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The Construction of the Transient Expression Vector for the Foreign Green Fluorescent Protein (GFP) and the Study of Its Protein Expression

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Abstract:

Transient gene expression facilitates the study and analysis in gene function and its short-term effect. GFP (Green Fluorescent Protein) has been studying for years as a marker of gene expression and protein targeting. Thus, establishing a transient expression vector for GFP is conducive to gene function research, cell process analysis, and drug screening. Through plasmid DNA isolation and purification, PCR amplification of GFP, and plasmid recombination, the vector is established.

Keywords: GFP; transient gene expression system; recombinant plasmid; plant expression; tobacco leaves

Introduction

Green fluorescent protein (GFP) is capable of self-catalyzing the formation of chromophores, which then fluoresce green when stimulated by blue or ultraviolet light. It has been genetically modified to facilitate the fusion with other proteins, thereby rendering otherwise invisible proteins visible. Agrobacterium-mediated transformation, which involves the penetration of an infection solution containing agrobacteria into plant tissues via syringe, has been shown to facilitate transient gene expression effectively. This method minimizes the transformation process's impact on plant cells and leverages the intercellular space, which constitutes up to one-third of plant tissue volume, to enhance t-DNA transfer efficiency. Multiple transient expression assays with various plasmids can be conducted on a single leaf. However, the complex cell structure poses challenges in accurately visualizing target proteins. Agrobacterium-mediated transient transformation is a prevalent approach for modifying plant genomes to study gene function and expression. Researchers have established different systems for various plant species, including Arabidopsis, grapevine, hybrid aspen, persimmon, and strawberry. Agroinfiltration is widely used to induce target gene expression and is also applied in cell biology research. GFP, discovered in 1962 in Aequorea Victoria, serves as a reporter gene and has diverse applications, including fusion tags, active indicators, protease action, transcription factor dimerization, and calcium ion indicators. GFP-based calcium ion indicators are versatile and can be targeted to specific tissues, cells, organelles, or proteins. The detectability and expression level of GFP depend on factors such as expression duration, gene copy number, mRNA splicing absence, protein degradation and export, temperature, oxygen availability, excitation and emission wavelengths, and competi-

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tion with noise and background signals. GFP's structural complexity, including primary, secondary, tertiary, and quaternary structures, has been studied, with mutations and chaperones aiding its folding, providing insights into chaperone function.

Previous studies have identified factors affecting transient gene expression efficiency, such as concentration, incubation time, and simple in-cell assays for subcellular localization. Common methodologies include cloning GFP into binary vectors as reporter genes, vacuum infiltration, and injection and infiltration methods. Despite these advancements, the internal molecular mechanisms underlying observed phenomena remain unclear and warrant further investigation. This study diverges from focusing solely on transient gene expression in plant tissues, instead providing a comprehensive examination of foundational technologies, including vector construction and target protein expression. The research involved plasmid recombination, verification, and transformation, with anticipated results demonstrating successful GFP expression in tobacco leaves. This study offers robust tools for assessing medicinal compounds and expressing foreign genes in a simple, efficient, and biosafe manner.

This study primarily investigates the construction of transient expression vectors and the integration of Green Fluorescent Protein (GFP). Transient gene expression is a technique that introduces foreign genes into cells without integrating them into the genome, allowing for high-level expression of target genes within a short timeframe. This method has gained widespread application in research areas such as foreign gene expression and protein function analysis. Specifically, transient gene expression technology is utilized in various domains, including the localization of protein products within plant cells, the study of physiological roles of gene products involved in plant growth and development, responses to biotic environmental factors, regulation mechanisms of plant metabolic pathways, and protein-protein interactions (PPI) along with their subcellular localizations. Plant systems employed in transient gene expression encompass protoplasts, calluses, suspension cultures of plant cells, intact plants, isolated plant organs, and specialized plant tissues. Common gene constructs used in transient expression include vectors containing Ti plasmids with t-DNA. Delivery methods for recombinant plasmids include transfection of protoplasts using polyethylene glycol (PEG), electroporation, biolistic techniques, and agroinfiltration. Researchers have extensively studied the efficacy of these methods. The aim of this experiment is to attempt to reproduce the GFP gene in bacteria and then transplant it into plants for expression. Try to correctly express GFP in plants, understand the general experimental principles of molecular biology, and master the basic experimental operations of molecular biology. The success of this experiment indicates that other, more practical gene fragments can be inserted into plants for expression through the following experimental methods. This will enable the reduction of plant growth drawbacks to the greatest extent possible and the constant optimization of plants that are more suitable for human consumption or enjoyment.

Materials and Methods

1. Plasmid DNA Isolation and Purification

Materials:

liquid bacterial culture; Resuspension buffer (RB); Lysis buffer (LB); Neutralization buffer (NB); Wash buffer (WB); Elution buffer (EB); Mini-plasmid spin columns with collection tubes.

Methods:

1) 4 μl of liquid bacterial culture was collected and centrifuged for 1 min.

2) 2250 μl of RB buffer was added to the liquid, resuspending the bacterial pellet.

3) 250 μl of LB buffer was added to the solution, with NAOH breaking the cell wall.

4) The solution was mixed by gentile inversion, and 350 μl of NB buffer was added, neutralizing the basic environment and forming PDS, which is insoluble.

5) Incubating at room temperature for 2 mins, the solution was centrifuged for 5 mins.

6) The supernatant was carefully transferred to a microcentrifuge tube, centrifuging the liquid 1 min and discarding the waste liquid.

7) 650μl of wash buffer was added and the solution was centrifuged for 1 min, discarding the waste liquid.

8) Pure water was added, and the liquid was incubated at room temperature for 1 min and then centrifuged for 1 min. We repeated this step to increase the concentration.

9) Then, the concentration was measured by the UV-Vis spectrophotometry.

2. Polymerase Chain Reaction (PCR)

Materials: 20bp of primer; Taq enzyme; dNTP; DNA template; PCR buffer

Methods:

1) 50μl of the reaction system was set up by adding 1μl of DNA template, 18μl of water, 2μl of forward primer, 2μl of reverse primer, 1μl of dNTP, 25μl of buffer, 1μl of Taq enzyme.

2) The reaction system was put in the PCR Machine. The temperature for denaturation, annealing, and extension

were 95 Celsius, 55 Celsius, and 72 Celsius. The cycle was repeated for 34 times. At last, an additional extension for 10 mins was completed to make sure that all DNA segments are synthesized.

3. Vector Linearization

Materials: Cut smart; vector; Brahmi; Pat; water
Methods:
1) 50μl of the reaction system was set up by adding 5μl of Cut smart, 5μl of the vector, 1μl of Brahmi, 1μl of Sac I, and 38μl of water.
2) Density gradient centrifugation was conducted.
3) The reaction mixture was heated at 37 Celsius for 1 hour using metal bath.

4. Gel Electrophoresis

Materials: 50X buffer, agarose; golden view (produce fluorescence); 10X loading buffer; marker; PCR product; restriction digestion product; plasmid without restriction digestion.
Methods:
1) Agarose gel was prepared by adding 20milliliter 50X buffer, 980milliliter water, 0.5grams of agarose. The solution was heated until it turned to be clear. 10μl of golden view was added in the solution. The gel solution was poured into the casting mold.
2) 5μl 10X DNA loading buffer was added to the 50μl PCR and restriction digestion product.
3) The 10μl marker was added to the first lane.
4) Two 50μl PCR products were added to the second and third lane respectively.
5) Two 50μl restriction digestion products were added to the fourth and fifth lane respectively.
6) The plasmid without restriction digestion was added to the sixth lane.
7) Electrophoresis was performed for 20 mins.
8) The gel was visualized under UV light.

5. DNA Recovery and Purification

Materials: the gel; buffer GDP; sol solution; elution buffer
Methods:
1) The gel was cut.
2) 200μl buffer GDP was added to the gel being cut.
3) The mixture was heated at 55 Celsius using metal bath for 20 mins.
4) Density gradient centrifugation was performed.
5) The mixture was added to the microcentrifuge tube and centrifuged for 1 min.
6) Discarding the waste liquid, 300μl of sol solution was added and the mixture was centrifuged for 1 min.
7) Discarding the waste liquid, 650μl of wash buffer (buffer GW) was added and the mixture was centrifuged for 30 seconds. We repeated this step for twice.
8) Discarding the waste liquid, centrifugation was performed for 2 mins.
9) The absorption column was changed to a new microcentrifuge tube, and the lid was opened to let the ethanol to vaporize.
10) The elution buffer was preheated to improve the elution efficiency.
11) 30μl of elution buffer was added, the mixture was centrifuged after standing still for 1 min.
12) The liquid was added to the absorption column and centrifuged for 1 min again.

6. DNA Concentration Measurement

Materials: PCR and restriction digestion products recycled and purified; UV-vis spectrophotometry; water.
Methods:
1) Water was added to the machine to perform blank correction.
2) PCR and restriction digestion products were added to the machine respectively to measure the concentration and the value of A260/A280.

7. Homologous Recombination

Materials: linear vector; PCR product; 5X cell buffer; Expanse II; water
Methods:
1) The 20μl reaction system was set up by adding 11.7μl of linear vector, 1μl of PCR product, 4μl of 5X cell buffer, 2μl of Expanse II, 1.3μl of water.
2) Density gradient centrifugation was conducted
3) The mixture was placed in the PCR machine to react at 37 Celsius for 30 mins

8. Chemical Transformation

Materials: competent cells; target DNA; LB culture medium; Kanamycin
Methods:
1) 50μl of competent cell was thawed on ice.
2) 5μl of target DNA was added and the mixture was iced for 30 mins.
3) Kanamycin with concentration of 100 milligram per milliliter, working concentration of 50 microgram per milliliter was added to the culture medium. The solution was mixed thoroughly, and a pour plate was performed.
4) After the ice bath, the competent cell with the target DNA was heat shocked at 42 Celsius for 45 seconds and then iced for 2 mins.
5) Liquid LB culture medium without resistance to the Kanamycin was added to the competent cell after transfor-

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mation to recover the cell.
6) The cell was incubated on the shaker at 37 Celsius, 200rpm for 1 hour.
7) The cell was centrifuged at 4000rpm for i min, because there was too much bacterial suspension. The precipitate was the bacteria.
8) 800μl of the bacterial suspension was removed, and 100μl was left.
9) The bacterial suspension left and the precipitate was mix thoroughly.
10) The mixture was transferred to the culture medium using pipette.
11) The cell spreader was sterilized and cooled, then the bacterial suspension was spread evenly on the agar plate by using cell spreader.
12) The agar plate was placed upside down and incubated at 37 Celsius overnight for bacterial growth.

9. Colony PCR Identification

Materials: positive colony; sterile water; forward prime; reverse prime; taq enzyme mix
Methods:
1) 3 positive colony were picked and added to 40μl of sterile waster separately. The negative control was the sterile water.
2) The PCR reaction system was set up by adding 40μl of taq enzyme mix, 4μl of forward prime, and 4μl of reverse prime, 48μl in total. The mixture was divided into 4 mixtures and added, each had a volume of 12μl. Each mixture was added to 8μl of bacterial suspension, forming a 20μl PCR reaction system.
3) Density gradient centrifugation was performed.
4) The reaction was placed in the PCR machine. The temperature for denaturation, annealing, and extension were 95 Celsius, 55 Celsius, and 72 Celsius. The cycle was repeated for 28 times.

10. Shake Culture

Materials: liquid LB culture medium, bacterial suspension that was confirmed to have correct clones, Kanamycin
Methods:
1) All lab supplies were exposed to the UV light in the biosafety cabinet for 20 mins for sterilization, except the bacterial suspension.
2) 3 milliliter of liquid LB culture medium was added to a 15 milliliter sterilized microcentrifuge tube.
3) Kanamycin with concentration of 100 milligram per milliliter, working concentration of 50 microgram per milliliter was added
4) 32μl of bacterial suspension that was confirmed to have correct clone and recombination was added to the mixture.
5) The mixture was incubated on shaker at 37 Celsius overnight.

11. Plasmid Extraction

Materials: bacterial suspension, RB, LB, NB, WB, EB
Methods:
1) 1 milliliter of shaken bacterial suspension was added to a centrifuge tube and centrifuged at 1200rpm for 1 min
2) Discarding the waste liquid, the bacterial pellet was collected
3) 250μl of RB buffer was added and mixed thoroughly
4) 250μl of LB buffer was added and mixed well
5) 350μl of NB buffer was added and stand for 1 min
6) The mixture was centrifuged for 1 min and the supernatant, which is approximately 700μl, was collected
7) The supernatant was transferred to a absorption column and centrifuged for 1 min.
8) Discarding the waste liquid, 500μl of WB buffer was added. The mixture was centrifuged for 1 min. Then, the waste liquid was discarded and this step was repeated.
9) Discarding the waste liquid, an empty centrifugation was performed for 2 mins. The lid was opened to allow the ethanol to evaporate.
10) EB buffer was preheated to 55 Celsius. 30μl of EB buffer was added to the centrifuge tube and stand for 1 min, and then the mixture was centrifuged for 1 min
11) The eluted liquid was transferred back to the centrifuge tube and centrifuged again for 1 min
12) The Nanodrop Spectrophotometry was used to measure the concentration of the plasmid

12. Restriction Enzyme Digestion Verification

Materials: enzyme, plasmid extracted, water, Cut smart buffer
Methods:
1) 1μl of the enzyme (Brahmi, Sac I), 13.05μl of plasmid, 5μl of 10X Cut smart buffer, and 30.95μl of water was added to set up the reaction system.
2) The system was incubated in metal bath at 37 Celsius for 2 hours.

13. Transformation

Materials: competent cells; plasmid that was confirmed to be correct; liquid nitrogen; liquid LB culture medium; triple antibiotic plate
Methods:
1) The competent cell was thawed on ice.
2) 20μl of competent cell was added to the microcentrifuge tube.
3) 2μl of the 35S-2300-GFP plasmid was added to the microcentrifuge tube.

4) The mixture was mixed thoroughly and iced for 5 mins.
5) Then, the mixture was place in the liquid nitrogen for 5 mins.
6) The mixture was heat shocked in the metal bath at 37 Celsius for 5 mins.
7) The mixture was placed in the ice again for 5 mins.
8) 500μl of LB culture medium was added to recover the cell.
9) The solution was incubated on the shaker at 28 Celsius for 2 hours.
10) Later, the solution was centrifuged for 1 min at 5000 rpm.
11) Plating was performed
12) The culture medium was incubated at 28 Celsius for 2 days.

14. Agrobacterium- mediated transformation

Materials: MES; MgCl2; ASG; spectrophotometry; centrifuge; disposable syringe
Methods:
1) Strain activation and culture expansion was performed.
2) 1 milliliter of MES, 100 milliliter of distilled water, 1 milliliter of MgCl2, 200μl of ASG was added to prepare the infection solution.
3) The bacterial suspension was centrifuged for 5 mins and the supernatant was discarded.
4) The bacterial pellet was resuspended by 10 milliliter of infection solution.
5) The mixture was diluted to obtain more accurate concentration when measuring. So, 100μl of mixture and 900μl of infection solution was mixed well.
6) The concentration was measured by using spectrophotometry.
7) To adjust the concentration to 1.43 μl of the infection solution was added to the 10-milliliter mixture.
8) The mixture was incubated at 28 Celsius for 1 hour.
9) The mixture was injected into the needle holes on the underside of the leaf where there are no veins by using 1 milliliter syringe.

Results

1. Cloning and recovery of target genes

PCR products in the second and third lane are 750bp. Restriction digestion products in the fourth and fifth are 10045bp, and they run slightly slower than the original plasmid. Theoretically, the restriction digestion product should run faster than the original plasmid due to smaller molecular size. To explain, the restriction digestion product is linear plasmid, while the original plasmid is supercoiled. The migration rate of the supercoiled DNA is the fastest than that of the linear DNA. Therefore, the phenomenon above occurred (Figure 1). The concentration of PCR products was 63.823 nanogram per μl, 136.002 ng per μl, with A260/A280 value of 1.97 and 1.98. Consequently, the DNA concentration is relatively high. However, the value of 63.823 is relatively low, because recycling the sample from the gel incurred losses, which may affect the final yield of DNA. The concentration of restriction digestion products was 17.155 nanogram per μl, 20.664 nanogram per μl.

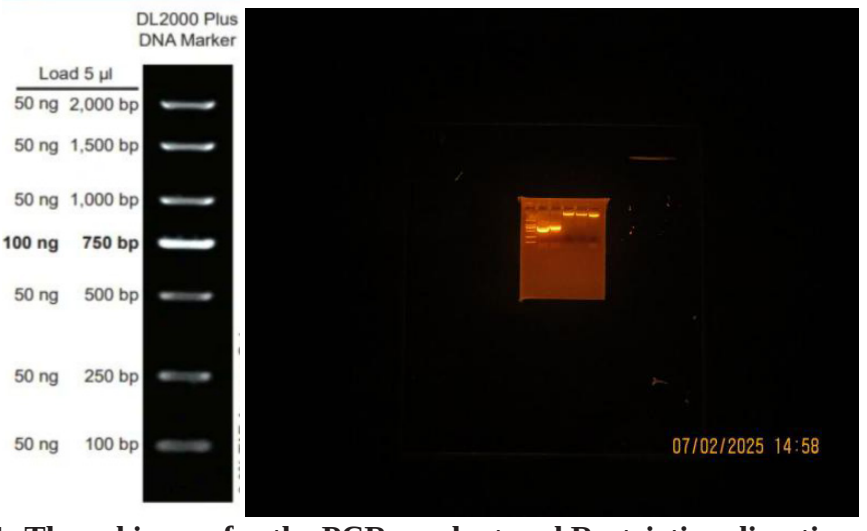

Figure 1: The gel image for the PCR product and Restriction digestion product

2. Colony PCR

Based on the marker, the products of colony PCR are 1000 bp, which confirms that the clone is correct. However, the negative control, water, in the fourth lane shows band,

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which means that the primer or taq enzyme mix polluted the negative control. No cloning due to unsuccessful connection might occurs due to primer design error, improper vector-to-insert ratio, impure vector and insert, improper operation. To avoid this, the homologous arm needs to be 15-25 bp, with GC concentration needs to be 40%-60%, and the the sequence specificity needs to be guaranteed. The best vector-to-insert ratio is 1:2-1:3. For single insert, vector should have 0.03mol, and insert should have 0.06-0.09mol. For multiple inserts, the best DNA concentration should be 0.03mol per segment. However, if the connection is success but there is no cloning, the result can be explained by low efficiency of competent cell or wrong type of antibiotics. The competent cell should have transformation efficiency greater than the seventh power of ten. False-positive is also very common, having 2 main cases. First, the clone does not contain target gene, caused by the incomplete linearization of the vector or circular plasmid with same resistance in the reaction system. Second, the clone contains wrong target gene, caused by non-specific PCR product or segment deletion. We can improve the specificity of the PCR system and conduct sequence alignment to solve this problem. No bands in colony PCR might incurred by 3 main reasons, including unsuccessful connection, improper PCR system, and wrong colony PCR primer. Multiple bands in restriction digestion verification might also happen, and the possible reason is that the recombinant vector contains multiple same restriction enzyme cut site, so we can use colony PCR or sequence screening to verify the result (Figure 2).

Figure 2: The gel image for the products of colony PCR

3. The DNA sequencing result of the recombinant plasmid

The detected sequence exhibits high accuracy and completeness, with a confirmed length of 720 base pairs (bp). This length aligns with the expected range for the target genomic region, as predicted by the reference genome, indicating successful capture of the entire region without significant truncation or elongation. The sequence's accuracy is supported by high-quality scores from the sequencing platform, reflecting minimal base-calling errors. Furthermore, the presence of all essential functional elements—such as promoter regions, coding sequences, and termination signals—confirms its completeness, ensuring proper gene expression and regulation. Overall, the 720bp sequence provides a reliable and comprehensive representation of the target genomic segment, making it suitable for downstream applications, including comparative genomics, functional annotation, and molecular cloning (Figure 3,4).

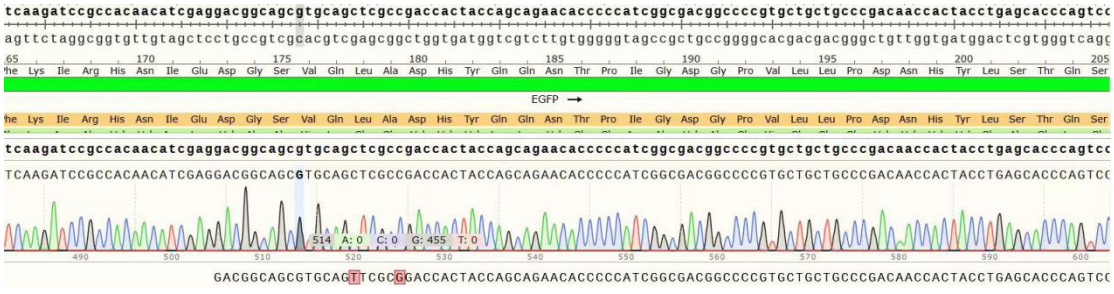

Figure 3: The DNA sequencing result of the recombinant plasmid

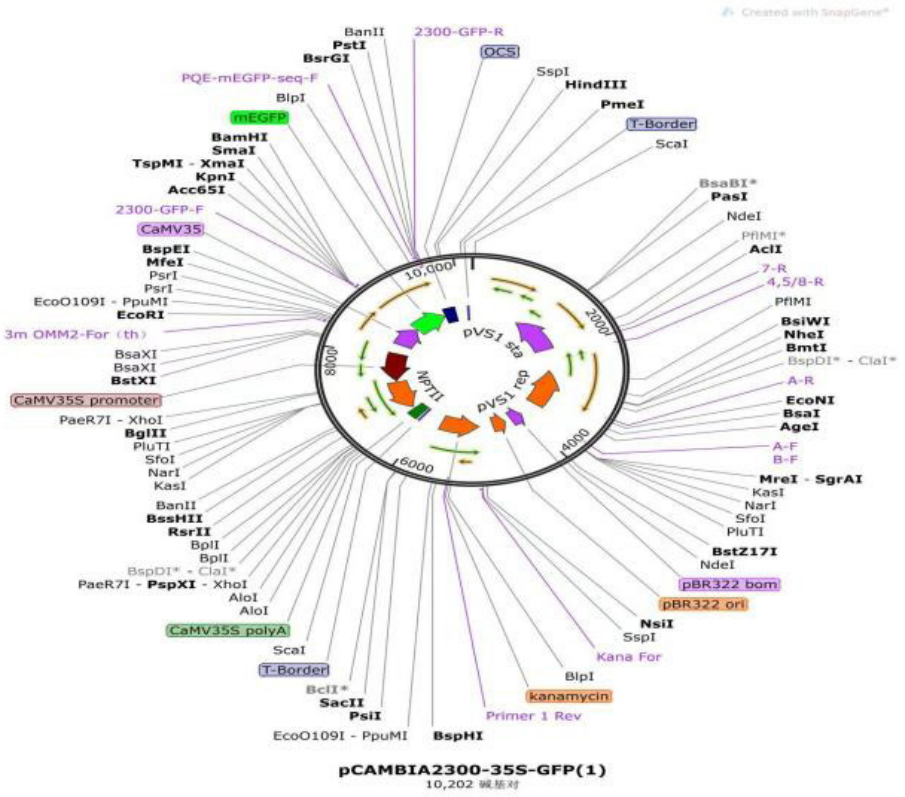

Figure 4: The recombinant plasmid

4. Plasmid digestion validation

The second to the fifth lane all show two bands, indicating the vector and the target gene. Consequently, the vector and the target gene were correctly connected. Multiple bands in restriction digestion verification might also happen, and the possible reason is that the recombinant vector contains multiple same restriction enzyme cut site, so we can use colony PCR or sequence screening to verify the result (Figure 5).

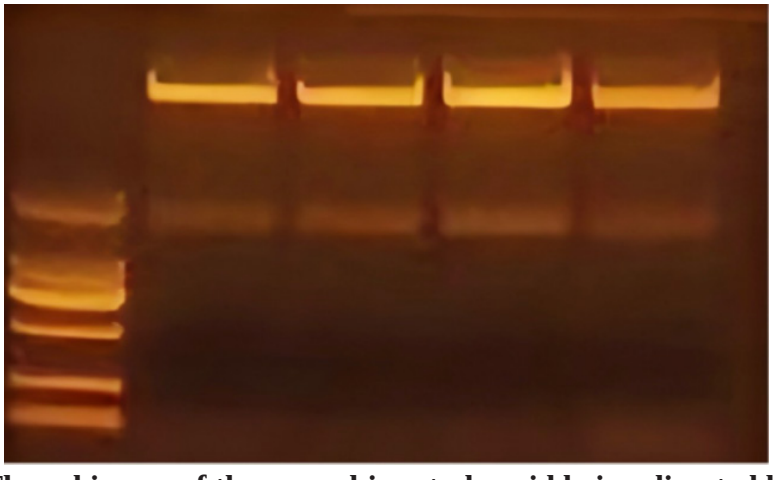

Figure 5: The gel image of the recombinant plasmid being digested by enzymes

5. Tobacco leaves emit green fluorescence

Implant the recombinant plasmid into Agrobacterium and use Agrobacterium as a vector for infection of tobacco leaves. Shake and coat plates with Agrobacterium, then place them in an incubator for cultivation. After cultivation, inject the bacterial solution into tobacco leaves and continue cultivation for 2~3 days. Subsequently, certain

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region of the tobacco leaf shows green fluorescent under the UV light illumination, indicating the success expression of GFP gene in tobacco leaves. (Figure 6,7).

Figure 6: the image of Agrobacterium infects tobacco leaves

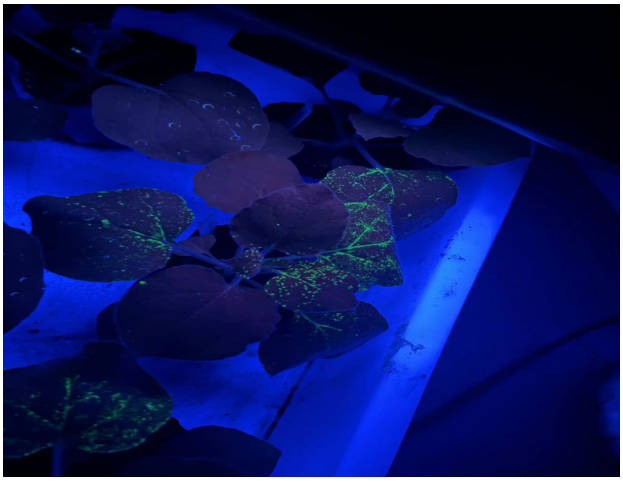

Figure 7: The image observed directly under UV light

Conclusion

The study investigated the construction of vector of the transient gene expression of GFP in plants and the protein based on the central dogma of biology and biological and chemical feature of DNA molecule. In this study, we isolated and purified Plasmid DNA by using alkaline lysis. This method uses the molecular size and chemical features of plasmid DNA and chromosome. To elaborate, plasmid DNA, a covalent supercoiled closed circular DNA, is independent from the chromosome, being able to perform replication without relying on chromosome. In the highly alkaline environment provided by the LB buffe, cell wall and cell membrane are broken, releasing the DNA inside. The hydrogen bond is broken. The NB buffer, an acidic solution, neutralizes the alkaline environment. Thus, the plasmid DNA and chromosome experience renaturation. However, two chains of the plasmid DNA entangle with each other while the hydrogen bond is broke and the plasmid DNA is smaller than the chromosome. As a result, the plasmid DNA will have a higher renaturation rate, while chromosome will entangle with the PDS protein, which is insoluble in water. The isolated plasmid needs purification, the silicon substrate membrane could absorb the plasmid. Consequently, a purified target DNA is obtained.

The target DNA, GFP, was then amplified using PCR. To explain, PCR includes 3 steps: denaturation, annealing, and extension. In denaturation, the temperature reaches 95 Celsius to break the hydrogen bond. In annealing, the temperature is usually 58 Celsius so that the primer combines with the DNA template. In extension, the temperature is 72 Celsius, which is optimized temperature for DNA polymerase. The After-vector linearization, the PCR and restriction digestion products were verified by using gel electrophoresis. DNA recovery and purification and homologous recombination was performed, forming the recombinant plasmid. The homologous recombination does not rely on restriction enzyme and DNA ligase, the target segment can be inserted into the vector at any position. In homologous recombination, the forward and reverse primers are introduced into the linear vector so that the PCR product contains sequences at its ends that match he sequences at the ends of the linear vector, and the PCR products are combined with the linear vector. Chemical transformation was conducted, transforming the plasmid into E. coli. Specifically, chemical transformation uses ice bath and heat shock, so the plasmid is absorbed on the surface of the competent cell and then enter the cell. The second ice bath in the experiment aims to form a complete cell by closing holes on the cell surface.

We did a series of experiment to confirm the accuracy and completeness of the recombination. Confirmed to be correct, the plasmid was transformed into Agrobacterium, which is able to integrate t-DNA on Ti plasmid into plant genome and express it stably under the stimulation of phenolic compound. Consequently, we used Agrobacterium-mediated transformation so that the plant can express GFP gene. The infection solution can enhance the absorption of the Agrobacterium on the recipient cells and increase the cell membrane permeability. The MES in the solution maintains the stability and activity of the Agrobacterium. The ASG is a phenolic compound that promotes the integration of t-DNA. MgCl2 adjusts the osmotic pressure across the membrane, increasing cell membrane perme-

ability. We collected the concentration of the PCR product, restriction digestion product, and the recombinant plasmid to make sure the purity of DNA. The gel images were also collected to make sure correct sequence length. DNA sequencing was performed and we compared the test sequence with the GFP sequence. The recombinant plasmid map was generated using Snap gene. The DNA sequencing shows that the target gene was correct, and the restriction digestion verification indicates that the recombination was success. The method used in the study was technically mature, but we need to improve the rigor of the experimental operation to minimize experimental variations, and the result should be observed under the confocal microscope, instead of direct UV light illumination, to generate more accurate image. Based on the successful recombination, this study provides safe and efficient method to study gene expression and function in plants. Transient gene expression of GFP will be widely utilized by pharmaceutical companies to evaluate medicines more efficiently. In addition, this technology is also conducive to Biomedical Science, by tracking cells, the GFP is able to shed light on the mechanisms of complex diseases and the treatment efficiency and safety. As a reporter gene, the GFP also contributes to the construction and improvement of gene circuit in synthetic biology. It is necessary to optimize the vector system, such as improved virus vectors and non-virus vectors, to refine the transfection efficiency and expression level. New regulatory elements including promoter, operator, enhancer, and silencer need to be studied to achieve more accurate spaciotemporal control.

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