Lab Report

Nature's dose of sunshine: engineering vitamin D in peppers*

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Vitamin D deficiency affected more than 15% of the worldwide population from 2000 to 2022. Vitamin D deficiency is often linked to a wide range of chronic conditions such as osteoporosis, muscle weakness, and fatigue. Hardly any foods naturally produce adequate vitamin D concentrations and vitamin D supplements do not fully alleviate the symptoms of the deficiency. In recent years, scientists have used biofortification to enhance vitamin D levels in various vegetables such as tomatoes. Last year a team of Western Reserve Academy (WRA) students proposed a CRISPR-based knockout method to produce vitamin D in bell peppers emulating a similar study done in tomatoes, which belong to the same Solanaceae family. Our team now has focused on the experimentation of the mentioned methods.

Keywords: Bell pepper, vitamin D, 7-dehydrocholesterol, CRISPR-Cas9, *Agrobacterium*-mediated transformation

Approximately 50% of the world's population suffers from insufficient vitamin D levels (Siddique et al, 2021). Vitamin D restores and maintains calcium in bones and improves phosphorus absorption in the body. (“Vitamin D”, 2022). One of the most common forms of vitamin D is vitamin D3, which is produced through the sun's ultraviolet rays. Insufficient amounts of vitamin D or vitamin D3 can cause fatigue, mood changes, muscle weakness, and dizziness (Mayo Clinic Staff, 2016). In some cases, bones become incredibly fragile, leading to osteoporotic–bones so delicate that mild stresses such as bending over or coughing can cause a fracture (De Martinis, et al., 2021).

Several countries report vitamin D deficiency within their population. India, Pakistan, and Iran's populations are most deficient, with more than 20% of the population having less than 12 ng/mL in their diet. In India alone, it has been estimated that 490 million people are deficient (Cashman KD et al., 2016). This can be attributed to the low concentration of vitamin D found in natural foods, combined with the resource constraints faced by many developed countries, preventing the acquisition of adequate amounts of vitamin D-rich food. Additionally, as veganism, which involves consuming only foods not derived from animals, grows in popularity globally, with an estimated 79 million adherents, it presents a risk because most natural sources of vitamin D are found in meat and dairy products (“*2022 Vegan and Plant-Based Diet Statistics”*, 2022). This shift towards veganism leaves more than 1% of the world's population at risk of a severe vitamin D

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deficiency.

Vitamin D supplements are commonly employed to address vitamin deficiency. However, recent meta-analyses show that supplements are not significant in preventing the effects of vitamin D deficiency, such as bone fragility (LeBoff et al., 2022). Thus, with vitamin D's lack of natural occurrence in foods and supplements that cannot fully target the deficiency, there is an unmet need for a natural, accessible, and inclusive food supplement.

In recent years, scientists have used biofortification to enhance vitamin levels in food. Biofortification is the process of breeding crops to contain higher levels of vitamins, minerals, and proteins. Some examples include rice, which has been biofortified to address iron deficiency, and sweet potatoes which have had their antioxidant properties increased (Garg et al., 2018). As a basis for our project, we followed the scientists who biofortified tomatoes — a fruit from the Solanaceous family — to target vitamin D deficiency. In this study, scientists used the gene-editing technology that modifies, deletes, or corrects specific DNA regions, CRISPR-Cas 9, to increase intracellular 7-dehydrocholesterol (7-DHC) concentrations, the precursor to vitamin D, in tomatoes to increase vitamin D3 levels (Li, 2022). They utilized a CRISPR-Cas9 mediated knockout method to deactivate the 7-dehydrocholesterol Reductase 2 (7-DR2) gene (Figure 1) since it converts 7-DHC into cholesterol if not altered or blocked, which allows vitamin D3 to accumulate in the fruit (CRISPR/cas9, n.d.). Once modified, the plants were exposed to sunlight to facilitate the conversion of 7-DHC to pre-vitamin D3 stored in the fruit (Li, 2022).

While the scientist's research solely

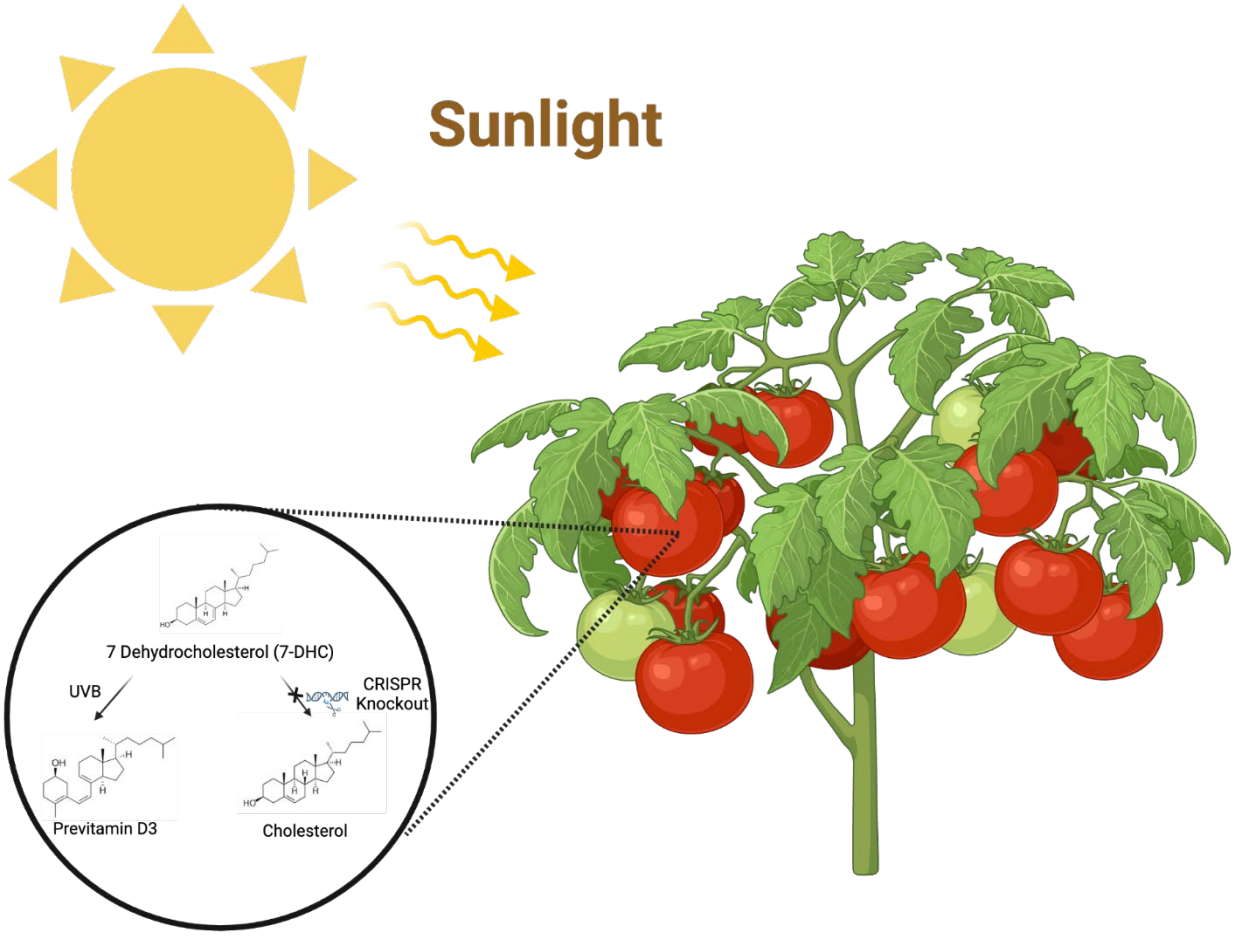

Figure 1. Vitamin D biofortification in the tomato plant. The figure shows the mechanism of CRISPR-Cas9 knockout acting on the tomato plant to increase the vitamin D level.

focused on tomatoes, this method could be applied to other foods in the solanaceous family, like peppers, chilies, and potatoes. All Solanaceae food pathways are similar, but the one closest to the tomato is the bell pepper (Sonawane et al., 2016). The bell pepper, *Capsicum annuum*, is also low in calories and high in vitamins A and C, making them a perfect addition to a healthy diet (Arnarson, 2019). They are greatly accessible as they are inexpensive, readily available, and easily grown in various climates (Williams, 2016).

Within the United States and the global market, bell peppers account for 70.51% of all vegetable production as of 2020 (Karst, 2022*)*. From 2000 to 2021, more than 70% of the world's bell peppers were produced in Asia, mostly in China (Biswas T et al, 2018). This is closely followed by Mexico, Spain, Indonesia, and the United States as fellow large producers. (Biswas T et al, 2018). The traditional price of the bell pepper is 0.55 USD per kilogram, which allows for it to be bought in bulk at low prices ("*2022 fresh bell pepper"*, 2022). Consequently, with the global scale of bell pepper production and availability in various climates, the bell pepper is an ideal vegetable to target for vitamin D biofortification. This project can spur economic and genetic growth in the $1.94 billion industry. ("*2022 fresh bell pepper"*, 2022).

7-ehydrocholesterol (7-DHC), a cholesterol, is the intermediate product of the steroidal glycoalkaloid (SGA) synthesis, which is a pathogen defense mechanism developed by the solanaceous species (Cárdenas et al., 2016, p. 1). In the pathway of SGA synthesis, 7-DHC is transformed into cholesterol and then into the SGA (Figure 2). In this process, an enzyme called 7-ehydrocholesterol Reductase 2 (7-DR2) makes the transformation reaction occur and controls the reaction rate (Sonawane et al., 2016, p. 2). The wavelengths of ultraviolet B (UVB) (290–315 nm) in sunlight induce the photolysis of 7-DHC and transform it into pre-vitamin D instead of cholesterol (Figure 1) (Jäpelt & Jakobsen, 2013, p. 2; Li, 2022). However, it is still unclear why regular solanaceous species do not have vitamin D when sunshine is available.

The CRISPR-Cas 9 complex comprises the Cas-9 enzyme and a single guide RNA (sgRNA). The sgRNA combines with the complementary target sequence by matching the paired nucleotide bases. Subsequently, the Cas-9 enzyme is activated by the combined structure between sgRNA and the target sequence and then cuts the target sequence, breaking the hydrogen bond between nucleotides so that the gene sequence is not transcribed and expressed (Ran et al., 2013, p. 2).

Agrobacterium-mediated transformation is a common technique scientists use to introduce genes into the plant's genome. *Agrobacterium* is a bacterium that induces tumor formation in infected plants (Nester, 2008). Scientists insert the desired gene into the *Agrobacterium*'s tumor inducing (Ti) plasmid and then infect the target plant with *Agrobacterium*. Hence, the *Agrobacterium* will incorporate the transgene into the plant genome, allowing for stable expression (Song et al., 2019).

Our team aims to use *Agrobacterium*-mediated transformation to insert the plasmid pHEE401E, which expresses CRISPR-Cas 9 and sgRNA, into a bell pepper to knock out the gene of the 7-DR2 enzyme (Figure 3). We chose this plasmid because it contains CRISPR-Cas9 gene already and also contains hygromycin and kanamycin resistant genes, which are used for selection. After the knockout, the 7-DR2 enzyme would downregulate in the bell pepper, which prevents the transformation of 7-DHC into cholesterol and makes 7-DHC accumulate in the bell pepper due to the lack of other

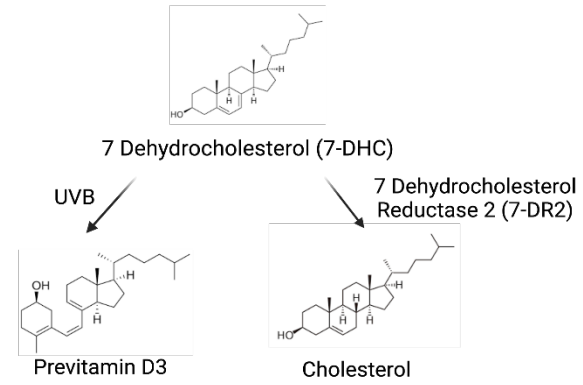

Figure 2. 7-DHC transformation pathway. The figure represents the two pathways through which 7-ehydrocholesterol can be transformed in the bell pepper.

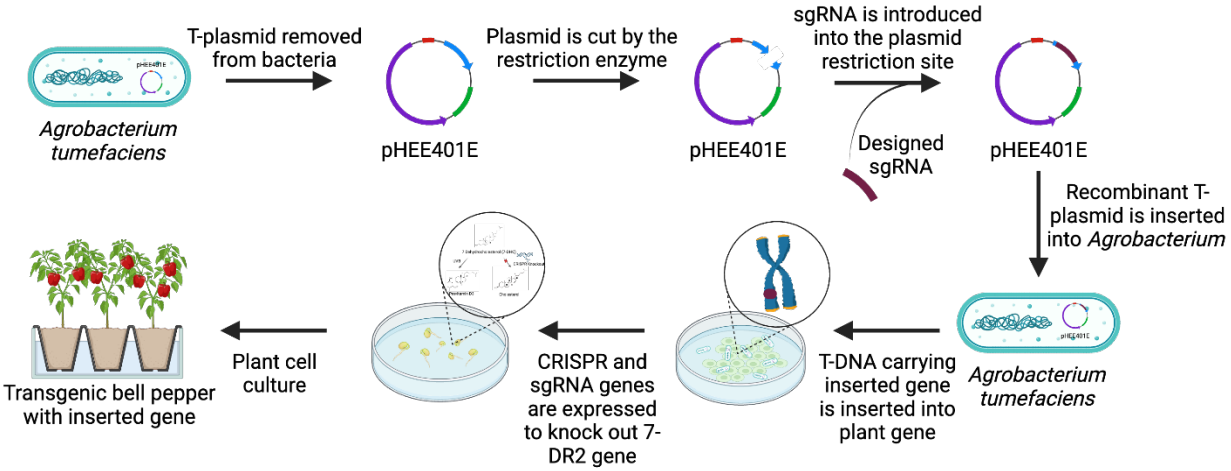

Figure 3.

mechanisms that can break down 7-DHC. The knockout can also decrease the synthesis of SGA, which is toxic to humans through disrupting cell membranes and cholinesterase in brain neurons (Dahlin et al., 2017). When accumulated, 7-DHC is exposed to UVB and transformed into vitamin D3. This process would enrich the concentration of vitamin D3 in the bell pepper. Thus, people who consume peppers can supplement the vitamin D concentration in their bodies.

For this study, we have decided to work on three different bell pepper cultivars supplied by the Southern Exposure Seed Exchange (Mineral, Virginia). These are Doe Hill Golden Bell, Carolina Wonder, and Charleston Belle. These cultivars were selected as they are commercially available and commonly bought in the U.S. Three different cultivars were chosen to create more precise bell pepper models and become less affected by the individual characteristics of a single cultivar. The peppers will be germinated *in vitro* in Murashige and Skoog media (MS media) containing the selectable marker agrimycin and after 3 weeks they will be transferred into the greenhouse (Kumar et al., 2012).

To measure the vitamin D level after the CRISPR Knockout, our study uses a MyBioSource (San Diego, California) Plant Vitamin D (VD) ELISA kit.

ELISA is a diagnostic assay which utilizes a series of antibodies to detect presence and concentration of vitamin D (Figure 4). The presence is indicated by a change in color of the media and how much the color changes indicate the vitamin D concentration (Parker et al., 2016).

Materials and methods

In order to select only the *Escherichia coli* that contains the plasmid and thus kanamycin resistance, a kanamycin concentrated solution was made. 0.25 g of kanamycin powder and 5 mL of distilled water were mixed by vortex and filtered through a 0.2 µm filter (Figure 5). Thus, obtaining a 50 mg/mL concentrated solution. The solution was then stored at -20 ºC.

LB Preparation

Luria-Bertani (LB) broth and agar were made in order to grow the *E. coli* containing the PHEE401E plasmid. 200 mL of LB broth was made by adding 195 mL of distilled water and 5 g of premixed LB powder and then mixed until dissolved. Then, 250 mL of agar was made by adding 241 mL of distilled water and 8.75 g of LB premixed powder and then mixed until dissolved. Both mixtures were autoclaved at 121 ºC and stored (Figure 6). The broth was stored in two 100 mL flasks.

Plasmid extraction

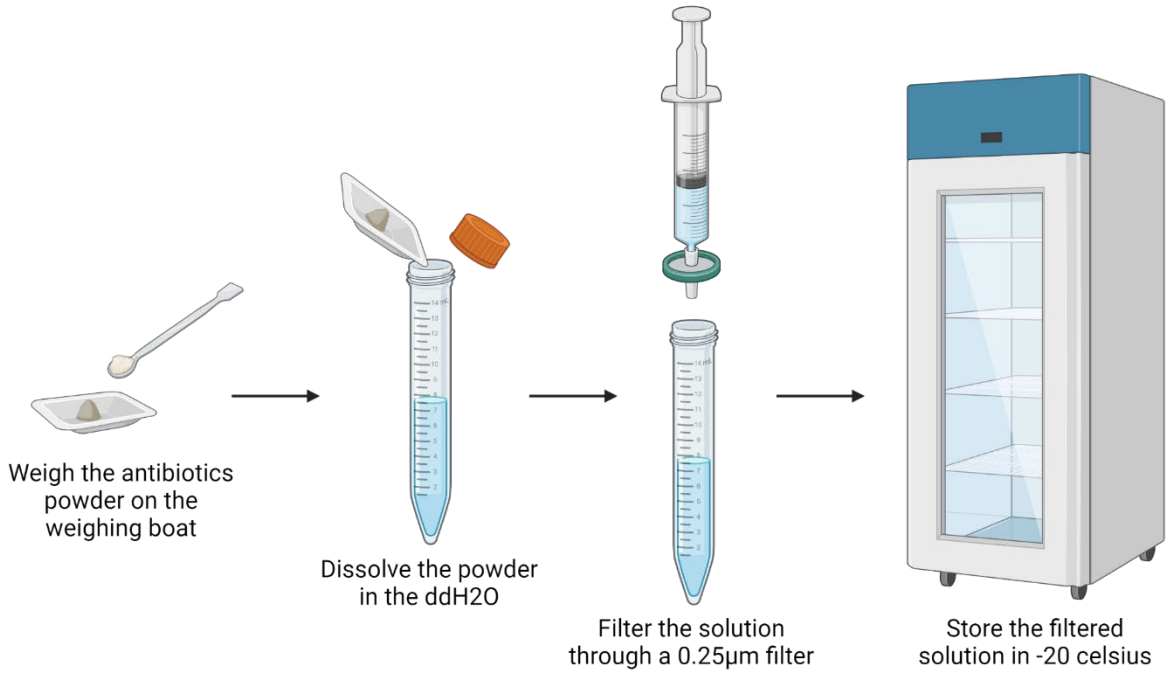

Figure 4. The complete procedure for antibiotic preparation. The diagram illustrates the detailed process of preparing kanamycin for usage in Luria-Bertani (LB) agar and LB broth.

The 250 mL of LB agar was melted and 250 µL of the previously made kanamycin concentrated solution was added and mixed. Then, 10 plates were poured from that mixture. Once the agar solidified The *E. coli* containing the pHEE401E plasmid from Addgene (Watertown, Massachusetts) was extracted from the vial it arrived in using a needle. pHEE401E was a gift from Qi-Jun Chen (Addgene plasmid #71287; http://n2t.net/addgene:71287; RRID:Addgene_71287). One of the LB agar plates was then streaked and incubated at 37 ºC for 24 h. The following day we added 100 µL of the kanamycin concentrated solution into one of the flasks containing 100 mL of LB broth and then mixed. 2 *E. coli* colonies were picked from the agar plate and placed into the LB broth and kanamycin mixture. The broth was incubated and shaken at 37 ºC

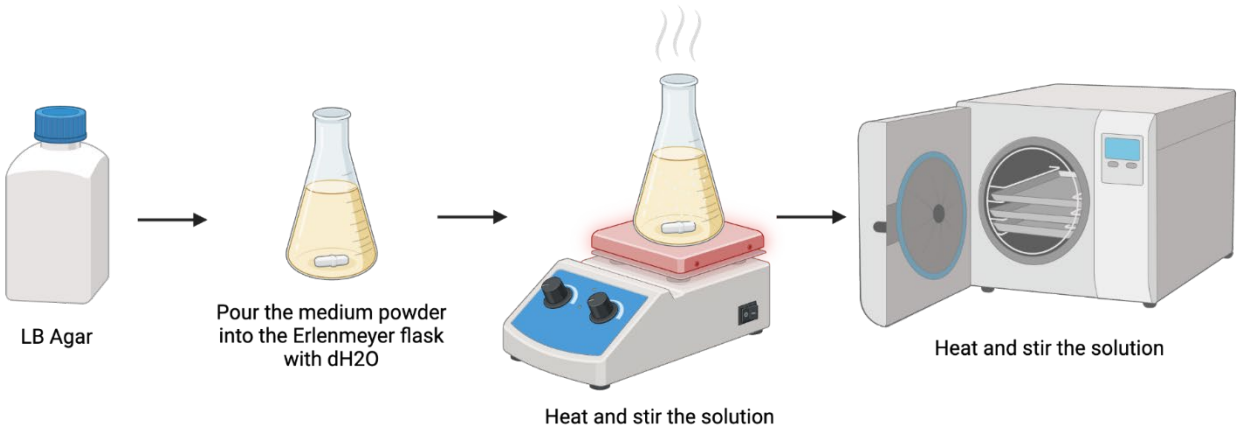

Figure 5. Procedures for LB agar and broth preparation. The diagram shows the procedure to produce LB agar and broth for bacteria streaking.

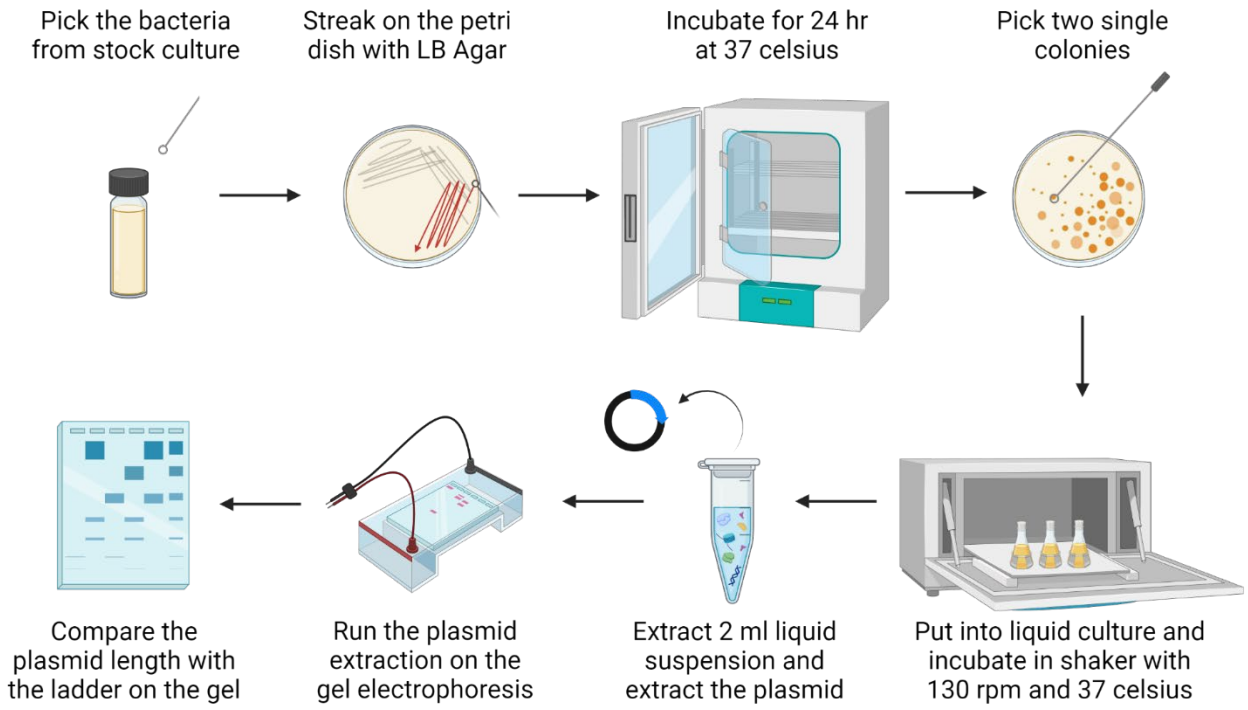

Figure 6. Complete procedure of plasmid extraction. The diagram illustrates the entire procedure of plasmid extraction from streaking the plasmid to the final gel electrophoresis.

overnight.

The following day we used a Bio-Rad Quantum miniprep kit in order to extract the plasmid. We first transferred 2 mL of the overnight broth culture into a microcentrifuge tube. We then pelleted the bacteria by centrifuging for 30 s at 12,000 × g. We removed all the supernatant and added 200 µL of cell resuspension solution and vortexed until the cell pellet was resuspended. We then added 250 µL of the cell lysis solution and mixed it gently. We added 250 µL of the neutralization solution and mixed gently creating a precipitate. The precipitate was pelleted, and the remaining supernatant liquid was placed in a 2 mL microcentrifuge wash tube along with 200 µL of Quantumprep matrix. This was mixed and centrifuged for 30 seconds. The filtrate was removed and 500 µL of wash buffer was added and centrifuged for 30 seconds. This step was repeated although this time we centrifuged for 2 minutes. We then placed the spin filter in a 1.5 mL collection tube. We added 100 µL of deionized water and eluted the DNA by centrifuging for 1 minute at full speed. The DNA was then stored at -20 ºC. (*Quantum Prep®*, n.d.)

ELISA

MyBioSource Plant Vitamin D (VD) ELISA kit is used to measure the VD expression level. We first collected the plant tissue and added 500 mg to 500 µL 1X PBS. It was put on ice for a half-hour and then homogenized with a homogenizer. After 10,000 rpm for 5 min, 50 µL was taken for analysis, and hen prepared with 1X Wash Buffer, 1X HRP-Conjugate, and VD Standard from the kit. After that, we started the experiment by removing excess microplate strips from the plate frame. Next, we set a blank well without any solution and added 50 µL of the standard of the sample per well. We also added 50 µL of HRP-conjugate to each well, and we mixed well. We covered it with the adhesive films and incubated for 60 minutes. We aspirated each well and washed, repeating the process of three washes using 250 µL wash buffer. We added 100 µL substrate solution to each well and incubated for 15-20 minutes

at 37 Celsius. After that, we added 50 µL of Stop solution to each well. At last, we determined the optical density of each well within 5 minutes, using a microplate reader set to 450 nm.

Choosing variants

Three sweet pepper variants were chosen to experiment with. Carolina Wonder, Charleston Bell, and Doe Hill Golden Bell. These variants were acquired through the Southern Exposure Seed Exchange. Three different variants were chosen to provide a larger data sample and make sure we will be able to perform this transformation in different variants of commercial peppers to cover a large portion of the market.

Growing peppers

Four plant peppers for each variant (12 in total) were grown in the lab under fluorescent grow lights. They were first grown in peat pellets and after growing 4 leaves they were moved to a 1-gallon pot for around a month. Then the largest 2 plants from each variant were kept and transplanted to a 10-gallon pot leaving us with 6 pepper plants.

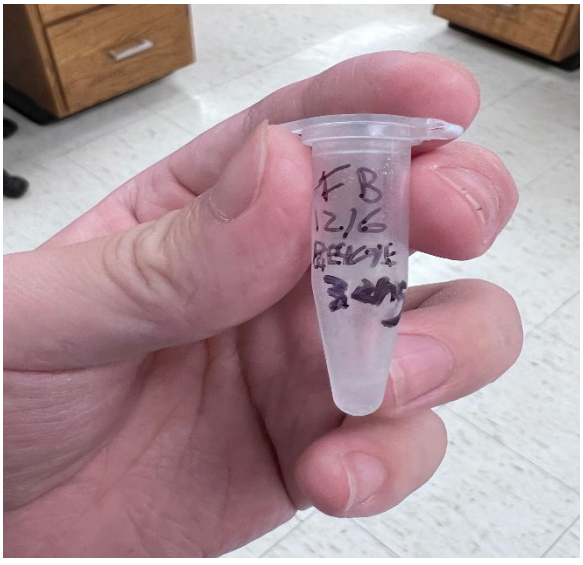

Figure 7. The photo of the microtube containing pHEE401E plasmid. The picture of extracted pHEE401E plasmid in the microtube.

Results

Plasmid extraction results:

We performed a plasmid extraction from the liquid culture of the DH5 Alpha bacteria containing the pHEE401E plasmid. As a result, we obtained two separate microtubes of pHEE401E plasmid; using Nanodrop 2000, we measured the plasmid concentration of two microtubes; one tube has 30 ng/µL of plasmid and the other contains 40 ng/µL.

Gel electrophoresis:

The plasmid extractions were run on gel electrophoresis shown in Figure 8. The bands on Lane 1 represent the 1 kb ladder. The bands on Lane 2, 3, 4, and 5 represent the extracted plasmid. We can see that the two bands on Lane 2, 3, 4, and 5 are above the ladder band of 10 kbps on Lane 1; the plasmid is about 16 kbp long and supposed to move slower than the 10 kbps band so the bands on Lane 2, 3, 4, and 5 show that plasmid extraction succeeded.

Vitamin D ELISA Kit result:

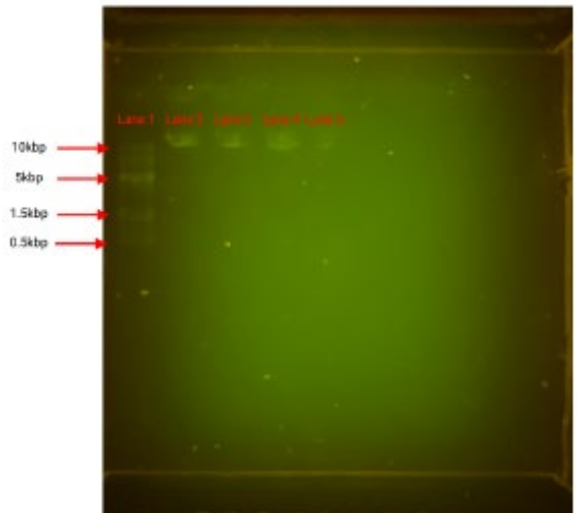

Figure 8. The photo of the gel electrophoresis result. Lane 1 represents the 1 kbp ladder, lanes 2, 3, 4, and 5 represent the plasmid extracted from experiments.

Pepper growth results:

Initially, we planted bell peppers in 12 pots and separated them into 3 groups equally. Two plants from each group were chosen to keep growing after random selection. After three months of watering and cultivation, we successfully obtained 6 pots of bell pepper plants which blossom and yield fruit.

Discussions

Although we successfully extracted the pHEE401E plasmid from the DH5 Alpha bacteria, the concentration of 40 ng/μL still fails to fulfill the lowest concentration of 100 ng/μL to perform the golden gate reaction. Therefore, we cannot finish our plasmid construct by inserting the designed sgRNA sequence into the pHEE401E plasmid. We repeated experiments several times, but the production is still low.

Because the initial plasmid extraction kit had expired for 1 year, we first purchased a new plasmid extraction kit named QIAprep Spin Miniprep Kit and repeated for two more times. However, the yield rate of the plasmid concentration is still low.

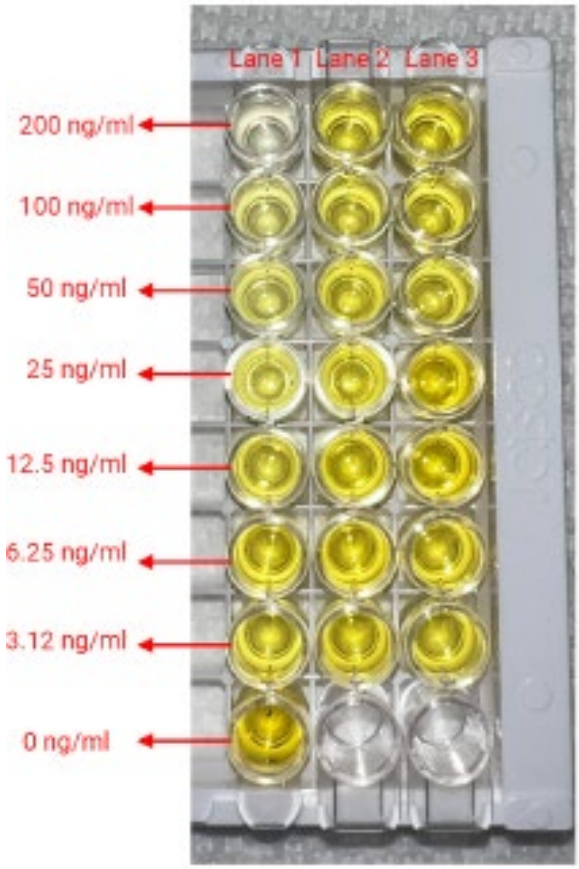

Figure 9.

The amount of liquid culture is another source of error considered. The number of bacteria in the solution can impact the performance of enzymes in the plasmid extraction. We performed the plasmid extraction with 1, 2, 3, and 4 mL of plasmid extraction. Still, the yield does not increase but decreases with the decrease in the liquid culture used in the plasmid extraction.

Therefore, we suspect the low yield rate was caused by the contamination while taking the bacteria out of the stock culture. Instead of streaking on the petri dish, the stock culture was first grown in the liquid culture. Because single colonies that contain the plasmid and resist antibiotics cannot be picked during liquid culture, other bacteria that do not contain plasmid were stored and used in later experiments. This error causes a large number of undesired bacteria used for plasmid extraction and causes a low yield rate. However, due to the lack of time and funds, this source of error cannot be authenticated. Moreover, the low yield rate can also be caused by the unoptimized protocol. DNA extraction protocol should be adjusted and optimized to improve the yield rate of the extraction process. Eluted buffers can be added to the final step of plasmid extraction to increase the yield. Furthermore, each spinning step should be extended so that we can remove any residual reaction buffer from each step to possibly increase the yield rate of the plasmid.

Another noteworthy point is the result of ELISA. Contrary to the expected result of light yellowish color, the vitamin D extracted from bell pepper seems to react strongly. Moreover, the VD Standard also follows a reverse pattern, in which the lower the concentration of vitamin D is the darker the yellow color. From the result, we can infer there is low amount of vitamin D or none in bell pepper because the wells all show a dark yellow color. However, this result conflicts with the regular ELISA experiment, where higher concentration of experiment protein yields a darker yellow color. Therefore, further experiment is needed to find out the

cause of the reversed reactions.

Next Steps

Due to the low production rate of the current bacteria stock, we will first optimize and improve our protocol to increase the plasmid concentration from plasmid extraction. After, the plasmid is extracted and used to synthesize the desired construct by inserting sgRNA sequence targeting on 7-DR2 gene into the plasmid pHEE401E using a golden gate reaction. We will follow the instructions of the golden gate reaction from the Addgene website. Then, the construct should be transformed into *Agrobacterium* using electroporation. The *Agrobacterium* then should be used to transfer the plasmid construct into the bell pepper cotyledon and thus perform CRISPR-Knockout inside the bell pepper plant. Following the yield of fruit on the pepper plant, vitamin D ELISA must be performed to measure the concentration of vitamin D in transformed bell pepper fruit. The concentration should also be compared with the fruit that is not transformed to confirm the increase after the CRISPR-Knockout.

Author contributions

J.H. had the idea and worked on the design last year. F.B. and J.H. worked on the background. J.H. created the images. F.B. worked on Material and Methods. J.H. worked on results and discussion. J.H. and F.B. worked on the various experiments conducted and collected and analyzed the data.

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Figure 10. The photo of the bell pepper. The picture of bell pepper plants blossoms and yielding fruits.

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