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Enhancing Tomato Fruit Quality: Application of CRISPR/dCas9-Mediated Methylation on the *PG* Gene Promoter Region

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Abstract:

Commercially produced tomatoes are typically harvested well before maturation in order to withstand domestic and international distribution. While this process ensures unspoiled goods, the nutritional value of the fruits have been curtailed as a result of premature harvesting (15). Previous research has found that the *polygalacturonase* gene (*PG* gene) is responsible for cell wall degradation of the tomato (11). In this study,CRISPR/dCas9 technology was employed to increase methylation levels in the promoter regions of the *PG* gene. This method is significant in that it epigenetically modifies the tomato genome using a technique that has the potential to develop non-genetically modified progeny with enhanced nutrient accumulation.
This alteration could lead to decreased *PG* expression, thus delaying the weakening of the cell wall of the tomato. By delaying this softening, farmers can harvest tomatoes at a later growth stage, thus improving the nutritional value of the product. Although my time to work on the project has been limited, Agrobacterium-mediated genetic transformation of tomato plants and various laboratory and greenhouse techniques were learned. More importantly, tomato plants that express GFP and dCas9-PG-gRNA have been produced, a significant step for the project. Characterization of the plants that express dCass9 and PG-gRNA will allow us to verify whether

dCas9 can be used to enhance DNA methylation levels of the promoter region of the *PG* gene, consequently extend the ripening period of tomatoes on the plant, and boost their nutritional value.

Introduction

Commercially available tomatoes fall prey to premature harvesting due to their short shelf life (*Solanum Lycopersicum*). The premature harvesting results in a lack of nutrients, aroma, and flavor in the commercially available produce when compared to home grown and locally sourced products. In many fruits and vegetables, but particularly tomatoes, vitamins accumulate as the fruit matures on the plant (13). Premature harvesting allows for a longer shelf-life but at the price of a nutritious product (14).

Recent research in the field of plant genetics has used transgenic technology to improve the nutritious value and quantity of commercial produce; however the consumer remains wary of the true health benefits of genetically modified organisms (GMOs). This social stigma exists even in populations relatively well educated on nutrition and genetic modification of produce (15). Society has serious concerns regarding the consumption of GMO, with about half of United States Citizens believing that GMOs are detrimental to human health (16). As the negative sentiment towards genetically modified fruits and vegetables among the North American population exists, non-GMO consumer goods are critical vessels to improving access to nutrients and antioxidants in communities without access to locally grown produce, such as urban communities.

Prior research has identified that the *Polygalacturonase* gene is responsible for the cell wall degradation and subsequently fruit softening as maturation commences. This gene affects the decomposition rate of the tomato, but has no reported result on nutrient accumulation or tomato lycopene values(11). Research has also been done to insert a constructed antisense RNA into the DNA sequence of the *PG* gene to reduce its expression in the tomato fruit. This approach was effective in decreasing expression of the *PG* gene, but resulted in a genetically modified tomato(22).

Epigenetic regions of the gene are attached to the DNA and can affect transcription of the gene. The promoter sequence is the region that RNA polymerase binds to transcribe the DNA of the gene. While the promoter is initially bound to RNA polymerase, that region of the gene is not transcribed by the enzyme and therefore not translated and expressed in progeny.

There has been research to genetically modify the gene using CRISPR technology, however this approach aims to alter the promoter region of the gene. This would result in an epigenetic modification, which potentially has the same effect as DNA modification without the use of a transgene. The targeting of an epigenetic region of the genome indicates that the T1 generation will not be considered transgenic (GM), yet could maintain the desired effect of the genetic modification.

Plants adopt methylation patterns to become more resistant to its ever changing environment. This adaptation allows the plant progeny to “remember” methylation patterns from the parent plant (23).

My approach addresses the lack of nutrients in commercial goods and seeks to ultimately offer a non-transgenic alternative to genetically modified organisms with enhanced traits. The experiment that I have proposed uses CRISPR/dCas9 technology to add methyl groups to the promoter region of the *PG*. It was hypothesized that the increased methylation level in the promoter region of the *PG* gene will decrease the gene's expression and delay the decomposition rate of the tomato fruit.

Methods and Materials

The tomato seeds should first be sterilized using 1% NaClO bleach solution and 2 drops of Tween 20 for 40 mL solution. Next sterilized seeds should be placed on solid germination media. The germination media should consist of 1 mol of Murashige and Skoog Basal Media (M524), 30 g/L glucose, and 7 g/L Agar. The final solution should be adjusted to a pH value of 5.8. LB-Agar media must be prepared to grow the stock of agrobacterium approximately 3-5 days prior to transformation. This media will consist of 100 mg/L Kanamycin, 10 g/L Tryptone, 10 g/L NaCl, 5g/L Yeast Extract, and 15 g/L Agar. This media should be adjusted to a pH value of 7.0 pH. Once the autoclaved LB media has been poured into petri dishes and cooled, it can be used to cultivate the agrobacterium necessary for transformation. The agrobacterium stock (stored at -80°C) should be thawed on ice, prior to spreading onto the petri dish. Using aseptic technique, dip a sterilized loop into the thawed stock of agrobacterium and dilute the concentration of cells onto the petri dish as shown in diagram below. Check the petri dish in the 28°C incubator daily to monitor the growth and possible contamination. The new stock should be sufficiently grown by the third day in incubation.

To cultivate agrobacterium for transformation, LB media should be prepared with 100 mg/L Kanamycin. Pour 6-8 mL of LB media into each of 3 test tubes. Using a pipette tip, collect a single colony from the new stock of agrobacterium, and place it in one of 3 test tubes. Repeat the process with a second colony into its own test tube and leave the third LB sample as the control. Allow the agrobacterium to oscillate at 28°C for approximately 16 to 18 hours. The OD value should range between 0.5 A - 0.9 A.

The agrobacterium should be spun down at 3000x rpm for 5 minutes. The infection media is composed of 30 g/L sucrose, 1 mol MS, pH adjusted to 5.8 pH. Following autoclaving, 1 mL/L acetosyringone (AS), and 0.5 ML/ L of 2-mercaptoethanol(2-ME) should be added to each of 4 50 mL conical tubes of the infection media.

1 mL of infection media should be used to resuspend the pellet after discarding the supernatant of the spun agrobacterium. Set aside the resuspended agrobacterium microcentrifuge tubes.

To perform the transformation, cotyledons should be at an ideal stage of growth to maximize infection efficiency.

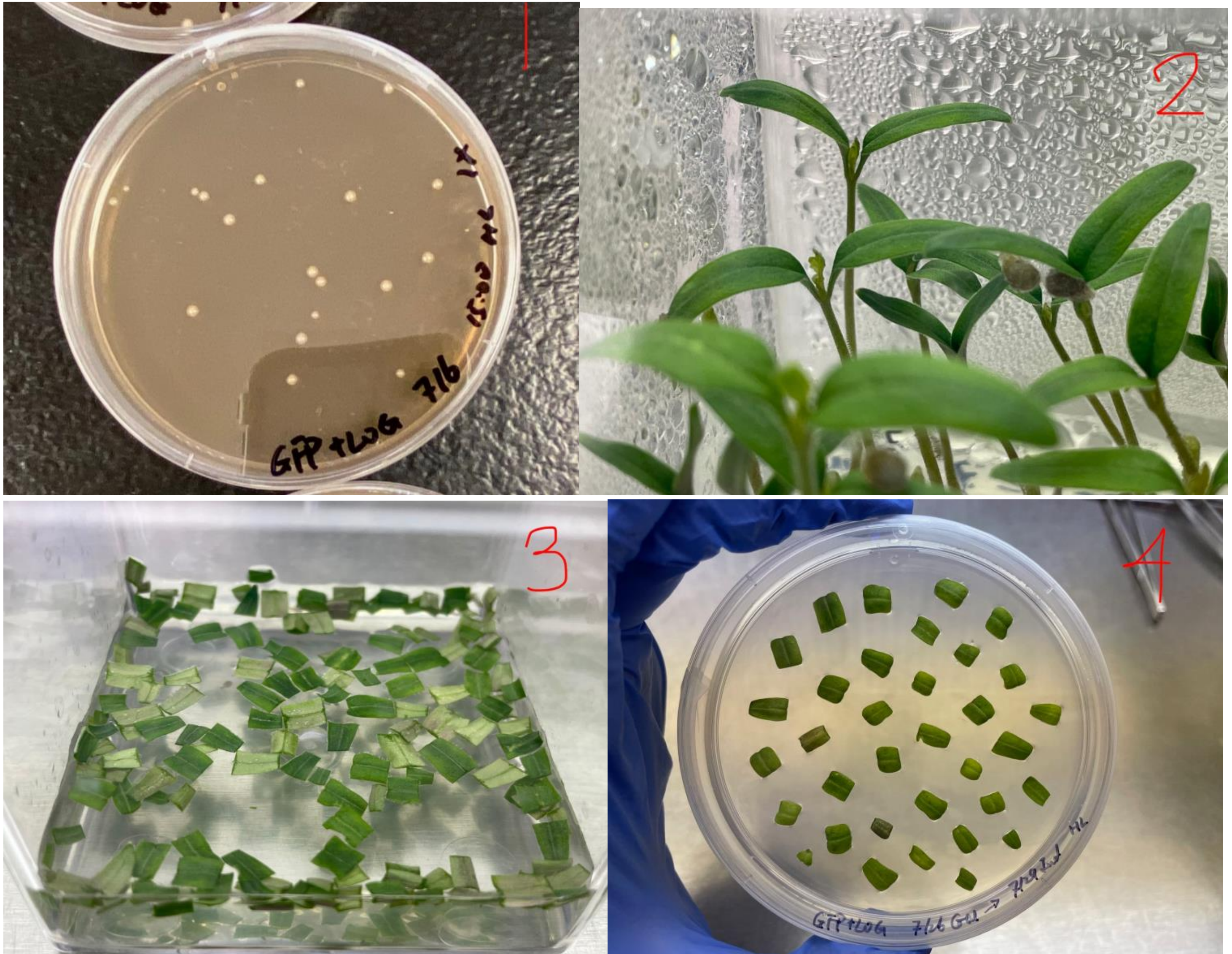

Fig. 1 Several stages of the transformation process. **(1.1)** The sufficient growth of agrobacterium with the GFP+LOG vector. **(1.2)** The ideal stage to perform transformation, when the first "true" leaf has begun to show. (**1.3)** The tomato cotyledon in infection media. (**1.4)** The recently infected cotyledons in cocultivation media.

The first true leaf begins to grow 5 to 6 days following seed germination. In preparing the explants for transformation, previous authors from the lab showed a technique using transverse cutting that allows for the greatest efficiency. The cotyledons are sampled on sterilized paper towels as shown below.

These cotyledon samples will then be carefully transferred with forceps to 40 mL of the infection media and 1 mL of the agrobacterium for approximately 10 minutes. The cotyledons will be

removed from the infection media agrobacterium with forceps and placed on dry sterilized paper towels. Once the excess solution has been removed, the cotyledons will be transferred to solid cocultivation media in a petri dish. Cocultivation media is composed of 30 g/L sucrose, 1 mol MS, 7 g/L agar, and pH adjusted to 5.8. After autoclaving, add 0.1 mL/L AS and 1.5 mg/L Zeatin. This process can be repeated with remaining cotyledons and agrobacterium solution in batches. The petri dishes of cocultivation media should then be allowed to cultivate in darkness for approximately 3 days, regularly observed for contamination and transient signal of GFP using a UV light. The transient GFP signal shows a basal level of efficiency in the transformation.

Following the cocultivation period, the cotyledons should be transferred to callus induction media. The callus induction media consists of 1 mol MS, 30 g/L sucrose, 7 g/L sucrose, and pH adjusted to 5.8. After autoclaving, 1.5 mg/L Zeatin, 10 g/L Hygromycin, and 200 mg/L Timentin should be added to the media. The cotyledons should be transferred to new media every 10-14 days until sufficient calli has been produced. At which time, the calli should be transferred to shoot regeneration media. The shoot regeneration media is prepared with 1 mol MS, 30 g/L sucrose, 7 g/L agar, 1.0 mg/L zeatin, 10 g/L hygromycin, and 200 mg/L timentin at pH adjusted to 5.8.

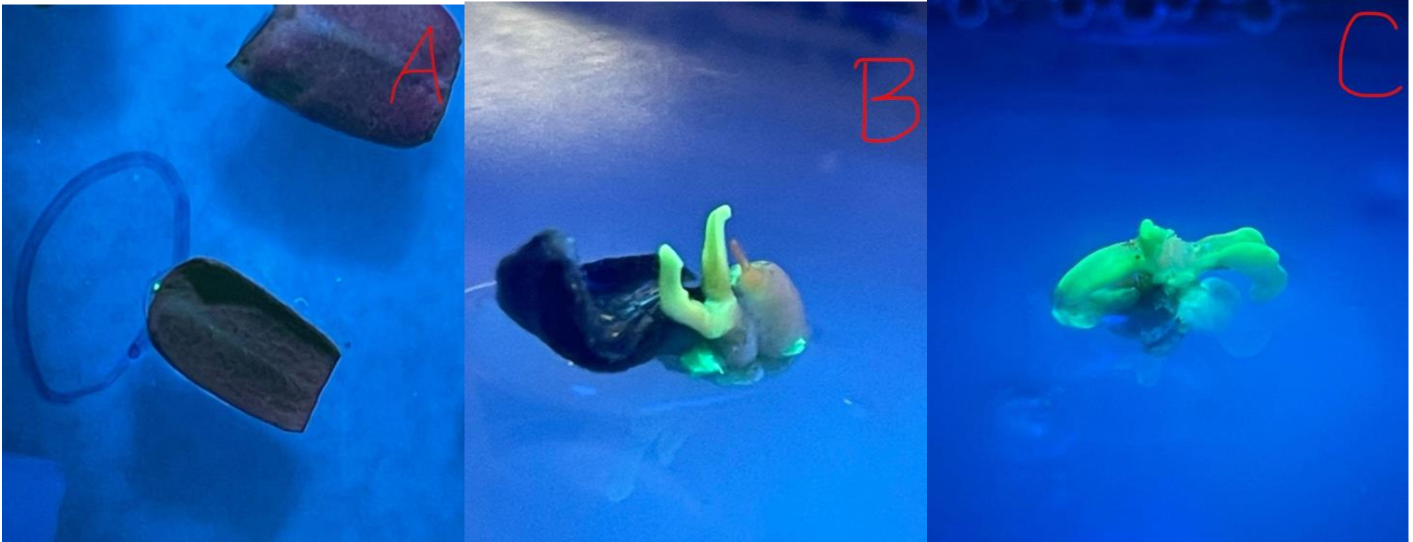

Fig. 2. Images of the callus growth process and shoot regeneration process with GFP. (A) Initial GFP positive callus in callus induction media. (B) Shoot beginning to form in shoot regeneration media. (C) Recently transferred shoot in root induction media.

Once an ideal shoot has formed, in approximately 3-4 weeks following its transfer, the shoot should be severed from the callus and transferred to root induction media. The root induction media is composed of ½ mol MS, 30 g/L sucrose, 7 g/L agar, 10 g/L hygromycin, 200 mg/L Timentin, and pH value adjusted to 5.8. Roots should begin to form within 2 weeks of transfer.

Discussion

There are no significant results to present due to the time constraints of the experiment which was limited to the four months; however, in this section, the author discusses their observations and explanations of what was learned over the time period spent working on this project.

The main focus of this experiment was to produce an epigenetically modified polygalacturonase gene that had the potential to affect pectin levels as a fruit ripens. The use of GFP as the indicator allows the visible confirmation of the successful transformation in the earlier stages of selection. GFP expression can be monitored with the unaided eye in the transformed cotyledons by

exposing the sample to UV light. GFP expression was viewed in several stages of the tomato growth process. In the transformations, it was observed that transient expression is visible under UV light in the 3 days following the transformation. This lesser but significant GFP signal was expressed by translating proteins in the cytosol prior to the tDNA insertion in the genome. It is worth noting that the transient signal was visible in nearly all cotyledons of attempted transformations. Expression of the GFP insertion in the genome is visible in any regenerated cells that has uptaken the epigenetic modification, these cells are prompted to regenerate in the form of calli with the encouragement of the cytokinin. Once transferred to the shoot regeneration medium the regenerated "shoot" cells also express the GFP signal, subsequently, the regrown tomato plant produces tomato with GFP expression.

A critical portion of the experiment was ensuring that the antibiotic and cytokinin used to select for the successful transformations in the media were effective. The success of the zeatin concentration and hygromycin concentration were compared qualitatively by condition of exposed cotyledon.

The zeatin concentration efficiency was observed by comparing the cotyledon health and callus reproduction in the cocultivation media and the callus induction media at concentrations of 1.5 mg/L and 2.0 mg/L. The cotyledons cultivated with 1.5 mg/L concentration of zeatin were more successful in reproducing initial calluses than the 2.0 mg/L group. Within 2 weeks of transformation, approximately 80% of the 100 cotyledons exposed to a concentration of 2.0 mg/L had significant and drastic changes in color, varying from dark brown to black. While in comparison to the 1.5 mg/L group, just 15% of the 100 cotyledons appeared darker and lacked

signs of prosperity. It is worth noting that though the 1.5 mg/L group appeared healthier, neither group produced a transgenic calli that reproduced shoots.

The hygromycin concentration was also compared in a similar fashion in media with hygromycin and without hygromycin. In samples that did not undergo successful transformation or the wild type group, it was observed that calli formed but did not grow out to reproduce shoots. The wild type sample in media with hygromycin deteriorated more rapidly than those in media without hygromycin. This observation shows the efficacy of the hygromycin as an instrument for selection.

Another important factor in the health of the transformed cotyledons is the effect that damage can have on the tissue in the weeks following the transformation. A particular issue was encountered in transferring the cotyledon from the infection media to the cocultivation media. To minimize the damage at this stage the size of forceps used was changed. The tomato cotyledon is easily susceptible to damage, so finding a method that minimizes the damage to the sample is critical for transformation efficiency. Though using small forceps benefitted in precision, it resulted in more damage to the cotyledons due to its sharper ends. The larger forceps, while less precise, allowed for a greater margin of error in selecting and placing the cotyledons onto fresh media.

The cotyledons damaged by forceps in transferring media appeared to negatively affect the health of other cotyledons in the same media. It was observed that the cotyledons seemed healthier when media was more frequently refreshed. It was clearer to determine which

cotyledons were selected for hygromycin resistance when the media was changed closer to 10 days than 14 days. It was also noticed that when cotyledons of poor health were not as frequently separated from healthy cotyledons, the healthy cotyledons appear less healthy than their separated counterparts. This is likely due to the effect of toxic gasses expunged by the dying cotyledons on the healthy tissue.

In addition to the time of the experiment spent with tissue culture in collaboration with the mentoring graduate student, the author had the opportunity to learn the basics of mature tomato plant maintenance. These steps in the process of transgenic tomato plant regeneration would have taken this experiment at a minimum of 6 months to reach. This involved the fertilization of plants once to twice a week, pruning, watering the plants, isolating the pollinating flowers, and overall management of the plants. Many of these plants were epigenetically modified in previous years and were being monitored for future experiments. While assisting with these experiments and maintenance, observations on the growth process of the plant were made, involving insect and pest prevention, watering moderation, and fertilizer requirements.

While the completion of the experiment initially proposed would have required a longer timeline due to human inefficiencies, the experiments that were executed within the timeline were successful in educating the transformation of plant cells, maintenance of tomato plants, tissue culture, agrobacterium culture, media and buffer constitution, and various other topics within plant genetics and its applications.

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