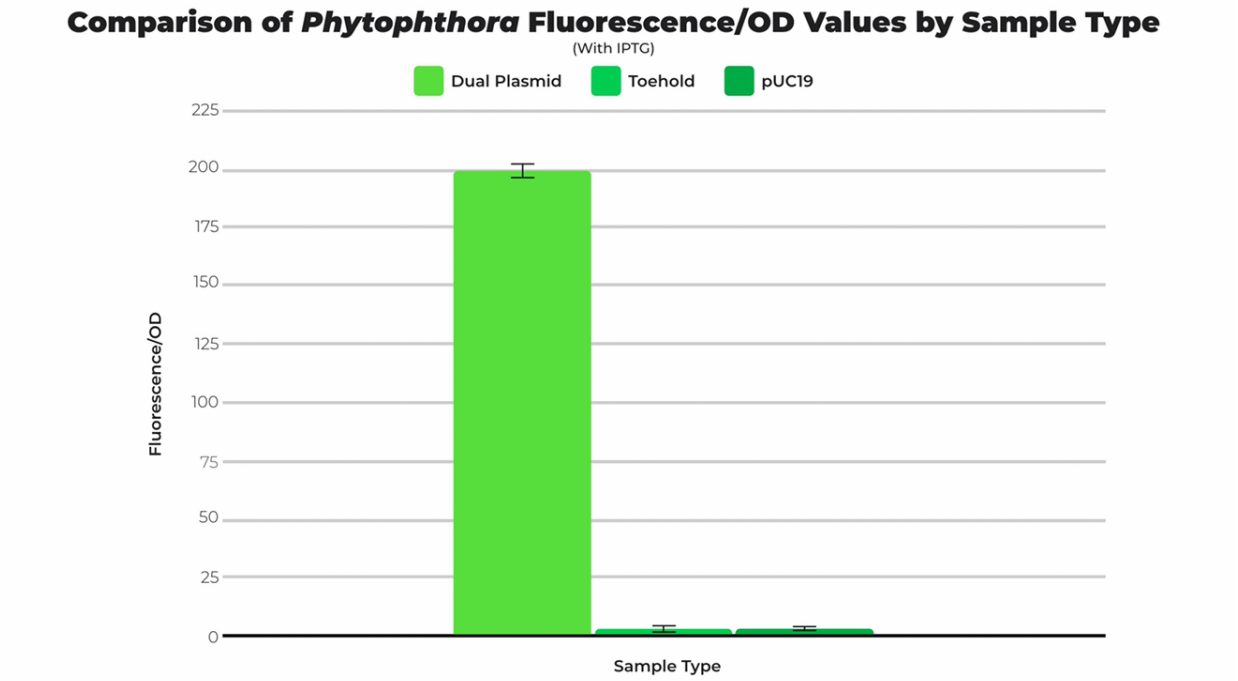

Figure 16. *Mean fluorescence/OD of IPTG-induced Phytophthora dual plasmid transformation compared to toehold and pUC19 with SEM error bars. DP stands for dual plasmid, TH stands for toehold only. Ran at gain of 60.*

Learn

Because the fluorescence/OD values for the dual plasmid transformations yielded statistically significant results, parts BBa_K3725022 and BBa_K3725070 for *Fusarium* and parts BBa_K3725010 and BBa_K3725040 for *Phytophthora* were deemed compatible. We believe that the lack of IPTG during the first phase of testing was the main cause of low fluorescence in the dual plasmid transformations. Furthermore, we had two main reasons that could potentially address the lower fluorescence levels in the Fusarium Dual Plasmid compared to the Phytophthora Dual Plasmid. The normalized ensemble defect rate for the Fusarium Toehold switch was approximately 10% higher than the Phytophthora Toehold Switch, which might factor into end GFP production from our data due to less optimal compatibility. Another reason might have been OD timing. To prepare the liquid cultures for quantification using the plate reader, we needed to add IPTG when the OD of the cultures reached 0.5, which is within the active growing range for *E.coli* cells. However, IPTG was added when the OD surpassed 0.5 and was around 0.8 for the Fusarium cells, which could result in poor uptake of IPTG, resulting in comparatively low fluorescence values.

Future

To safely implement our toehold biosensors, we are planning on putting our biosensors in a cell-free paper strip. We also hope to develop an accessible and affordable kit of toehold biosensors to detect a wide variety of common plant pathogens.

References

[1] Arie, Tsutomu. “Fusarium Diseases of Cultivated Plants, Control, Diagnosis, and Molecular and Genetic Studies.” *Journal of Pesticide Science*, vol. 44, no. 4, 2019, pp. 275–281., https://doi.org/10.1584/jpestics.j19-03.

[2] Caltech. *Help*. NUPACK. http://www.nupack.org/utilities/info?page_name=main