(this research has not been conducted, so all outcomes and results are hypothetical)

**Development of a Transformation Protocol for Expression of Green Fluorescent Protein in Whole Geranium Plants**

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*Key words:* GFP, Transformation, Agrobacterium, Recombinant DNA, Geranium

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**Abstract**

**Background and Objective:** Green fluorescent protein (GFP) is readily used in plants as a reporter protein, but recently there is an increasing interest for using GFP to fluoresce all of the plant for ornamental purposes. Geraniums are common garden plants that are not heavily researched. The purpose of the experiment is to express GFP in all parts of the geranium that is bright enough to be seen with the naked eye. **Method:** *gfp* gene is combined with the Ti plasmid through recombination. Then the recombinant vector is transformed in *Agrobacterium tumefacien.* Petiole explants were inoculated in Murashige and Skoog (MS) media with a constant concentration of IAA and different concentrations of TDZ plant hormone. The petiole explants then get exposed to the transformed culture of bacteria. **Results:** 10 μM of TDZ proved to be most efficient for shoot regeneration. As expected, the results were varied. Transformation did not occur in all samples. 4 of the samples expressed GFP in different degrees, some of the explants exhibited brighter fluorescence in leaves under UV. Other explants had a more even expression of GFP throughout the plant but not as bright fluorescence. **Conclusions:** These findings suggest that geraniums are capable of transformation and cultivation. However, levels of GFP expression are not as high as desired. The discrepancy might have happened due to the transformation of the geraniums. The error might also lay in the recombinant plasmid that was originally designed. Using genes modified for use in plants might result in brighter expression of GFP.

**Introduction**

Green fluorescent protein (GFP) is a protein produced by the jellyfish, *Aequorea victoria* that absorbs light to exhibit a green fluorescence7. The fluorescence is easily detectable which made GFP valuable as a marker protein. Therefore, this means that GFP can be used to track the presence of other proteins or genes. GFP is mainly used in bacteria, but its use in mammals and plants is increasing. In plants, GFP is becoming a vital marker and can be used to track a virus or a transgene throughout the plant1. GFP is often used as a tool to confirm or deny the results of another experiment. Usually, only a specific part of the plant will fluoresce. Fluorescing all parts of the plant is difficult due to the different levels of gene expression.

GFP in plants is expressed through recombinant DNA technology. The *gfp* gene that expresses the green fluorescent protein can be combined with the gene of interest that is being studied. This complex, or recombinant DNA, is then inserted in the cell. If the GFP is expressed, then the target gene was successfully transferred in the cell and is also expressed. This process saves money and time by eliminating the need for gene sequencing to confirm results. For this project, the target gene is the *gfp* gene itself.

The bacteria Agrobacterium tumefaciens is the medium that delivers the recombinant DNA fragment into the nucleus of the plant cell. In nature, Agrobacterium causes crown gall disease. It does so by integrating a part of its own DNA, called T-DNA into the plant genome. This triggers rapid cell growth in plants which results in the galls. T- DNA can be modified so that the agrobacterium transfers the gene of interest into the plant.

The 35S promoter is a key factor in allowing the artificially inserted gene to be expressed by the host plant. It is derived from the cauliflower mosaic virus and is a component in more than 80% of the genetically modified (GM) plants4. Once the *gfp* gene is engineered to accommodate plant cells, it is inserted in the T-DNA region of the Ti plasmid. This is performed through bacterial transformation which allows bacterial cells to integrate the foreign DNA into their genome3. After that, the agrobacterium is now ready to infect the plants and transfer the recombinant DNA to the plant. The heat-shock method was used for transforming agrobacterium since it is the simplest method, thus easier to replicate

Before attempting to transfer *gfp* into geraniums, first the growth media needs to be optimized for callus tissue generation2. Different concentrations of the plant growth hormones, auxin and cytokinin, are responsible for initiating callus growth. Callus tissue refers to plant cells that are undifferentiated, which makes them ideal for in vitro propagation. It’s important to confirm that geraniums can be regenerated in vitro before attempting transformation. Fortunately, previous research has been conducted regarding geranium shoot regeneration2. Winkelmann et al (2005) provide a protocol that indicates the hormone concentrations for optimal shoot regeneration for transformation. To confirm this research, we were able to recreate the shoot regeneration protocol and achieved similar results.

This paper presents a protocol that enables all parts of the geranium to express GFP at the same time. GFP has been expressed in other plants to varying degrees; however, every plant has different factors that will either make it harder or easier to express GFP. When it comes to physiology, geraniums are hardy plants. They also have thick leaves and stems that allow them to withstand sterilization. Attempting to make the entire geranium plant fluoresce, could result in new insight about the internal mechanisms and gene expression of geraniums. Engineered fluorescent geraniums also have potential to be used as ornamental garden plant or in public spaces as a source of light.

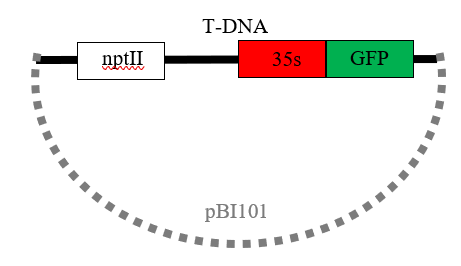
**Material and Methods**

Recombinant DNA Design

The pBI101 vector is commonly used in transformation experiments and is available in lab. The vector will serve as the backbone which will house the foreign genes. The components that need to be engineering in the vector are the *gfp* gene and the 35s promoter. There are multiple variants of the *gfp* gene, but the one that’s available is pGLO-GFP variant. This variant is mostly used in bacterial transformation, specifically E. coli. There are other variants that were modified to work better in plants, with the most similar one being pMG.d2.r8. The reason for these differences is because organisms have codon biases. For the sake of simplicity, the easily acquired pGLO will be used. The differences in sequence lead to different codons. Adapted from Guisinger et al (2010) research, table 1 illustrates the codons that are different between pGLO and pMG.d2.r, along with the percent use of these codons in geraniums. For contrast, the codon usage in E. coli is also presented.

The sequence for the 35s promoter had to be extracted from a transgenic papaya. Although there are different variants of the 35s promoter, the beginning and end of the sequence are conserved. DNA was extracted from the papaya and the 35s promoter region was amplified using PCR.

The *gfp* gene and the 35s promoter gene were added to the pBI101 backbone through Gibson Assembly. Gibson Assembly is a molecular cloning technique that’s favored when 2 or more sequences need to be combined. In our case there were 3 fragments and a total of 6 primers were designed. The primers were ordered through “ThermoFisher” Scientific9. The reaction was carried out at X°C. Figure 1 shows the completed recombinant plasmid. The *nptII* is an antibiotic resistant gene, used as a selective marker.

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***Figure 1****: shows the designed recombinant DNA*

nptII

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Type of  GFP | Codon | Code | Amino  Acid | % Geranium  (plastid DNA) | % E. Coli |
| pMG.d2.r | AGT | S | Ser | 19.8 | 13 |
| pGLO | AGC | S | Ser | 8.3 | 27 |
| pMG.d2.r | GCA | A | Ala | 28.5 | 22 |
| pGLO | GCT | A | Ala | 41.6 | 19 |
| pMG.d2.r | AAA | K | Lys | 67.7 | 76 |
| pGLO | AAG | K | Lys | 21.7 | 24 |
| pMG.d2.r | ACT | T | Thr | 41.3 | 21 |
| pGLO | TCT | S | Ser | 25.9 | 19 |
| pMG.d2.r | TCA | S | Ser | 18.5 | 12 |
| pGLO | TCC | S | Ser | 17.8 | 17 |
| pMG.d2.r | AGA | R | Arg | 22.8 | 4 |
| pGLO | CGT | R | Arg | 23.1 | 42 |
| pMG.d2.r | TAC | Y | Tyr | 16.1 | 47 |
| pGLO | TAT | Y | Tyr | 77.3 | 53 |
| pMG.d2.r | CCA | P | Pro | 28.5 | 20 |
| pGLO | CCG | P | Pro | 16.6 | 55 |
| pMG.d2.r | AAG | K | Lys | 21.7 | 24 |
| pGLO | AAA | K | Lys | 67.7 | 76 |
| pMG.d2.r | CAC | H | His | 26.6 | 48 |
| pGLO | CAT | H | His | 52.7 | 52 |
| pMG.d2.r | TTC | F | Phe | 29.5 | 49 |
| pGLO | TTT | F | Phe | 69.1 | 51 |
| pMG.d2.r | AGC | S | Ser | 8.3 | 27 |
| pGLO | AGT | S | Ser | 19.8 | 13 |
| pMG.d2.r | CCT | P | Pro | 32.3 | 16 |
| pGLO | CCC | P | Pro | 17.1 | 10 |
| pMG.d2.r | GAG | E | Glu | 26.5 | 30 |
| pGLO | GAA | E | Glu | 67.0 | 70 |
| pMG.d2.r | GGA | G | Gly | 34.5 | 9 |
| pGLO | GGT | G | Gly | 29.3 | 38 |
| pMG.d2.r | GTG | V | Val | 14.1 | 34 |
| pGLO | GTA | V | Val | 36.7 | 17 |
| pMG.d2.r | GAG | E | Glu | 26.4 | 30 |
| pGLO | GAA | E | Glu | 67 | 70 |
| pMG.d2.r | AGG | R | Arg | 10.8 | 3 |
| pGLO | CGC | R | Arg | 8.8 | 37 |
| pMG.d2.r | ACC | T | Thr | 18.5 | 43 |
| pGLO | ACT | T | Thr | 41.3 | 21 |
| pMG.d2.r | ATC | I | Ile | 18.8 | 46 |
| pGLO | ATA | I | Ile | 30.6 | 7 |
| pMG.d2.r | GAC | D | Asp | 21.8 | 41 |
| pGLO | GAT | D | Asp | 67.5 | 59 |
| pMG.d2.r | ACA | T | Thr | 29 | 1 |
| pGLO | ACG | T | Thr | 9.8 | 23 |
| pMG.d2.r | GGA | G | Gly | 34.5 | 9 |
| pGLO | GGT | G | Gly | 29.3 | 38 |
| pMG.d2.r | CTC | L | Leu | 6.8 | 10 |
| pGLO | CTT | L | Leu | 20.6 | 10 |
| pMG.d2.r | GTC | V | Val | 14.1 | 20 |
| pGLO | GTT | V | Val | 35.8 | 29 |
| pMG.d2.r | AAC | N | Asn | 24.7 | 61 |
| pGLO | AAT | N | Asn | 63.3 | 39 |
| pMG.d2.r | AGG | R | Arg | 10.8 | 3 |
| pGLO | CGT | R | Arg | 23.1 | 42 |
| pMG.d2.r | CTT | L | Leu | 20.6 | 10 |
| pGLO | TTA | L | Leu | 31.8 | 11 |
| pMG.d2.r | ATC | I | Ile | 18.8 | 46 |
| pGLO | ATT | I | Ile | 50.6 | 47 |

***Table .1****: the different codons in GFP and precent of codon use*

Transformation of Agrobacterium

First step was to prepare competent cells. Agrobacterium was inoculated in 2 mL of liquid growth media (LB) and grown in a shaker overnight at 28°C. 2 mL of the culture was inoculated in 50mL of the same medium. The culture was incubated until the opacity level was around 1.0. the culture was chilled on ice for 10 min. the cells were pelleted by centrifuge at 4°C for 8 min. the supernatant was discarded, and the cells were resuspended in 7mL of cold 20mM CaCl2. Solution was pelleted again, and supernatant was discarded. Resuspend cells in 1mL of CaCl2 and make 100 μL aliquots. The aliquots were stored in a -80°C freezer. The competent cells were ready to be transformed. The cells were placed on ice and 1μL of the recombinant plasmid DNA was added before cells thawed. The cell/DNA mixture was placed in liquid nitrogen for 5 minutes then left to thaw at room temperature for 10 min. The cells were transferred to a 2mL growth media and were incubated for 4 hours at 28°C. Cells were pelleted for 2 min and resuspended in 1mL of growth media. 200μL aliquots were spread on agar plates that contain 50 mg of kanamycin.

Plant Culture Media

The basal medium used was Murashige and Skoog (MS) which only contains nutrients necessary for plant growth. To optimize callus and shoot growth,0.57 μM of the auxin hormone, IAA were used and10 μM of the cytokinin hormone, TDZ were used.

Plant Material

8 petiole cultivars were collected from a nearby geranium plant. the samples were taken from young plants. The sterilization steps took place in a glass box. The samples were surface sterilized in ethanol solution for 30 sec and then they were placed in a 2% chlorine solution for 10 min. the samples were washed in sterile was 3 times, separated into intervals of 1, 2, and 5 min. the petioles were cut into 5mm segments and then placed horizontally in culture medium. the cultures were incubated at room temperature in 16 hr of light

Plant Transformation Experiment

The agrobacterium culture should be inoculated in 20 ml of LB medium and then incubated in shaker at 28 °C for 24hrs. The bacterial solution is then centrifuged at 3000 rpm for 10 min, the supernatant will be discarded and replaced with liquid plant culture medium (MS) to suspend the pellet. The petiole explants are incubated with the bacterial solution for 10 minutes. The petioles are then blotted dry and placed on basal media containing only 10 μM of TDZ. After 3 days the petioles were transferred to a medium of the same composition but with the addition of cefotaxime, an antibiotic, to combat the agrobacterium. Results should be seen after about a week. Additionally, the protocol could be modified based on the results.

**Results and Discussion**

Plant Regeneration

There was minor contamination which means our surface sterilization procedure was effective. 7 of the petioles grew shoots which also indicates that the culturing protocol was successful.

Plant Transformation

Transformation did not occur in all samples. Out of the 8 samples 4 of them confidently expressed GFP. The expression was also in different degrees, explants 2 and 3 exhibited brighter fluorescence in leaves under UV. while explants 5 and 8 had a more even expression of GFP throughout the plant but not as bright fluorescence. The other samples had little to no activity of GFP. this was expected since the recombinant DNA was not completely refined for plant cells. A simpler and easier to replicate protocol was favored. The presence of the fluorescent plants also confirms that the agrobacterium transformation protocol was effective.

Recombinant DNA Design

We were able to confirm that using pGLO-GFP and wildtype 35s promoter was sufficient to allow geranium plants to fluoresce

**Conclusion**

The results were as expected given the limited resources available. This indicates that geraniums are relatively flexible and can be transformed with less technical equipment or skills. There are multiple factors that could have interfered with the results. Using pGLO-GFP and wildtype 35s promoter most likely limited the levels of GFP expression. Using pMG.d2.r-GFP and an enhanced variant of 35s promoter might result in brighter and more consistent GFP expression.

The pBI101 vector already contains a different kind of reporter gene called the GUS reporter. It is unnecessary and makes the vector larger than needed. In this experiment the GUS reporter gene was untampered with; however if the reporter gene was removed, GFP could have had higher levels of expression. The different levels of GFP expression that were exhibited could be due to agrobacterium integrating the T-DNA in random regions of the plant.

This research gave us a better understanding of geraniums’ internal mechanisms and gene expression. It was implied that geraniums are less strict about codon usage. They were able to express GFP from genes that did not have specific modifications for better expression in plants. These factors could suggest that geraniums are good model organisms for expressing genes of interest. Further research to improve GFP expression in geraniums could allow for the use of fluorescent geraniums as decorative plants in private or public spaces.

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