Journal of Modern Crop Science (2025)
Clausius Scientific Press, Canada

DOI: 10.23977/jmcs.2025.040103
ISSN 2616-2075 Vol. 4 Num. 1

Construction of an Ectopic GFP Gene Transient Expression Vector and Study on Protein Expression

Rongan Xu

Shoudu Normal University Affiliated High School, No. 33 Beiwa Road, Haidian District, Beijing, China
xurongan2026@163.com

Keywords: Green Fluorescent Protein (GFP); Transient Expression Vector; Tobacco Leaves; Agrobacterium-Mediated Transformation; Gene Expression

Abstract: This study developed a rapid tool for plant gene function research by constructing a transient ectopic expression vector for GFP—an essential reporter gene—and validating its expression in tobacco leaves. Using gene cloning techniques, GFP was inserted into a plant expression vector, transformed into tobacco via Agrobacterium-mediated delivery, and verified through PCR, gel electrophoresis, and enzyme digestion. The system achieved high-efficiency transient GFP expression in tobacco cells, confirmed by distinct green fluorescence under microscopy. Results demonstrate that this method provides rapid, effective technical support for plant gene expression studies, with significant application value; future optimizations may further enhance its stability and expression efficiency for broader molecular biology applications.

1. Introduction

Green fluorescent protein (GFP) is a widely used reporter gene in biomedical and plant science research. Due to its ability to emit green fluorescence within cells without the need for additional substrates, GFP has been extensively utilized in the study of gene expression, protein localization, and cellular dynamics. In plant research, GFP technology is not only used to mark the expression of specific genes but also helps scientists understand the roles of genes in plant growth, development, and responses to stress. Additionally, GFP has been applied to study protein interactions and subcellular localization within plant cells. Therefore, constructing a transient expression vector for the GFP gene is of significant importance for rapidly verifying gene functions and protein expression. In recent years, with the continuous development of molecular biology techniques, the application scope of GFP has expanded, making it an indispensable tool in plant gene function research.

In recent years, the application of GFP in plant gene function research has attracted widespread attention. Studies have shown that GFP can be used as a reporter gene to monitor gene expression and protein localization in plant cells. For example, "GFP fusion protein technology has been successfully applied to study signal transduction and protein interactions in plant cells" [2]. Additionally, "GFP has also been used to study changes in gene expression under stress conditions in plants" [3]. In terms of plant gene expression vector construction, transient expression systems have been widely used for the preliminary validation of plant gene functions due to their rapid and efficient

nature. Transient expression technology introduces the target gene into plant cells via Agrobacterium-mediated transformation, achieving high-level expression without integrating into the plant genome. This method not only saves time but also avoids the complex selection processes required for stable transformation. Tobacco, with its high efficiency in gene transformation and expression, has become an important model plant in the fields of molecular biology and bioengineering. For example, "Zhang et al. (2022) studied the effects of different transformation conditions on the transient expression of GFP genes from three types of Agrobacteria in Nicotiana Benthamian and found that optimizing transformation conditions can significantly improve GFP expression efficiency" [1]. In addition, "Zhao et al. (2013) reviewed the main methods and progress of plant transient expression technology, highlighting its significant application value in plant gene function research" [2]. At the same time, "Liu et al. (2024) constructed a rice sheath protoplast transformation system and successfully achieved transient expression of Pik-H4 and AvrPik-H4 proteins, providing new ideas and methods for the study of rice disease resistance genes" [3]. This study not only expanded the application scope of transient expression technology but also offered references for the functional research of other plant genes. Furthermore, "Wu et al. (2024) explored the multi-pathway applications of tobacco transient expression technology, further enriching its application patterns in plant gene function research" [4]. In plant gene function research, Agrobacterium-mediated transformation is a commonly used technique. Huo et al. (2016) optimized the experimental conditions for Agrobacterium-mediated transient expression in tobacco, improving the expression efficiency of the GFP gene [5]. In addition, Liu et al. (2009) successfully localized the Masr protein in onion epidermal cells using Agrobacterium-mediated methods, further demonstrating the reliability and effectiveness of this approach in plant gene function research [6].

This study aims to construct a transient expression vector for the ectopic GFP gene and verify its expression in tobacco leaves. By employing gene cloning and vector construction techniques, the GFP gene will be inserted into a plant expression vector and introduced into tobacco leaves via Agrobacterium-mediated transformation. The results will be observed under a fluorescence microscope to monitor GFP expression and validated through molecular biology methods to confirm GFP protein expression. The goal of this study is to develop a rapid and efficient tool for plant gene expression research, providing technical support for plant gene function studies. Through this research, we hope to offer a new method for the rapid validation of plant gene functions and protein expression, thereby advancing the development of plant science research. Additionally, this study will provide references for the transient expression of other plant genes, promoting the development of plant molecular biology techniques.[7,8]

2. Materials and Methods

(1) Extraction and Concentration Determination of Plasmid DNA

Materials:

Overnight bacterial culture, Pipette, Centrifuge tubes, Resuspension Buffer (RB), Lysis Buffer (LB,Blue), Neutralization Buffer (NB, Yellow), Wash Buffer(WB), Elution Buffer(EB), RNase A(10 mg/ml), Mini-Plasmid Spin Columns with Collection Tubes, Centrifuge, UV spectrophotometer.

Methods:

1) Take the overnight bacterial culture and divide it into two portions in centrifuge tubes. Centrifuge at 10,000 ×g for 1 minute and remove the supernatant (try to remove it completely). Add the colorless Resuspension Buffer (RB, containing RNase A) and vortex to resuspend the bacterial pellet, ensuring no small bacterial clumps remain.

2) Add the blue Lysis Buffer (LB) and gently invert the tube 4-6 times to allow complete lysis of the bacterial cells, resulting in a clear blue solution. The change from semi-transparent to clear blue

indicates complete lysis (do not exceed 5 minutes).

3) Add the yellow Neutralization Buffer (NB) and gently mix 5-6 times (the color should change completely from blue to yellow, indicating thorough mixing and neutralization). Allow the mixture to stand at room temperature for 2 minutes until a tight yellow precipitate forms.

4) Centrifuge at 12,000×g for 5 minutes. Carefully transfer the supernatant to the mini-spin column.Centrifuge at 12,000×g for 1 minute and discard the flow-through. If the supernatant volume exceeds 800 μL, it can be added to the column in multiple portions and centrifuged as described above,discarding the flow-through each time.

5) Add 650 μL of Wash Buffer (WB) to the column and centrifuge at 12,000×g for 1 minute. Discard the flow-through.

6) Centrifuge at 12,000×g for 1-2 minutes to completely remove any residual WB.

7) Place the spin column into a clean centrifuge tube. Add pre-warmed deionized water (heated in a 60-70℃ water bath) to the center of the column and allow it to stand at room temperature for 1 minute.

8) Centrifuge at 10,000×g for 1 minute to elute the DNA. Transfer 2 μL of the eluate to a UV spectrophotometer to determine the concentration. Store the eluted DNA at -20℃.

(2) PCR Amplification

Materials:

Primers: Approximately 20 bases long, determining the starting points and specificity of amplification Enzyme: A DNA polymerase used for synthesizing new DNA strands, dNTPs: The building blocks for DNA synthesis, DNA Template, Reaction Buffer: Contains Mg^{2+}, providing an optimal environment for the PCR reaction.

Methods: (Setting up a 50 μL Reaction System)

1) Preparation of the Reaction Mixture: In a centrifuge tube, add the following reagents in sequence of buffer,1 μL of dNTPs,2 μL of each primer,1 μL of DNA template,18 μL of water,1 μL of enzyme (add last)

2) PCR Cycling Conditions: Place the centrifuge tube into the PCR instrument and set the following program: Heat to 95℃ for 3 minutes (initial denaturation). Heat to 95℃ for 30 seconds (denaturation). Cool down to 55℃ for 30 seconds (annealing). Heat to 72℃ for 1 minute (extension). Repeat steps 2-4 for 34 cycles. Maintain at 72℃ for 5 minutes (final extension). Post-PCR Processing: After the PCR is complete, remove the centrifuge tube from the PCR instrument and add 5 μL of 10x loading dye.

(3) Preparation of Agarose Gel

Materials:

TAE Buffer (Tris-Acetate-EDTA Buffer): 20 ml of 50x concentrated solution and 980 ml of water (used to prepare 1 L of 1x TAE buffer), Agarose: 0.5 g, for preparing a 1% agarose gel, Nucleic Acid Stain for Agarose Gel: Add 5 μL to 50 ml of agarose gel.

Methods:

1) Take 50 ml of TAE buffer and add 0.5 g of agarose.

2) Heat in a microwave until completely dissolved.

3) Add the nucleic acid stain for agarose gel (add 5 μL to 50 ml of agarose gel).

4) Pour into a gel mold and allow to set.

(4) Vector Linearization

Materials:

10×Cutsmart, BamHI, PstI, Vector(Plasmid DNA), H_2O

Methods:

1) Add following the reagents to a centrifuge tube in sequence:5 μL of 10× CutSmart Buffer,5 μL of Vector (Plasmid DNA),1 μL of Brahmi enzyme,1 μL of Pusti enzyme,36 μL of H_2O

2) Place the centrifuge tube in a metal bath at 37 ℃ and incubate for one hour.

(5) Agarose Gel Electrophoresis

Materials:

Electrophoresis apparatus, PCR products, Plasmid after digestion, Undigested plasmid, UV transilluminator

Methods:

1) Place the prepared agarose gel into the electrophoresis apparatus and fill the wells with the following samples: Well 1: 10 μL of DNA marker, Well 2: 50 μL of PCR product, Well 3: 50 μL of PCR product, Well 4: 50 μL of digested plasmid, Well 5: 50 μL of digested plasmid, Well 6: 50 μL of undigested plasmid

2) Set the voltage to 180 V and run the electrophoresis for 15 minutes.

3) After electrophoresis, place the gel on the UV transilluminator to observe the results.

(6) Recovery and Concentration Determination of DNA Fragments and Linearized Vectors

Materials:

Buffer GDP (Gel Dissolving Solution), Buffer GW (containing anhydrous ethanol),Centrifuge, Centrifuge tubes, Buffer, Adsorption column, Pipette, Collection tube.

Methods:

1) Under UV light, use a scalpel to cut out the gel containing the DNA fragment (two portions: one from PCR and one from digested plasmid).

2) Place the excised gel on a balance to weigh it.

3) Transfer the weighed gel into a centrifuge tube and add 200 μL of Buffer GDP. Place the tube in a heat block and heat at 55 ℃ for 15 minutes.

4) After heating, briefly centrifuge to remove any liquid from the walls and lid of the tube.

5) Transfer the dissolved gel solution to the adsorption column placed in a collection tube. Centrifuge at 12,000 rpm for 1 minute and discard the flow-through.

6) Add 650 μL of Buffer GW to the column and centrifuge for 30 seconds.

7) Repeat step 6.

8) Centrifuge for 2 minutes to remove any residual wash buffer. Leave the lid open for an additional 2 minutes to allow the wash buffer to evaporate completely.

9) Place the adsorption column into a new centrifuge tube. Add 30 μL of pre-warmed elution buffer (55 ℃) to the center of the column. Allow it to stand for 1 minute, then centrifuge for 1 minute.

10) Transfer the eluate back onto the adsorption column and centrifuge for another minute to maximize recovery.

11) Measure the concentration of the recovered PCR product (63 ng/μL) and the recovered digested plasmid (20 ng/μL) using a UV spectrophotometer.

(7) Ligation of Recombinant DNA

Materials:

1.5×T4 DNA Ligase Buffer: 4 μL, Exo SAP-IT: 2 μL, PCR Product (m = cv, 63 ng/μL, 25 ng, add 1 μL), Digested DNA (m = cv, 20 ng/μL, add 10 μL),H_2O: 3 μL (to make up to 20 μL total volume)

Methods:

1) Add the above reagents to a centrifuge tube in sequence, adding the enzyme last.

2) Place the centrifuge tube in a PCR instrument and incubate at 37 ℃ for 20 minutes.

(8) Homologous Recombination

Materials:

Escherichia coli competent cells, Target DNA, Ice bath, LB liquid medium, Kanamycin sulfate, Laminar flow hood, Petri dishes, Heat block, Shaking incubator, Centrifuge Spreader, Alcohol lamp, Parafilm, Incubator

Methods:

1) Remove the competent E. coli cells from the -80 ℃ freezer.

2) Take 50 μL of competent cells and add 5 μL of target DNA. Incubate on ice for 30 minutes.

3) Allow the LB liquid medium (200 mL) to cool to a non-scalding temperature (not hot to touch), then add 100 μL of kanamycin sulfate (100 mg/mL). The working concentration of kanamycin will be 50 μg/mL.

4) Pour the medium into Petri dishes, adding 20 mL per dish.

5) After the ice bath, heat shock the cells at 42 ℃ for 45 seconds.

6) Place the cells back on ice for 2 minutes.

7) Add 500 μL of antibiotic-free LB medium.

8) Incubate the cells in a 37 ℃ shaking incubator at 300 rpm for 1 hour.

9) Centrifuge at 4000 rpm for 1 minute to collect the bacterial cells.

10) Remove 800 μL of the supernatant and mix the bacterial pellet by pipetting.

11) Sterilize the spreader by heating it over the alcohol lamp.

12) Aspirate all the bacterial suspension and spread it evenly on the Petri dish.

13) Label the dish and sterilize the spreader.

14) Cover the dish and seal it with parafilm.

15) Invert the dish and incubate at 37 ℃ for 12 hours.

(9) Colony PCR Verification

Materials:

Sterile water, Forward primer (Primer-F), Reverse primer (Primer-R), Taq enzyme mix.

Methods:

1) Take four centrifuge tubes and add 40 μL of sterile water to each.

2) Use a pipette tip to pick a single colony and resuspend it in the tube by pipetting.

3) Repeat this step for the remaining three tubes and label them as Colony 1, Colony 2, Colony 3, and Water 4 (negative control).

4) In one of the centrifuge tubes, add 4 μL of Primer-F, 4 μL of Primer-R, and 40 μL of Taq enzyme mix. Mix thoroughly by pipetting.

5) Prepare three additional centrifuge tubes, each containing 12 μL of the PCR reaction mixture, and label them as 1, 2, and 3.

6) Add the bacterial suspensions from Colony 1, Colony 2, and Colony 3 to the respective tubes, and place the tubes in the PCR machine. Set the PCR program as follows: 95 ℃ for 3 minutes (initial denaturation), 95 ℃ for 30 seconds, 55 ℃ for 30 seconds, 72 ℃ for 30 seconds, Repeat steps 2-4 for 28 cycles, 72 ℃ for 5 minutes (final extension)

(10) Bacterial Culture Shaking.

Materials:

LB liquid medium and Kanamycin sulfate

Methods:

1) Place 15 mL centrifuge tubes, LB medium, and pipettes in a laminar flow hood and sterilize with UV light for 15 minutes.

2) Add 2 mL of LB liquid medium and 1 mL of kanamycin sulfate to the centrifuge tube.

3) Based on the colony PCR results, all three tubes of bacterial suspension showed successful recombination and homologous recombination. Therefore, randomly select one tube of bacterial suspension and add it to the centrifuge tube.

4) Place the centrifuge tube in a shaking incubator at 37 ℃ for 12-16 hours.

(11) Enzymatic Digestion Verification

Materials:

Plasmid DNA (concentration 59.8 ng/μL, extracted from bacterial culture), Brahmi enzyme, PstI enzyme, 10 × CutSmart Buffer and Water

Methods:

1) Extract plasmid DNA and measure its concentration.

2) Add 16.72 μL of plasmid DNA to a centrifuge tube (calculated using the formula:1000 ×enzyme volume (1 μL)/plasmid concentration (59.8 ng/μL)=16.72)Add 1 μL of enzyme (0.5 μL each of Brahmi and PstI), 5 μL of 10× CutSmart Buffer, and 27.28 μL of water to make up to 50 μL.

3) Incubate the mixture in a heat block at 37 ℃ for 2-3 hours.

4) Run the digested plasmid on an agarose gel. If the band length matches the expected PCR product length (around 750 bp), it confirms that the plasmid contains the desired PCR insert.

(12) DNA Sequencing

Methods:

Send the plasmid DNA to a DNA sequencing company for sequencing to verify that the base pairs of the gene match the designed sequence.

(13) Transformation of Agrobacterium tumefaciens (Freeze-Thaw Method)

Materials:

Agrobacterium tumefaciens competent cells (pv3101), LB liquid medium, LB solid medium (with three antibiotics: kanamycin sulfate, rifampicin, gentamicin), Liquid nitrogen, Petri dishes, Heat block, Shaking incubator, Forceps, Spreader, Incubator, Parafilm

Methods:

1) Remove the Agrobacterium tumefaciens competent cells (pv3101) from the freezer and gently tap the tube.

2) Pipette 20 μL of Agrobacterium tumefaciens competent cells into a centrifuge tube.

3) Add 2 μL of the 35S2300-GFP plasmid (from E. coli)

4) Gently tap the tube to mix the contents and incubate on ice for 5 minutes.

5) Place the centrifuge tube in liquid nitrogen for 5 minutes.

6) Using forceps, transfer the centrifuge tube to a 37 ℃ heat block for 5 minutes.

7) Remove the tube from the heat block and place it back on ice for 5 minutes.

8) Pipette 510 μL of liquid LB medium slowly into the centrifuge tube.

9) Place the centrifuge tube in a shaking incubator at 28 ℃ for 2-3 hours.

10) Centrifuge at 5000 rpm for 1 minute to collect the bacterial cells.

11) Remove approximately 400 μL of the supernatant and mix the bacterial pellet by pipetting.

12) Use the spreader to evenly distribute the bacterial suspension on the plate, and seal the plate with parafilm. Then place the plate in a 28 ℃ incubator for 2-3 days.

(14) Inoculation of Tobacco Plants

Materials:

Agrobacterium tumefaciens bacterial suspension, MES (2-Morpholinoethanesulfonic acid), Magnesium chlorides (Acetosyringone), Distilled water spectrophotometer, Incubator, Syringe, Tobacco plants.

Methods:

1) Centrifuge the Agrobacterium tumefaciens bacterial suspension at 5000 rpm for 5 minutes to collect the bacterial cells. Discard the supernatant.

2) Prepare the inoculation medium by mixing 1 mL of MES, 100 mL of distilled water, 1 mL of magnesium chloride, and 200 μL of ASG.

3) Add 10 mL of the inoculation medium to the centrifuge tube and mix by shaking.

4) Take 100 μL of the bacterial suspension and 900 μL of the inoculation medium and mix in a centrifuge tube.

5) Measure the optical density of the liquid in the centrifuge tube using a UV spectrophotometer (target OD: 0.162 Abs).

6) Calculate the volume of inoculation medium needed to adjust the concentration to 1 Abs (e.g.,

if the measured OD is 0.162, add 6.2 mL of inoculation medium to achieve an OD of 1 Abs).

7) Place the centrifuge tube in a 28 ℃ incubator for 1 hour.

8) After incubation, use a syringe without a needle to inject the bacterial suspension into the tobacco leaves. First, make a small hole in the leaf with a needle, then gently inject the bacterial suspension into the leaf through the hole. Multiple leaves can be inoculated. The inoculated parts of the leaves will express the green fluorescent protein (GFP) gene.

3. Results

(1) Plasmid Extraction and Concentration Determination Results

Plasmid DNA was successfully extracted with a concentration of approximately 59.8 ng/μL, as measured by UV spectrophotometry. The purity was high, meeting the requirements for subsequent experiments. The extraction process was strictly followed to ensure the integrity and purity of the DNA. The extracted plasmid DNA showed a clear band in 1% agarose gel electrophoresis, indicating good quality suitable for subsequent enzymatic digestion and ligation reactions.

(2) PCR Amplification Results

The PCR products were detected by agarose gel electrophoresis, showing specific bands with sizes consistent with expectations, indicating successful amplification of the GFP gene. The PCR amplification process optimized reaction conditions, including annealing temperature and time, to ensure the specificity and yield of the products. The optimal number of cycles was determined by comparing the amplification products from different cycle numbers to obtain sufficient product quantities (Figure 1).

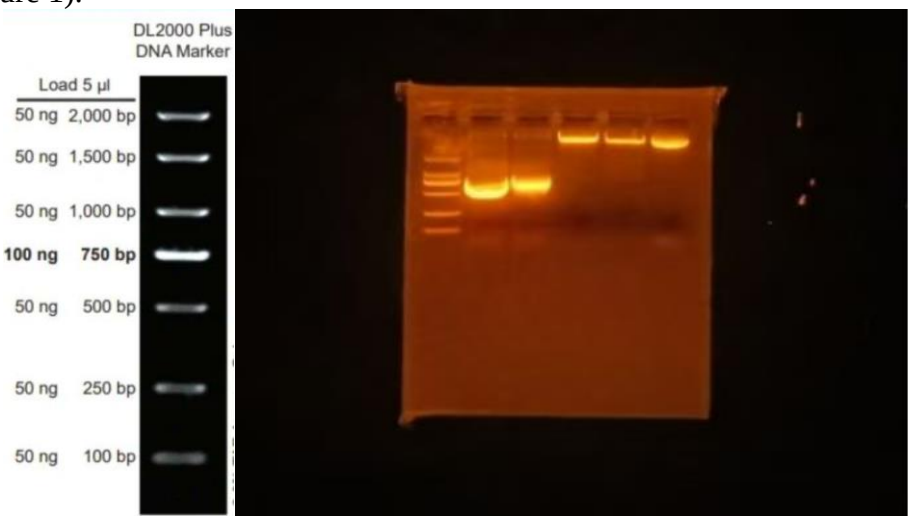

Figure 1: The image of gel electrophoresis result

(3) Vector Linearization Results

The vector was successfully linearized after digestion with Bahm and Pusti, as evidenced by the appearance of a band corresponding to the expected size of the linearized vector in agarose gel electrophoresis. The digestion reaction strictly followed the enzyme usage instructions to ensure complete and specific digestion. The recovery and purification of the linearized vector were also optimized to improve the recovery rate and purity. The PCR products, after electrophoresis detection, showed specific bands with sizes consistent with expectations, indicating the successful amplification of the GFP gene (Figure 2).

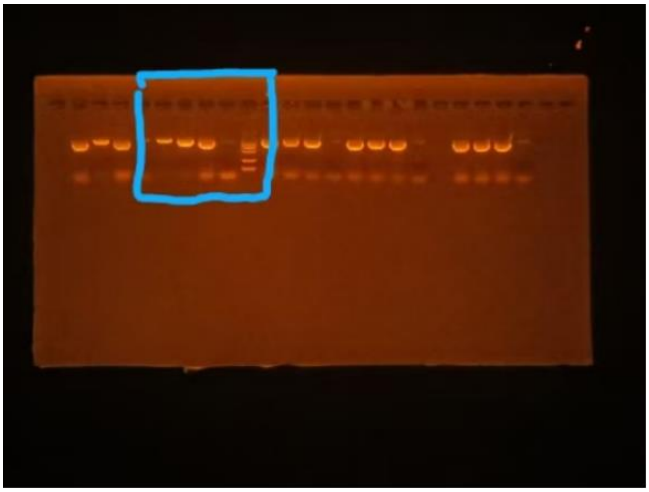

Figure 2: The image of colony PCR verification

(4) Recombination and Homologous Recombination Results

Figure 3: The image of enzyme digestion validation

Successful recombinant positive clones were obtained, as verified by colony PCR, indicating that the GFP gene had been successfully inserted into the vector. The homologous recombination process optimized reaction conditions, including temperature and time, to improve recombination efficiency. The screening and validation of recombinant clones were also optimized to ensure the accuracy and reliability of positive clones. The results of gel electrophoresis showed that in the fourth lane, the blank control group also had tiny bands. This might be due to the pipette tip not being changed, leading to contamination of the blank control group. The result of the Colony PCR Verification shows that, after enzyme digestion and gel electrophoresis, it was found that the length of the bands was the same as the length of the PCR product (around 750 bp). This indicates that the PCR in the plasmid is the one we intended to introduce, rather than a false positive. Finally, the plasmid was sent for DNA sequencing to confirm whether the base pairs of the gene were the ones we designed (Figure 3, 4).

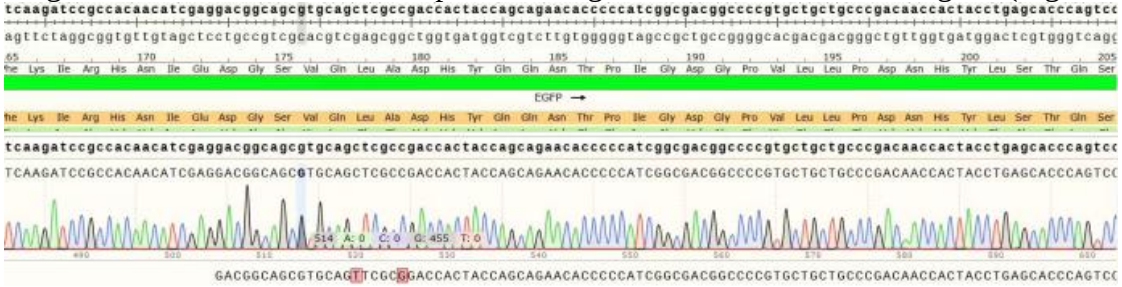

Figure 4: The image of gene sequencing result

(5) Agrobacterium Transformation Results

Agrobacterium successfully formed single colonies on LB solid medium containing three antibiotics, indicating successful transformation. The transformation process optimized conditions, including ice bath and heat shock times, to improve transformation efficiency. The concentration of the bacterial culture was confirmed by OD value measurement after shaking culture (Figure 5).

Figure 5: The image of infected tobacco

(6) Tobacco Inoculation Results

After injecting Agrobacterium suspension into tobacco leaves using a syringe, green fluorescence was observed in the inoculated leaf areas under a fluorescence microscope, indicating successful expression of the GFP gene in tobacco cells. The inoculation process optimized injection methods and bacterial suspension concentration to ensure uniform and efficient inoculation. Under confocal microscopy, GFP expression was observed in various parts of the leaves, indicating that the GFP gene was successfully expressed in tobacco cells and had a high level of expression (Figure 6).

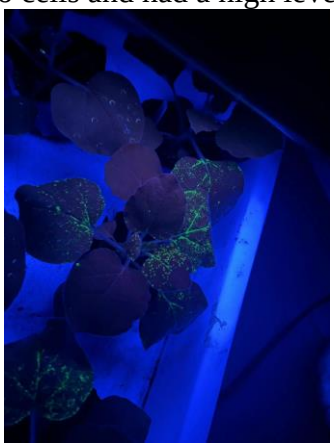

Figure 6: The image of tobacco observed under ultraviolet light.

4. Conclusion

This study successfully constructed a transient expression vector for the exogenous GFP gene and introduced it into tobacco leaves using Agrobacterium-mediated transformation, achieving transient expression of the GFP gene. This accomplishment not only confirms the effective expression of the GFP gene in plant cells but also provides a rapid and efficient tool for plant gene function research. The following sections will provide a detailed summary and outlook from the aspects of the main research achievements, optimization measures during the experimental process, and future research directions. The core achievement of this study is the successful construction of a transient expression vector for the GFP gene and its introduction into tobacco leaves using Agrobacterium-mediated transformation, thereby achieving transient expression of the GFP gene. The green fluorescence observed under the fluorescence microscope directly proves the successful expression of the GFP

gene in tobacco cells. This result not only confirms the feasibility of the transient expression system but also demonstrates its great potential in plant gene function research. As an important reporter gene, the successful expression of GFP in plant cells provides strong technical support for studying the expression patterns of plant genes, protein localization, and cellular dynamics.

During the experimental process, we optimized several key steps, significantly improving the efficiency and success rate of the experiment. First, in the plasmid extraction process, we strictly followed the operating procedures to ensure the integrity and purity of the DNA. By optimizing the use of lysis buffer and neutralization buffer, as well as adjusting the centrifugation conditions, we successfully extracted high-quality plasmid DNA, laying a solid foundation for the subsequent experiments. Second, in the PCR amplification process, we optimized the reaction conditions, including annealing temperature and time, to ensure the specificity and yield of the amplification products. By comparing the amplification products of different cycle numbers, we determined the optimal number of cycles to obtain a sufficient amount of product. Additionally, in the process of carrier linearization, we strictly followed the enzyme usage instructions to ensure the completeness and specificity of the enzyme digestion. By optimizing the recovery and purification process of the linearized carrier, we improved the recovery rate and purity, providing high-quality material for the subsequent recombination connection.

In the process of recombination connection and homologous recombination, we optimized the reaction conditions, including temperature and time, significantly improving the recombination efficiency. Through colony PCR verification, we successfully obtained recombinant positive clones, ensuring that the GFP gene had been successfully inserted into the carrier. In the Agrobacterium transformation process, we optimized the transformation conditions, including ice bath time and heat shock time, significantly improving the transformation efficiency. The transformed Agrobacterium formed single colonies on the LB solid culture medium containing three antibiotics, indicating successful transformation. Finally, in the tobacco infiltration process, we optimized the injection method and bacterial liquid concentration to ensure the uniformity and efficiency of the infiltration. The green fluorescence observed under the fluorescence microscope proved the successful expression of the GFP gene in tobacco cells.

Although this study has achieved significant results, there are still many aspects that can be further optimized and expanded. First, the culture conditions of Agrobacterium and the infiltration method can be further optimized to increase the expression level of GFP. By adjusting parameters such as the composition of the culture medium, cultivation temperature, and time, the transformation efficiency of Agrobacterium and the expression level of GFP can be further improved. Additionally, this technology can be extended to the functional research of other plant genes, providing a broader application prospect for plant molecular biology research. For example, this transient expression system can be applied to the study of plant disease resistance genes, drought tolerance genes, and other functional genes, providing technical support for plant genetic improvement. At the same time, combined with other molecular biology techniques, such as CRISPR/Cas9 gene editing technology, the functions of plant genes and their roles in growth, development, and environmental responses can be further explored. Through gene editing technology, target genes can be precisely knocked out or edited, and combined with the transient expression system, the functions and expression patterns of genes can be quickly verified. In addition, future research can also explore the subcellular localization of GFP in plant cells to gain a deeper understanding of its expression and function within plant cells. Through subcellular localization analysis, the specific sites of action of GFP in plant cells can be revealed, providing important clues for the study of signal transduction and metabolic pathways in plant cells.

This study can also serve as a reference for the transient expression of other reporter genes, promoting the development of plant gene function research. For example, this transient expression

system can be applied to the study of other fluorescent proteins and enzyme reporter genes, providing more options for the rapid verification of plant gene functions and protein expression research. In summary, this study not only successfully constructed a transient expression vector for the GFP gene but also significantly improved the efficiency and success rate of the experiment through the optimization of experimental steps, providing a rapid and efficient technical means for plant gene function research. In the future, by further optimizing and expanding this technology, a broader application prospect can be provided for plant molecular biology research.

References

[1] Zhang YJ, Li Y, Wang JJ, Pang HL, Jia LY, Feng HQ. Effects of different transformation conditions on transient expression of GFP gene in three Agrobacterium strains in Nicotiana benthamiana. Plant Research, 2022, 42(1): 121-129.

[2] Zhao WT, Wei JH, Liu XD, Gao ZH. Advances in the main methods and applications of plant transient expression systems. Biotechnology Communications, 2013, 24(2): 294-300.

[3] LIU Wei, LIU Hao, DONG ShuangYu, GU FengWei, CHEN ZhiQiang, WANG JiaFeng, WANG Hui. Construction of Rice Leaf Sheath Protoplast Transformation System and Transient Expression of Pik-H4 and AvrPik-H4 Proteins. Scientia Agricultura Sinica. 2017, 50(23): 4575-4584

[4] Wu RR, Wang YZ, Pan XL, et al. Multi-pathway applications of tobacco transient expression technology. Chinese Tobacco Science, 2024, 45(5): 114-120.

[5] Huo L, Ji XY, Wang YC. Optimization of transient expression conditions of tobacco transformation mediated by Agrobacterium. Molecular Plant Breeding, 2016, 14(1): 80-85.

[6] Liu HY, Feng DR, Liu B, He YM, Wang HB, Wang JF. Subcellular localization of MpASR in onion epidermal cells mediated by Agrobacterium. Journal of Tropical and Subtropical Botany, 2009, 17(3): 218-222.

[7] Zhai C, Zhang Y, Yao N, Lin F, Liu Z, Dong Z, Wang L, Pan Q. Function and interaction of the coupled genes responsible for rice blast resistance[J]. PLoS ONE, 2014, 9(6): e98067.

[8] Caplan J L, Kumar A S, Park E, Padmanabhan M, Hoban K, Modla S, Czymmek K, Dinesh-Kumar S. Chloroplast stromules function during innate immunity[J]. Plant Cell, 2015, 34(1): 45-57.