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

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Abstract: This study aims to construct an exogenous green fluorescent protein (GFP) gene expression vector and verify its expression in tobacco leaves. First, the GFP gene was inserted into a plant expression vector containing a strong promoter to drive the expression of GFP by molecular cloning technology. Subsequently, the constructed vector was transformed into Escherichia coli for amplification, and the correctness of the inserted gene was verified by restriction endonuclease analysis and sequencing. Next, the recombinant vector was transferred into tobacco cells and infected by Agrobacterium-mediated transformation. The GFP expression in tobacco leaves was observed by fluorescence microscopy. The results showed that the transgenic tobacco plants emitted strong green fluorescence under ultraviolet light, proving that the GFP gene was successfully expressed. This study provides effective tools and methods for plant genetic engineering and lays a foundation for further research on plant cell biology.

1. Introduction

Green fluorescent protein (GFP) was originally isolated from jellyfish and is a fluorescent marker widely used in cell biology and molecular biology research. Due to its high stability, strong fluorescence, and easy detection, GFP has become an important tool for protein localization and gene expression regulation. In recent years, with the development of plant genetic engineering, the application of GFP in plant cells has gradually increased, becoming an important means and auxiliary tool for studying biological processes in plant cells. In addition, green fluorescent protein (GFP) can emit green fluorescence under the excitation of blue-violet light, which is easy to track. It is often made into a fusion protein and is also widely used in cell biology and molecular biology research [1].

This study aims to construct an efficient GFP gene expression vector to achieve stable expression of GFP in tobacco, and to review it. Tobacco is an important model plant widely used in biological research [2]. By introducing the GFP gene into tobacco, gene expression and gene characteristics can be observed intuitively, and at the same time, it lays the foundation for subsequent research related to gene function.

The reason why Agrobacterium-mediated transformation was used in the experiment is that the

wild-type plasmid is too large to be inserted, contains genes that are involved in plant nodules, has no directly available restriction sites, is difficult to insert the target gene, and has no screening markers to determine whether the transfer is successful. Agrobacterium is a type of bacteria that lives on the surface of plant roots. It is rod-shaped, has a cell wall, and is 2-3μm long. It can infect the injured parts of plants under natural conditions and induce crown galls or hairy roots [3]. The principle of the mediated method is to use the Ti plasmid as a vector based on the infection characteristics of Agrobacterium, insert the target gene into the T-DNA region of the Ti plasmid, and then transform it into Agrobacterium tumefaciens. By injection, the Agrobacterium is brought into contact with the plant leaf cells, thereby introducing the target gene into the plant cells. The expression of the target gene can be detected within 1-2 days.

In addition, many scholars have conducted in-depth research on the cultivation and visualization of green fluorescent protein. For example, the article "Cultivation of Tomatoes with Visual Mitochondrial Morphology Based on Green Fluorescent Protein" published by the College of Food Science and Nutritional Engineering of China Agricultural University has expanded its content to the field of agricultural products. Through a series of studies such as plasmid extraction, Agrobacterium-mediated genetic transformation, gene determination, and trait determination, Mt-GFP was transferred into tomatoes for the first time, realizing the visualization of the mitochondrial morphology of tomato plants. It also laid the foundation for further research on gene-edited tomato hybridization and contributed new evidence theory to agricultural science and technology [4].

This article is based on the construction of the green fluorescent protein (GFP) gene expression vector based on the technology of DNA cutting, recombination and ligation, and introduces it into tobacco plants through Agrobacterium-mediated transformation to verify its expression in tobacco leaves. It is hoped that this will provide new ideas and methods for plant genetic engineering research.

2. Materials and Methods

2.1. Plasmid extraction

Materials:

Culture medium, centrifuge, colorless solution RB, blue solution, blue solution LB, pipette, yellow solution NB, EP tube, centrifugal column, water buffer.

Methods:

1) We take the culture medium and centrifuge it for 1 minute to remove the supernatant.

2) We add 250 microliters of colorless solution RB and oscillate to disperse the suspended bacterial sediment without leaving small bacterial lumps.

3) We add 250 microliters of blue solution LB and invert the tube 4-6 times. The alkaline solution ruptures the membrane, lysing the bacterial DNA and converting it from double-stranded to single-stranded.

4) We add 250 microliters of yellow NB solution (acidic) and mix 5-6 times. The solution changes from blue to yellow as it neutralizes the alkali, forming yellow agglomerates.

5) We separate the DNA by centrifuging for 5 minutes, carefully aspirate the supernatant, and transfer it to the centrifuge column.

6) We purify the sample by centrifuging the column for 1 minute, then discard the effluent.

7) We add 650 microliters of deionized WB (preheated in a 60-70 degree water bath), let it stand for 1 minute to recover DNA.

8) We centrifuge for 1 minute to elute the DNA.

9) We preheat the Elution Buffer, add 30 microliters to the centrifuge column, and let it stand for 1 minute to release the adsorbed DNA. We then centrifuge for 1 minute.

10) We aspirate the post-centrifugation precipitate, return it to the centrifuge column, and

centrifuge again for 1 minute to concentrate the DNA.

11) Finally, we store the DNA at -20 degree and measure its concentration.

2.2. PCR amplification

Materials:

Primers, Taq enzyme, dNTP, DNA template, reaction buffer, PCR instrument, pipette, 0.2ml thin-walled centrifuge tube.

Methods:

1) We add 25 microliters of reaction buffer to the centrifuge tube.

2) We add 1 microliter of dNTP.

3) We add 2 microliters of each primer.

4) We add 1 microliter of dna template.

5) We add 18 microliters of water.

6) After adding everything, we put it into the pcr instrument to amplify the dna fragment. This cycle is repeated more than 30 times at high temperature and takes more than an hour to complete.

2.3. Agarose gel preparation

Materials:

Agarose powder, 50 microliters of buffer 1xTAE, nucleic acid cold gel dye, and pipette.

Methods:

1) We mix 20 ml of 50xTAE with 980 ml of water.

2) We take 50 ml of 1xTAE after preparation.

3) We add 0.5 grams of agarose powder, heat it to mix it completely, and the solution becomes transparent.

4) We add a small amount of nucleic acid cold gel dye, shake well, and pour it into the mold to condense.

2.4. Agarose gel electrophoresis

Materials:

Prepared agarose gel, 50 micro PCR products x2, 50 micro enzyme digestion products x2, electrophoresis instrument, equal amount of uncut plasmid, 10 micro marker, 5 micro loading buffer x5, pipette.

Methods:

1) We add 5 micro loading buffer to each of the uncut plasmid, two PCR products and two enzyme digestion products.

2) Then we put the marker, two PCR products, two enzyme digestion products and uncut plasmid into the small grid of agarose gel respectively, turn on the power, and take out after electrophoresis for 20 minutes at a voltage of 180V, a current of 175mA and a power of 0.31W.

3) We observe with ultraviolet light instrument.

2.5. Vector linearization

Materials:

1 micro BamHI enzyme, 5 micro 10xcustsmart, 1 micro plasmid Vector, 1 micro PstI enzyme, 38 micro water, metal bath, pipette.

Methods:

1) We add 1 micro BamHI enzyme, 5 micro 10xcustsmart, 1 micro plasmid Vector, 1 micro PstI enzyme and 38 micro water to a 1.5ml EP tube and mix. Note that the enzyme should be added last.

2) We put it in a 37 degree metal bath and wait for one hour.

2.6. Purification and recovery of linearized vector

Materials:

Two tubes of enzyme digestion products, two tubes of PCR products, Buffer GDP, pipette, metal bath, centrifuge, Buffer GW, centrifugal column, 1.5ml EP tube, Elution buffer.

Methods:

1) We add 200 microliters of buffer gdp to each of the pcr and enzyme digestion products to obtain 4 tubes.

2) We put the sol into a 55-degree metal bath together, and then put it into a centrifuge for centrifugation.

3) We put the sol into the adsorption column, put the four together into the centrifuge for centrifugation for 1 minute, and pour out the waste liquid.

4) We add 300 microliters of sol buffer gdp, put it into the centrifuge and centrifuge for 1 minute, and pour out the waste liquid.

5) Then we add 650 microliters of buffer gw, centrifuge for 30 seconds, pour out the waste liquid, and repeat.

6) We centrifuge for 2 minutes, discard the tube, put the centrifuge column into a new 1.5 ep tube with the lid open and let it stand for 2 minutes.

7) We add 30 microliters of elusion buffer elution solution for elution, let it stand for 1 minute, and then centrifuge for 1 minute.

8) We aspirate the eluate and put it back into the ep tube and centrifuge for 1 minute.

2.7. Recombination Linkage

Materials:

200 μL PCR tube, 5xcell Buffer, linear recovery vector, PCR product, water, instant centrifuge, PCR instrument, Exnasell, pipette.

Methods:

1) We take a new 200 μL PCR tube, add 4 μL 5xcell Buffer, 11.7 μL linear recovery vector with a concentration of 136.002 ng/μL, 1 μL PCR product with a concentration of 17.155 ng/μL and 1.3 μL water, and finally add 2 μL Exnasell. 2.7.2.2 Take a new 200 μl PCR tube, add 4 μl 5xcell Buffer, 10 μl linear recovery vector with a concentration of 63.823 ng/μl, 1 μl PCR product with a concentration of 20.664 ng/μl and 3 μl water, and finally add 2 μl Exnasell.

2) We centrifuge at low speed using a flash centrifuge.

3) We place in a PCR instrument at 37 degrees and wait for 30 minutes.

2.8. Preparation of LB culture medium

Materials:

Yeast extraction, Tryptone, NaCl, Agar, water, magnetic stirrer, pH meter, sterilizing pot, and pipette.

Methods:

1) We add 1 gram of yeast extraction, 2 grams of tryptone, 2 grams of nacl, 3 grams of agar and 200ml of water.

2) When preparing liquid culture medium, we add 1 gram of yeast extraction, 2 grams of tryptone,

2 grams of nacl and 200ml of water.

3) After adding everything, we use a magnetic stirrer to vortex the solution to mix it evenly.

4) We use a ph meter to measure the ph value of the liquid culture medium, which is neutral.

5) We add deionized water to the autoclave until it is ready to run, put the two cans in it, and sterilize at 121 degrees for 20 minutes.

2.9. Chemical transformation

Materials:

E. coli competent cells, gene recombination connection product 2, metal bath, ice bath, non-resistant LB culture medium, shaker, centrifuge, and pipetting.

Methods:

1) We take out 50 microliters of e. Coli competent cells from the -83 degree freezer and add 5 microliters of connection product

2) We mix gently and place in an ice bath for 30 minutes. 2. heat shock in a 42℃ water bath for 45 seconds, then transfer the tube to an ice bath for 2 minutes. Do not shake the centrifuge tube during this process.

3) We add 700 microliters of non-resistant lb culture medium to the tube, mix well, and place in a shaker at 37℃ for 1 hour.

4) Finally, we collect the bacteria with a centrifuge and centrifuge at 4000rpm for 1 minute.

2.10. LB medium pouring and coating experiment

Materials:

Kana antibiotics, liquid LB culture medium, sterile chamber, alcohol lamp, applicator, pipette, culture dish, transformed competent cells, incubator, sealing strip.

Methods:

1) We pour 100 microliters of kana antibiotics into liquid lb culture medium and prepare a culture medium with a concentration of 50 micrograms per milliliter.

2) We pour the culture medium into the culture dish under a sterile environment and wait for solidification.

3) We burn the applicator in the sterile chamber, suck away the supernatant of the transformed competent cells, leaving 100 microliters and bacterial blocks, and then blow to dilute and mix.

4) We pour the mixed liquid into the culture dish.

5) We use the heated applicator to evenly spread the liquid on the lb culture medium.

6) We invert the plate, write a mark and seal it, and invert it in a 37-degree incubator for 12-16 hours.

2.11. Colony PCR identification

Materials:

Pipette, water, cultured colonies, centrifuge tubes, Taq enzyme mix, primer-F, primer-R, centrifuge, PCR instrument.

Methods:

1) We add 40 microliters of water to each of the 4 centrifuge tubes.

2) The fourth one is water for control.

3) We add 40 microliters of taq enzyme mix and 4 microliters of primer-f and primer-r to the new 4 centrifuge tubes.

4) We divide the prepared solution into 4 tubes, 12 microliters each.

5) We draw 8 microliters of bacterial solution from each of the previous 3 tubes, and 8 microliters of water from the fourth tube, and add them to the 4 12 microliter solution tubes respectively.

6) We centrifuge in an instant centrifuge.

7) We use a pcr instrument at 55 degrees p for one and a half hours.

8) We identify by agarose gel electrophoresis.

2.12. Shaking bacteria

Materials:

Pipette, centrifuge tube, kanamycin, LB liquid culture medium, shaker.

Methods:

1) First we put the instruments to be used into the sterile sterilization table for 15 minutes.

2) After disinfection, we enter the sterile table, add lb liquid culture medium to a 15ml centrifuge tube, and add 7.5 microliters of kanamycin at a concentration of 50 micrograms per milliliter.

3) Then we pour 3 ml of the prepared liquid into a new 15ml tube.

4) We add all the remaining 32 microliters of the correctly identified bacterial solution in a centrifuge tube.

5) We put it into a 37-degree shaker and shake for 12-16 hours.

2.13. Extraction of E. coli plasmid

Materials:

Centrifuge tubes, centrifuge columns, centrifuges, bacterial solution, RB solution, NB solution, LB solution, WB solution, Elusion Buffer, and pipettes.

Methods:

1) We use a pipette to suck 1ml of shaken bacterial solution and inject it into the centrifuge tube. Centrifuge at 1200 rpm for 1 minute, pour out the supernatant, and leave the bacterial mud.

2) We add 250 microliters of colorless solution rb and oscillate to disperse the suspended bacterial precipitate, leaving no small bacterial lumps.

3) We add 250 microliters of blue solution lb and invert 4-6 times. The solution itself generates alkalinity, which will rupture the membrane, thereby lysing the dna of the bacteria and changing the dna from double-stranded to single-stranded.

4) We add 35 microliters of yellow nb solution, which is acidic, and mix 5-6 times. The solution will completely change from blue to yellow, which means that it is completely mixed and neutralized with alkali to form a yellow agglomerate. Let it stand for one minute.

5) We centrifuge for 10 minutes, keep the supernatant and remove the precipitate, put the supernatant into the centrifuge column, put it into the centrifuge tube, centrifuge for 1 minute, and pour out the waste liquid.

6) We add 500 microliters of wb and centrifuge for 1 minute, and repeat this step once.

7) We leave it empty for 2 minutes, open the lid and let it stand to allow ethanol to evaporate.

8) We preheat elusion buffer and add 30 microliters to the centrifuge column, elute the dna adsorbed on the centrifuge column, let it stand for 1 minute, and then centrifuge for 1 minute.

9) We aspirate the precipitate after centrifugation and put it back into the centrifuge column for centrifugation for 1 minute to make its concentration higher.

10) We determine its concentration.

2.14. Enzyme digestion verification

Materials:

Pipette, plasmids from E. coli, cutsmart buffer, BamHl enzyme, Pstl enzyme, water, metal bath, centrifuge tube.

Methods:

1) We use a pipette to suck out 13.8 micro plasmids with a concentration of 72.5.
2) We add 5 micro cutsmart buffer.
3) We add 0.5 micro bamhl enzyme, 0.5 micro pstl enzyme and 30.2 micro water and mix.
4) We put in a 37 degree metal bath and digest for 2-3 hours.

2.15. Agrobacterium-mediated transformation and plating

Materials:

Agrobacterium competent cells, ice, liquid nitrogen, metal bath, pipette, restriction plasmid, 2300-35s-gfp plasmid, lb medium, shaker.

Methods:

1) We take out the agrobacterium competent cells from the -80 degree refrigerator and melt the competent cells in an ice bath.
2) We add 20 micro competent cells to the restriction plasmid yesterday.
3) We add 2 micro 2300-35s-gfp plasmid and ice bath for 5 minutes.
4) We quickly freeze in liquid nitrogen for 5 minutes, put in a metal bath at 37 degrees for 5 minutes.
5) We ice for another 5 minutes.
6) After that, we add 500 microliters of liquid lb culture medium and shake it in a 28-degree shaker for 2-3 hours.
7) We centrifuge at 5000 rpm for 1 minute.
8) On a sterile bench,we pour out 400 microliters of the supernatant and dilute the precipitate with the remaining 100 microliters of lb culture medium.
9) We use a pipette tip to suck it into the triple-antibiotic culture medium of kanamycin, rifampicin, and gentamicin, and spread it evenly with a spreader.

2.16. Agrobacterium infection

Materials:

Agrobacterium with the target plasmid, LB liquid culture medium, MES, ASG, magnesium chloride, centrifuge, centrifuge tube, spectrophotometer, incubator, and pipette.

Methods:

1) We activate the strain. Pick a single colony of agrobacterium containing the target plasmid and place it in 3ml lb liquid culture medium containing the corresponding antibiotics, and culture it at 28 degrees and 200rpm for 24h.
2) We culture for 10-12h.
3) We prepare the infection solution, add 1ml mes, 1ml magnesium chloride and 200μ asg and mix.
4) We place the activated bacterial solution in a 5000rpm centrifuge and centrifuge for 5min.
5) After centrifugation, we pour out the waste liquid, then pour in 10ml infection solution, and shake and mix.
6) We pipette 100μ of agrobacterium infection solution and 900μ of infection solution into a 2ml centrifuge tube for dilution.
7) We use a spectrophotometer to measure the od value, first measure only the infection solution, and then measure the absorbance value of the bacterial solution to be 1.69.
8) We add 7ml of infection solution to dilute and mix, and dilute the bacterial solution to a concentration of 1.

9) Finally, we place it in an incubator at 28 degrees for 1 hour.

2.17. Infect tobacco

Materials:

Needles, syringes, tobacco plants, and Agrobacterium bacterial solution.

Methods:

1) We use a small needle to gently poke the flat area on the back of the leaf.

2) We use a syringe to suck out the bacterial solution prepared in the previous step, press your finger on the back of the leaf hole, aim the syringe at the hole poked by the needle, and inject the bacterial solution.

3) The leaves are obviously wet, and can be observed with ultraviolet light after one or two days.

3. Results

3.1. DNA concentration detection

The DNA concentration of the first group is 585.402 ng/μl, and the concentration of the second group is 453.066 ng/μl (Figure 1).

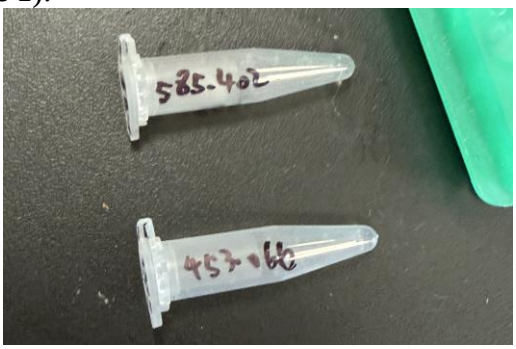

Figure 1: The concentration of DNA

3.2. Linearized vector concentration detection

The concentration of the first group of PCR is 136.002 ng/μl, the concentration of the second group of PCR is 63.823 ng/μl, the concentration of the first group of enzyme digestion is 17.155 ng/μl, and the concentration of the second group of PCR is 20.664 ng/μl. These phenomena show that the concentration of the first group of PCR is correct, while the concentration of the second group is too low. The reason is that the PCR product overflows the small grid during the gel run, resulting in a decrease in the quality of the final recovered product, thereby reducing the concentration. From this, we can conclude that accuracy is particularly important when doing experiments.

3.3 Electrophoresis of PCR products and enzyme digestion products

Figure 2 is an electrophoresis gel run of PCR products and enzyme digestion products. From left to right, they are marker, PCR product 1, PCR product 2, enzyme digestion product 1, enzyme digestion product 2, and uncut plasmid. And the PCR product runs faster. These phenomena indicate that the DNA fragment may be small, because the speed of running the gel determines the size of the fragment. The small DNA fragment may be caused by improper operation during production, using a pipette to blow away the required liquid and causing less liquid. From this, we can conclude that when operating the experiment, we must be careful to operate, otherwise a small mistake will affect

the experimental results.

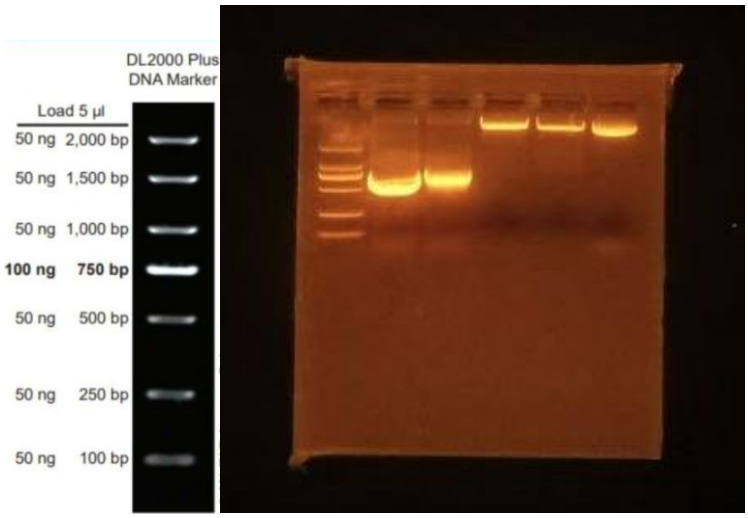

Figure 2: The image of gel electrophoresis result

3.4. Colony culture

The colonies grow more evenly on the culture medium (Figure 3). These phenomena indicate that the spreader is used to spread the bacteria more evenly, so the colonies grow well. When making, it is necessary to wait until the colonies are evenly spread and air-dried before turning the culture dish upside down, otherwise the colonies will grow together. The colonies grow more evenly on the culture medium. The spreader is used to spread the bacteria more evenly, so the colonies grow well.

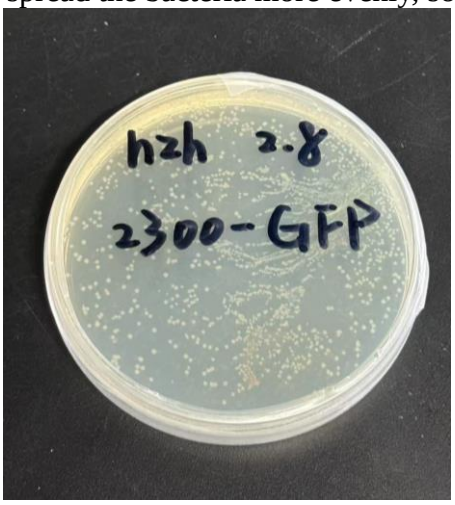

Figure 3: The image of colonies

3.5. Colony PCR Identification

The colony PCR was verified after running the gel, but the fourth group of water control group had light strips. These phenomena indicate that the colony culture is good and the PCR operation is correct, so they are all identified correctly. However, the water was identified with light strips, which may be caused by contamination of the gun tip when adding water or the water itself is not pure enough (Figure 4).

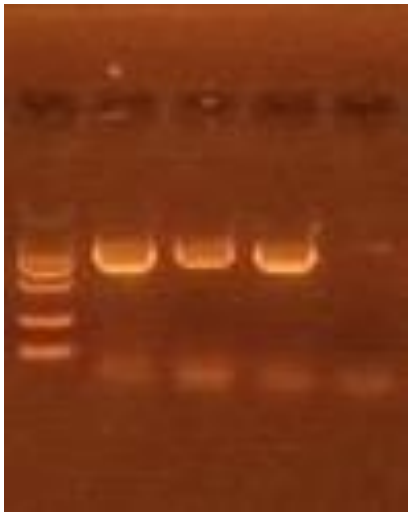

Figure 4: The image of colony PCR verification

3.6. Enzyme Digestion Verification and Sequencing

The target gene has been successfully transferred (Figure 5). The process is to take a restriction site at each end of the target fragment and measure the fragment between the two enzyme sites. As shown in the figure 5, each gel run has two bands in the figure, indicating that the transfer is successful. The gel run speed is not very fast, which means that the fragment is large. In addition, if multiple bands appear, it may indicate that the restriction site is repeated. The Figure 6, 7 is the sequencing result, which reflects that the bases of the fragment after enzyme digestion are all consistent with those of the template, indicating that the sequencing is correct (Figure 6, 7).

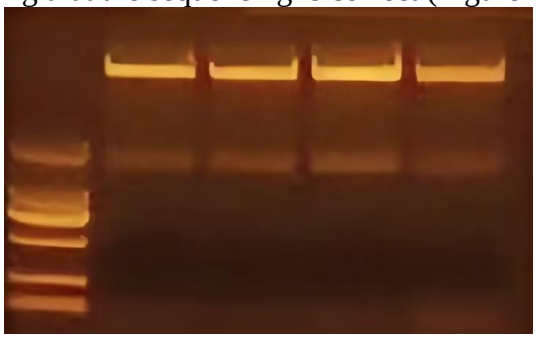

Figure 5: The image of enzyme digestion validation

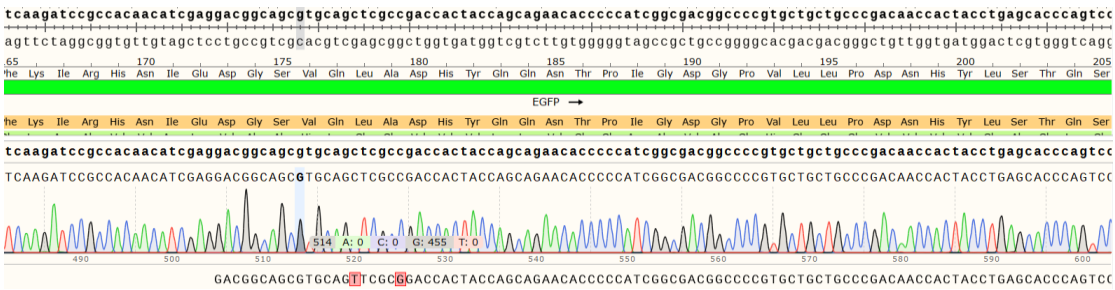

Figure 6: The DNA sequencing result of the recombinant plasmid

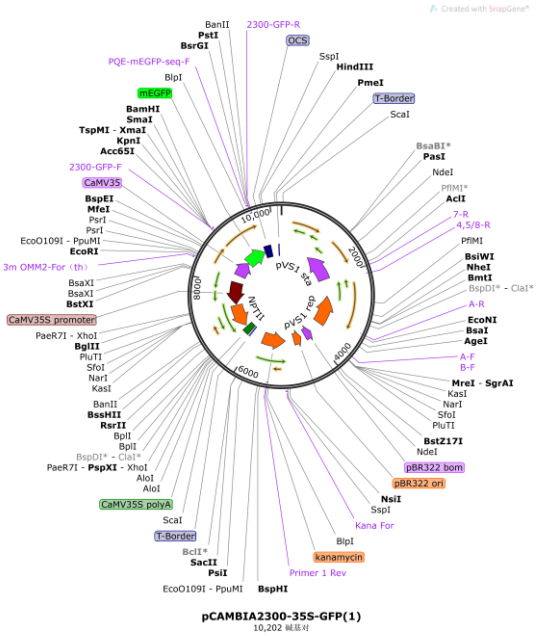

Figure 7: The recombinant plasmid

3.7. OD value of Agrobacterium colony

The OD value of the Agrobacterium colony is 0.169. Since the sample was diluted tenfold, the actual OD value (undiluted) is 1.69. Higher absorbance values correlate with higher bacterial concentrations.

3.8. Tobacco infection

Figure 8: The image of infected tobacco(left)and observed under ultraviolet light (right)

The tobacco leaves after local injection infection are obviously moist and darker in color, indicating that the Agrobacterium colony has been injected into the leaves (Figure 9, left). After one to two days, it can emit strong green fluorescence under ultraviolet light, , indicating successful expression of the GFP gene in tobacco cells (Figure 8, right).

4. Conclusion

This paper describes the construction of an exogenous green fluorescent protein (GFP) gene expression vector and the principle and process of its expression in tobacco leaves. The GFP gene was inserted into a plant expression vector through gene cloning technology, transformed and amplified, and the connection was successfully identified by agarose gel electrophoresis, sequencing, Agrobacterium-mediated transformation, and tobacco infection. The experiment was completed and explained using bioinformatics methods, tobacco transgenic technology, and real-time quantitative PCR for sequence, function, and tissue expression analysis [5]. Finally, the results of GFP-specific primer PCR identification and fluorescence observation showed that the GFP gene could be expressed normally [6]. The GFP expression in tobacco leaves was observed under a fluorescence microscope, which proved that the successful expression of the GFP gene was that the transgenic tobacco plants emitted strong green fluorescence under ultraviolet light.

The shortcomings of this study suggest the direction for future improvement: First, expand the scope of domestic literature research, read and compare carefully, and conduct more in-depth research. Second, accurately refine the research steps and be proficient and accurate in the experiment. For example, in the application process of plasmid DNA, its purity has a relatively large impact on the experimental results such as enzyme digestion and PCR amplification. Therefore, it is necessary to ensure the purity and efficiency of plasmid DNA extraction [7]. Therefore, in the future, more precise breakthroughs should be made in experiments to reduce errors and ensure the success rate of experiments. In addition, the current experiment takes a long time. If the Agrobacterium transformation technology is improved, the transformation efficiency may be further improved, so that more tobacco cells can successfully receive and express the GFP gene. Transformation technology plays an important role in plant gene function verification, shape improvement, and new variety breeding, thereby promoting the development of genetic engineering [8]. In short, research on the development of genetic engineering is still in the growth stage. There is a huge academic space for advancement.

Looking to the future, with the development of gene editing technology, the application prospects of GFP will be broader. We can further explore the expression characteristics of GFP in different plant species and study its different roles in plants when responding to different environments. At the same time, we will continue using GFP labeling to study the expression patterns of other plant genes, enabling visual observation and further research. In addition, future research can also consider optimizing transformation methods. For example, transgenic technology is one of the fastest-growing and most widely used high-tech technologies in the agricultural field [9]. Improving the transformation efficiency and stability of transgenic plants can further improve the expression level of GFP. In summary, this study provides new ideas for the application of GFP in plants. It is hoped that further research in the future will promote the development of plant biotechnology and provide more innovative possibilities for agriculture, medicine and other fields.

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