International Journal of Biology and Life Sciences
ISSN: 2957-9511 | Vol. 12, No. 3, 2025

Construction of an Exogenous Green Fluorescent Protein (GFP) Gene Expression Vector and its Expression Verification in Tobacco Leaves

Sicong Ge *

Guangdong Country Garden School, Foshan, Guangdong, 510000, China
*** Corresponding author Email:** elsa_ge_eg@outlook.com

Abstract: This study employed genetic engineering techniques to design and construct a specific expression vector containing the GFP gene. Subsequently, the constructed vector was transformed into Agrobacterium GV3101 via Agrobacterium infection of tobacco leaves. Experimental results demonstrated that the constructed exogenous GFP gene vector successfully achieved transient expression in tobacco leaves, which emitted distinct green fluorescence. This study not only validated the effectiveness of the gene expression vector construction but also provided crucial practical experience for further investigating gene functions within plants and advancing plant genetic improvement efforts.

Keywords: Green Fluorescent Protein (GFP); Gene Expression; Vector Construction.

1. Introduction

Vector construction technology is a technique that utilizes molecular biology and genetic engineering methods to incorporate specific substances into vectors such as plasmids or proteins to achieve certain functions. This technology relies on restriction enzymes to cleave vectors and target genes, creating complementary ends. These ends are then joined by DNA ligase to form recombinant vectors, which are ultimately introduced into host cells to enable gene replication and expression. The high efficiency, versatility, and precision of this technology enable its broad application. For instance, constructing prokaryotic or eukaryotic expression vectors facilitates efficient expression of target genes in bacteria or mammalian cells, serving protein function studies or industrial production. Vector construction technology also holds broad prospects in plant genetic improvement and gene function studies. Chen Sa et al. [1] designed specific primers to amplify soybean chloroplast homologous recombination sequences, then cloned these alongside selection marker genes and target genes into plasmid vectors. This approach provided critical technical support for resolving the contamination issue between sterile lines and maintainer lines in hybrid soybean seed production.

Green Fluorescent Protein (GFP), a bioluminescent protein discovered in jellyfish, consists of approximately 238 amino acids. It emits green fluorescence when excited by blue or ultraviolet light [1]. Its luminescence primarily results from a luminescent protein in jellyfish—aureochrom—emitting blue light upon calcium ion stimulation. This energy is then transferred to GFP, which absorbs it, becomes excited, and subsequently emits green fluorescence. This protein was first discovered in 1962 by Japanese chemist Osamu Shimomura and colleagues from the Victoria jellyfish [2]. In 1994, Martin Chalfie's team successfully expressed GFP in E. coli and nematodes, demonstrating its utility as a universal fluorescent marker [3]. In 1995, the team led by Yong-Jian Chen engineered GFP through site-directed mutation (S65T), enhancing its fluorescence intensity sixfold compared to the wild-type and simplifying its excitation spectrum to better match commercial lasers [4]. These studies collectively established GFP's status as a "universal marker" in molecular biology. Its ability to stably express without exogenous assistance and its heritable nature have made it a core tool for gene function studies, cell tracing, and biomedical imaging. It finds extensive application across biological research, genetic engineering, drug development, developmental biology, and optogenetics.

Tobacco, valued for its short growth cycle, ease of propagation, and large, abundant leaves, is a widely used plant model in plant biology and pathology research. Mediated by Agrobacterium, tobacco exhibits high genetic transformation efficiency, making it an early model species in plant genetic engineering [5-7]. This study employed vector construction techniques to build a plasmid vector. Using Agrobacterium infection, the green fluorescent protein (GFP) gene was transferred into tobacco leaves for expression. This approach investigated how the target gene enters tobacco leaves via the vector for expression.

2. Methods and Materials

2.1. Materials

2 mL test tubes, pipettes, primers, plasmids, Tap enzyme, double-distilled water, PCR reaction mixture, LB medium, linearized vector, Resuspension Buffer (RB), Lysis Buffer (LB, blue), Neutralization Buffer (NB, yellow), Wash Buffer (WB), Elution Buffer (EB), RNase A (10 mg/mL), Buffer GDP, Buffer GDP+, Buffer GW, Elution Buffer, DH5α, PCR, 5× CE II Buffer, Exnase II, etc.

2.2. Experimental Methods

2.2.1. Plasmid Extraction

(1) Take overnight bacterial culture, centrifuge at 10,000 rpm for 1 minute, and remove supernatant. If the bacterial culture volume is too large, centrifuge in multiple batches to collect.

(2) Add 250 μL of colorless solution RB (containing RNase A). Vortex to resuspend bacterial pellet, ensuring no small clumps remain.

(3) Add 250 μL of blue solution LB. Gently invert 4–6 times to thoroughly lyse cells, forming a blue translucent solution. The color should change from semi-translucent to vivid blue.

(4) Add 350 μL of yellow solution NB. Gently mix 5-6 times (the color changes completely from blue to yellow, indicating thorough mixing and complete neutralization) until a compact yellow floc forms. Let stand at room temperature for 2 minutes.

(5) After standing, centrifuge at 12,000 rpm × 5 min. Carefully aspirate the supernatant and add it to the spin column. Allow to stand for 5 min, then centrifuge at 12,000 rpm × 1 min. Discard the flow-through. Repeat the centrifugation process. If the supernatant volume exceeds 800 μL, add it to the column in multiple portions, centrifuging and discarding the flow-through as described above.

(6) Add 650 μL WB solution, centrifuge at 12,000 rpm × 1 min, and discard the flow-through.

(7) Centrifuge again at 12,000 rpm × 2 min to completely remove residual WB. Open the tube and let it stand for 5 min to evaporate the ethanol.

(8) Place the spin column in a clean centrifuge tube. Add 50 μL of EB buffer or deionized water to the center of the column and let it stand at room temperature for 1 minute.

(9) Transfer the filtered liquid from the centrifuge tube back into the spin column and let it stand for 1 minute.

(10) After standing, centrifuge at 10,000 × g for 1 minute to elute the DNA. Store the eluted DNA at -20°C.

2.2.2. Amplification PCR

(1) Add 1 μL of 2300-GFP-F, 1 μL of 2300-GFP-R, 1 μL of plasmid, 7 μL of double-distilled water, and 10 μL of Tap enzyme to a 2 mL tube. Add 1 μL of 2300-GFP-F, 2300-GFP-R, 1 μL water, 7 μL double-distilled water, and 10 μL Tap enzyme into a 2 mL tube as a negative control.

(2) Simulate the catalytic action of DNA polymerase in living organisms using a PCR machine to perform specific DNA sequence polymerization and amplification in test tubes. Denature at 94°C for 30 seconds, anneal at 55°C for 30 seconds, extend at 72°C for 30-60 seconds, repeating approximately 30 cycles.

2.2.3. Gel Recovery

(1) After DNA electrophoresis, quickly cut out the band containing the target DNA fragment under UV light. Blot off surface liquid with paper towels, chop the gel into small pieces, and remove as much excess gel as possible.

(2) Add an equal volume of Buffer GDP. Incubate at 50–55°C for 7–10 minutes, adjusting time based on gel size to ensure complete dissolution. Invert and mix twice during incubation to accelerate dissolution.

(3) Briefly centrifuge to collect droplets from the tube walls. Place the adsorption column into a 2 ml collection tube. Transfer 700 μl of the solute solution into the adsorption column and centrifuge at 12,000 rpm for 60 seconds. If the solute volume exceeds 700 μl, place the adsorption column into a recovery tube and transfer the remaining solution into the adsorption column. Centrifuge at 12,000 rpm for 30–60 seconds.

(4) Discard the filtrate. Place the adsorption column in the collection tube. Add 300 μl of Buffer GDP to the adsorption column. Incubate for 1 min. Centrifuge at 12,000 rpm for 30–60 seconds.

(5) Discard the filtrate. Place the adsorption column in the collection tube. Add 700 μL of Buffer GW to the adsorption column. Centrifuge at 12,000 rpm for 30–60 seconds.

(6) Repeat step 5.

(7) Discard the filtrate. Place the adsorption column in the recovery collection tube. Centrifuge at 12,000 rpm for 2 minutes.

(8) Place the adsorption column in a 1.5 ml sterile centrifuge tube. Add 20–30 μl Elution Buffer to the center of the column. Incubate for 2 min, then centrifuge at 12,000 rpm for 1 min. Discard the column and store the DNA at -20°C. For recovery of fragments larger than 3 kb, preheat the Elution Buffer to 55°C to enhance recovery efficiency.

2.2.4. Homologous Recombination

(1) Calculate the required DNA amount for the recombination reaction based on the formula.

(2) Prepare the reaction mixture on ice: 13.6 μL linearized vector, 0.4 μL PRC, 4 μL 5× CE II Buffer, and 2 μL Exnase II.

(3) Gently mix by pipetting up and down, then briefly centrifuge to collect the reaction mixture at the bottom of the tube.

(4) Incubate at 35°C for 30 min, then place in a 4°C refrigerator.

2.2.5. Transformation of Competent E. coli with Recombinant Vectors

(1) Take 50 μL of thawed competent cells from an ice bath, add the target DNA, gently mix, and incubate on ice for 30 minutes.

(2) Heat shock at 42°C for 60 seconds, then promptly transfer the tube to ice for 2 minutes. Do not agitate the centrifuge tube during this process.

(3) Add 500 μl of LB medium to each centrifuge tube. Mix thoroughly and incubate on a 37°C shaking incubator at 200 rpm for 1 hour to allow bacterial recovery.

(4) Pipette 200 μl of the transformed competent cells onto LB agar plates containing kanamycin. Spread the cells evenly, place the plates at 37°C until the liquid is absorbed, then invert the plates and incubate at 37°C overnight.

2.2.6. Identification of Recombinant Products

(1) After overnight incubation, hundreds of monoclonal bacteria colonies form on the recombinant reaction transformation plate.

(2) Pick several clones from the recombinant reaction transformation plate for colony PCR identification, using at least one universal sequencing primer from the vector as an amplification primer.

2.2.7. Transformation of Agrobacterium

(1) Remove Agrobacterium competent cells stored at -80°C and allow them to partially thaw at room temperature or in an ice-water bath. Once in a slushy state, transfer the tube to an ice bath.

(2) Add 200 ng plasmid DNA to each 20 μl of competent cells. Mix by gently tapping the tube bottom. Sequentially incubate on ice for 10 min, in liquid nitrogen for 5 min, in a 37°C water bath for 5 min, and on ice for 5 min.

(3) Add 800 μl antibiotic-free LB liquid medium. Incubate at 28°C with shaking for 2–3 hours.

(4) Centrifuge at 5000 rpm for 1 minute. Resuspend the pellet in approximately 100 μl of the supernatant. Gently pipette to resuspend the cell pellet and spread it onto LB plates containing the appropriate antibiotics (kanamycin, gentamicin, rifampicin). Incubate the plates upside down in a 28°C incubator for 2–3 days.

3. Experimental Results and Analysis

The experimental vector selected for this experiment was plasmid DNA extracted from Escherichia coli. The figure below shows the concentration measurement graph for the extracted 2300GFP plasmid, indicating a plasmid concentration of 93.09 nanograms per microliter (as shown in Figure 1). The wavelength at 260 nanometers confirms the accuracy of the test results.

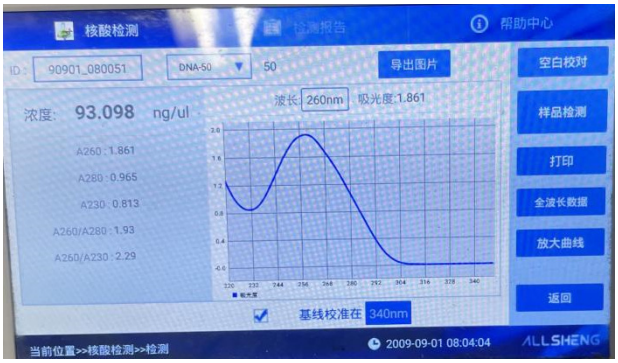

Figure 1. Plasmid concentration profile

After plasmid extraction is completed, primers must be designed. Using the target DNA as a template, PCR technology is employed to amplify the target gene fragment in large quantities. The figure below shows the PCR program (as shown in Figure 2). Based on the principle of semi-conservative DNA replication, it achieves rapid in vitro amplification of the target DNA fragment through three cycled steps: high-temperature denaturation, low-temperature annealing, and extension at the optimal temperature.

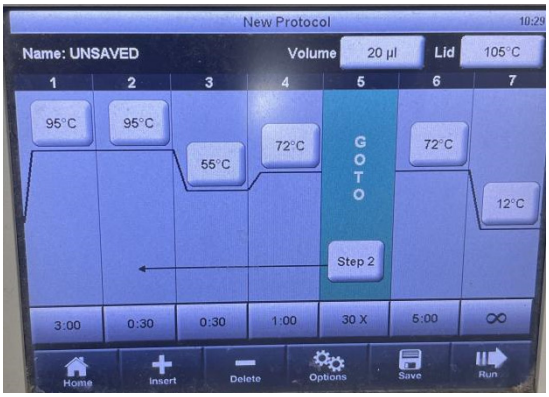

Figure 2. PCR instrument parameters

After PCR amplification of the target DNA, agarose gel electrophoresis is used to separate DNA fragments of different sizes. In agarose gel electrophoresis, negatively charged DNA molecules migrate toward the positive electrode under an electric field. DNA fragments of different sizes encounter varying resistance and thus exhibit distinct migration rates, enabling their separation. After staining with a nucleic acid dye, DNA bands become visible under UV light. as shown in the figure below (Figure 3). The image clearly displays the expected-sized GFP gene band, confirming successful amplification of the target sequence via PCR. This result establishes a solid foundation for subsequent experiments.

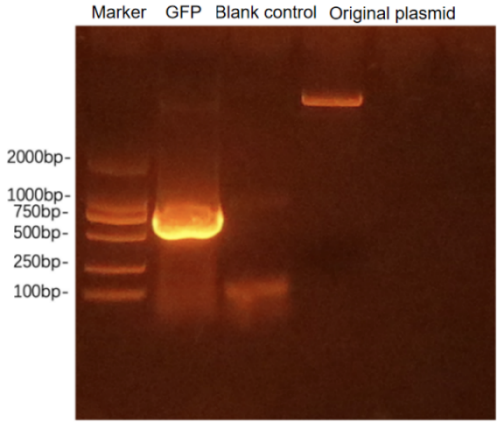

Figure 3. DNA bands observed under UV light

After successfully amplifying the target gene and extracting it into a plasmid, the extracted plasmid must be digested with restriction enzymes. These enzymes recognize and cleave specific DNA segments on the plasmid, creating nicks that facilitate subsequent ligation. The digested plasmid vector is then subjected to agarose gel electrophoresis to separate fragments of different sizes. After successful separation, the desired vector fragment is excised for subsequent recovery. The figure below shows the band pattern before and after plasmid digestion following electrophoresis (as shown in Figure 4).

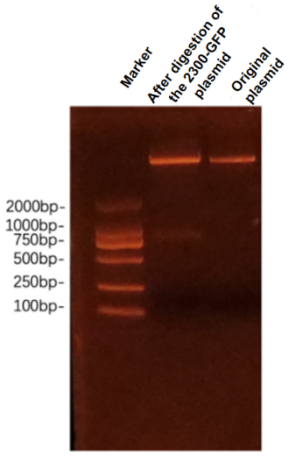

Figure 4. Agarose gel electrophoresis of plasmid vectors

After gel recovery of the vector, the recovered target gene fragment was ligated with the vector fragment using Exnase II. The recombinant plasmid was then transformed into host E. coli for cultivation. The resulting E. coli culture is shown in Figure 5.

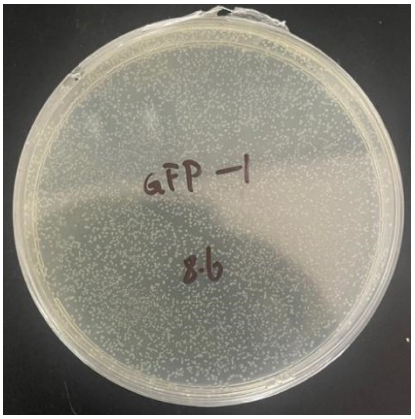

Figure 5. E. coli culture plate

After culturing E. coli, single colonies were picked, subjected to PCR amplification, and then analyzed via agarose gel electrophoresis to confirm whether the GFP-containing vector was correctly introduced into E. coli. The electrophoresis results are shown in Figure 6.

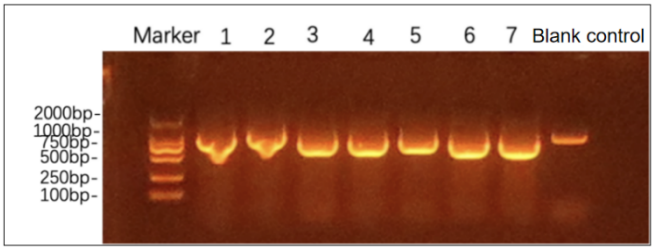

Figure 6. Agarose gel electrophoresis following PCR amplification

After confirming that the target gene was correctly replicated, the GFP-containing vector was transferred to Agrobacterium and cultured on solid medium containing kanamycin for screening. The culture results are shown in Figure 7.

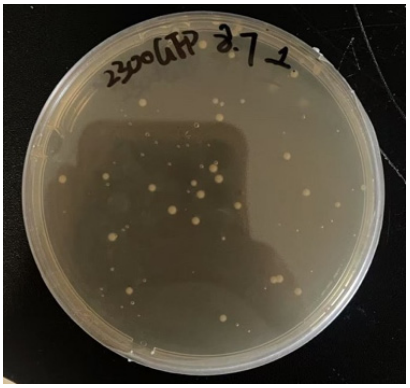

Figure 7. Agrobacterium cultured using the streaking method

4. Summary and Outlook

This study not only investigates the visual expression of exogenous genes but also technically validates the reliability of the plasmid transformation system, offering an innovative solution to practical agricultural challenges such as hybrid seed purity. By employing Agrobacterium-mediated transformation, we successfully achieved GFP gene expression in tobacco leaves. Fluorescent microscopy revealed distinct green fluorescence signals in transformed tissues, providing direct evidence of the method's efficacy. As a highly efficient reporter gene, GFP's unique fluorescent properties make it an ideal marker for studying gene expression in living cells. Despite successful GFP expression, experimental results indicate that transformation efficiency remains an area for improvement. Transformation efficiency, a critical indicator in genetic engineering, directly impacts the integration and expression of exogenous genes. Multiple factors influence this efficiency, including: selection of Agrobacterium strains, product recovery during experiments, physiological state of recipient material, and experimental procedures. Systematic optimization of these variables holds promise for significantly enhancing gene transfer efficiency in subsequent experiments.

Notably, this study did not thoroughly investigate the long-term stability of GFP gene expression. Gene silencing, a common phenomenon in plant transgenic research, may result from epigenetic regulation, integration site effects, or host defense responses. Future studies should focus on monitoring the expression dynamics of transgenes across different developmental stages, providing crucial evidence for assessing transgene persistence and application potential. The findings of this study establish a technical foundation for plant gene function research and transgenic crop development. The GFP labeling system offers unique advantages in gene regulation analysis, protein subcellular localization, and cellular process tracing. Furthermore, the results reinforce the reliability of Agrobacterium-mediated transformation, providing technical support for more complex genetic manipulations.

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