EXPERIMENTAL ARTICLES

UDC 602.6:577.213.3:577.112:582.926.2:633.15

https://doi.org/10.15407/biotech16.04.043

PECULIARITIES OF GREEN FLUORESCENT PROTEIN TRANSGENE DETECTION IN TOBACCO AND MAIZE PLANTS BY PCR

I. O. Nitovska[1]
D. Yu. Palekha[2]
B. V. Morgun[1]

[1]Institute of Cell Biology and Genetic Engineering of the sNational Academy of Sciences of Ukraine, Kyiv
[2]ESC "Institute of Biology and Medicine" Taras Shevchenko National University of Kyiv, Ukraine

E-mail: iranit@ukr.net

Received 2023/05/20
Revised 2023/08/14
Accepted 2023/08/31

The aim of the work was to investigate detection of different modifications of the green fluorescent protein gene (*gfp*) in the transgenic tobacco and maize plants by polymerase chain reaction (PCR).

Methods. Total DNA isolation, PCR, electrophoresis of DNA in agarose gel, bioinformatic resources.

Results. Three pairs of primers were used for PCR analysis of tobacco and maize containing wild-type *gfp* or mutant synthetic gene *S65Tpgfp*. The primer pair gfp1F-gfp1R interacted with the wild-type *gfp* gene only. The gfp2F-gfp2R primers interacted with the *gfp* gene of different modifications both in tobacco and maize. The gfp3F-gfp3R primer pair interacted with the modified *S65Tpgfp* gene in tobacco DNA, but not with maize samples.

Conclusions. Primers for detection of heterologous *gfp* gene, which were both narrowly specific (only one gene modification could be detected), and universal (more than one gene modification could be detected), were verified. It was shown that the primer pair gfp2F-gfp2R was universal for *gfp* gene detection both in tobacco and maize plants by PCR. The results obtained with gfp2F-gfp2R were reliably reproducible, so this primer pair was recommended for general use.

Key words: *gfp; S65Tpgfp*; *Zea mays* L., *Nicotiana tabacum* L.; PCR; transgene detection; molecular markers.

Cultivation of genetically modified varieties of important agricultural crops, which have useful traits of resistance to herbicides, pests, etc., leads to a significant reduction in the cost of final products in large scale [1], which is important in view of the constant growth of the human population and, accordingly, the need for food. Maize (*Zea mays* L.) occupies the third place among cereal crops in the world and the second place in European agricultural production [2, 3]. For efficient expression of genes and accumulation of their products in maize plants in the required amount, appropriate tissues and stage of plant development, components or genetic elements within the integrated DNA can come from various sources, such as: plants, bacteria, viruses [4]. To investigate the effect of transformation conditions and regulatory nucleotide sequences on gene expression, reporter genes are used, which facilitate visualization and quantitative measurement of transgenic protein. One such reporter gene that is often used in research on genetic transformation of plants is the green fluorescent protein gene (*gfp*) of jellyfish *Aequorea victoria* [5–7]. In addition to the wild-type gene, synthetic for plants (*pgfp*) and mutant synthetic genes that have a replacement of serine for threonine (*S65Tpgfp*) or cysteine (*S65Cpgfp*) in the 65th

position of the *pgfp* gene are also used in the studies [8]. Expression of synthetic *gfp* genes usually results in brighter fluorescence compared to the wild-type *gfp* gene.

The purpose of our study was to develop an optimal technique for detecting the *S65Tpgfp* transgene in the genome of maize plants obtained after *Agrobacterium*-mediated transformation with the pCB271 vector containing a mutant gene for green fluorescent protein [9]. The developed technique would allow us to investigate the influence of transformation conditions and regulatory nucleotide sequences on the expression of the *S65Tpgfp* reporter gene, in order to further use the acquired knowledge to construct efficiently working vectors that contain the desired gene of interest, as well as to develop transgenic maize plants with a certain trait.

Material and Methods

Plant material. Transgenic plants of maize hybrid F_1 KP7×PRZh5 of Ukrainian breeding were obtained after *Agrobacterium*-mediated transformation of cultivated immature embryos using the vector pCB271. Tobacco plants of cultivar Petit Havana were obtained after *Agrobacterium*-mediated transformation of leaf disks by the pCB271 or pICH5290 vectors [9]. Total DNA was isolated with CTAB and PVP-40 [10] from the plant leaves. The *gfp* sequence in pICH5290 was deposited within the synthetic construct GFP::LicBM3 (GFP::licBM3) (GenBank KX458181.2) while the sequence of *S65Tpgfp* in pCB271 was as in the cloning vector pNC-GFP (GenBank EU257522.1) [11-13].

PCR of plant DNA for the presence of the gfp gene. To exclude the contamination of the plant material by *A. tumefaciens*, an amplification of the bacterial *vir*-D1 gene was carried out prior to the PCR assay on the transgene [14]. To analyze the presence of the *gfp* gene in plant DNA by PCR, three pairs of primers were synthesized by Metabion (Germany) (Table).

The reaction was carried out as previously described [9]. The amplification program for detecting the *gfp* gene was set as follows: denaturation at 94 °C — 4 min, 34 cycles (denaturation at 94 °C — 30 s, annealing at 56 °C — 30 s, elongation at 72 °C for 45 s), completion of elongation at 72 °C — 4 min. For the gfp1F-gfp1R primer pair, the standard program was used without modification. When performing the analysis with primers gfp2F-gfp2R in the program, the annealing temperature was increased to 59 °C and the elongation time was reduced from 45 to 30 s. For the gfp3F-gfp3R primer pair, the temperature at the annealing stage was reduced to 55 °C, and the elongation stage time was reduced to 20 s. Electrophoresis of amplification products was performed in 1.0% or 2.0% agarose gel prepared in lithium borate (LB) buffer [15] with bromide ethidium (0.5 μg/ml) at 6 V/cm for 40 min.

An advanced software package CLC Main Workbench v. 6.9.2 (Qiagen) and the NIH genetic sequence database GenBank® were used for a comprehensive analysis and alignment of nucleotide sequences.

Results and Discussion

Analysis of plant DNA for the presence of the *gfp* gene by PCR using three pairs of primers revealed the following regularities. With the help of the gfp1F-gfp1R primer pair, it was possible to detect only the wild-type *gfp* gene. The amplicon of the expected size was detected in the DNA of transgenic tobacco, obtained with the help of the pICH5290 vector (Fig. 1). When the primer annealing temperature was increased to 59 degrees, the presence of an amplicon of weak intensity of the expected size was detected in the DNA of tobacco samples containing the mutant *S65Tpgfp* gene.

Table. **Features of primers used in the study**

Primer name	Nucleotide sequence	Melting temperature (Tm), °C	Amplicon size, bp	Reference
gfp1F,	5′-ATG GTG AGC AAG GGC GAG-3′	53.9	703	[7]
gfp1R	5′-CCA TGC CGT GAG TGA TCC-3′	51.3		
gfp2F,	5′-GAC GTG AAC GGC CAC AAG TTC A-3′	56.9	311	[9]
gfp2R	5′-CGA TGC GGT TCA CCA GGG TGT-3′	57.9		
gfp3F,	5′-ATG CCA CCT ACG GAA AGC TC-3′	54.3	263	this research
gfp3R	5′-GAT GCG GTT CAC CAG GGT AT-3′	52.8		

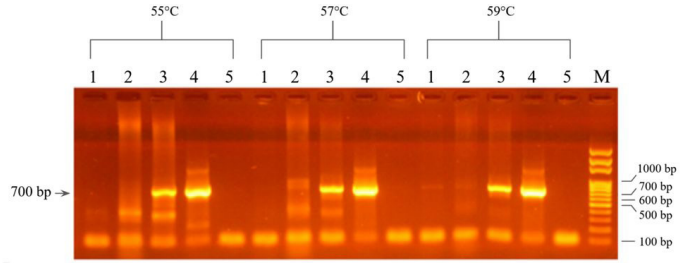

Fig. 1. **Electrophoregram of PCR products of tobacco plant DNA for *gfp* gene using gfp1F-gfp1R primer pair at different annealing temperatures in 1% agarose gel**

Lane 1–2, DNA samples of tobacco plants obtained as a result of *Agrobacterium*-mediated transformation with pCB271 vector, which contained the *S65Tpgfp* mutant gene of green fluorescent protein; 3–4, DNA samples of tobacco plants, obtained after *Agrobacterium*-mediated transformation by the pICH5290 vector, which contained the wild-type *gfp* gene; 5 — DNA no template control; M, the 1 kb DNA Ladder molecular weight marker, Solis BioDyne. The expected fragment size was 703 bp.

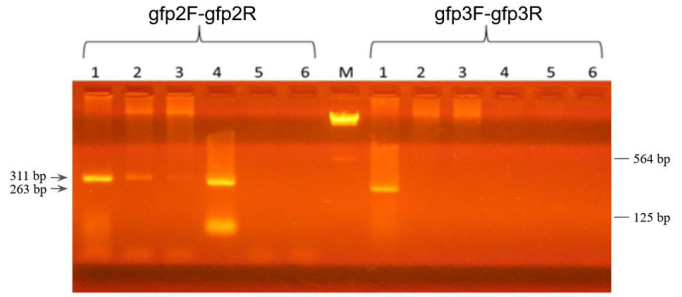

Fig. 2. **Electrophoregram of products of PCR analysis of plant DNA of tobacco and maize for the presence of *gfp* gene using primer pairs gfp2F-gfp2R or gfp3F-gfp3R in 2% agarose gel**

Lane 1, DNA sample of a tobacco plant transformed with the pCB271 vector, which contained the *S65Tpgfp* mutant gene of green fluorescent protein; 2–3, DNA samples of maize plants obtained as a result of *Agrobacterium*-mediated transformation with the pCB271 vector, which contained the *S65Tpgfp* gene; 4, DNA sample of a tobacco plant, obtained as a result of *Agrobacterium*-mediated transformation with the pICH5290 vector, which contained the wild-type *gfp* gene; 5, negative control (DNA sample of untransformed maize); 6, no DNA control; M, Lambda DNA/HindIII molecular weight marker.

A pair of primers gfp2F-gfp2R interacted with the *gfp* gene of various modifications. Amplicons of the expected size were visualized after the analysis of DNA samples of both tobacco and maize obtained with the pCB271 vector, as well as in the tobacco samples obtained after transformation with the vector pICH5290 (Fig. 2). Under ultraviolet light the amplicons from tobacco DNA samples were brighter compared to the amplicons obtained from the analysis of maize samples. This phenomenon may be due to either nonspecific priming with other sequences of maize DNA, or to a partial disruption of primer access to the gene's nucleotide sequence because, for example, its methylation [16]. Performing a touchdown PCR resulted in improved visualization of the expected size amplicon but it was rather negligible.

The gfp3F-gfp3R primer pair interacted only with the modified *S65Tpgfp* mutant gene found in tobacco DNA, but not with maize samples. This fact may indicate a strong affinity of this primer pair to other areas of the maize genome. An amplicon of the desired size was not observed either when analyzed with this primer pair of tobacco DNA containing the wild-type *gfp* gene. Since the expected size of the amplicon when using the gfp3F-gfp3R primer pair is small (Table) and may be too close to the common leading edge of the low molecular weight fraction in the gel. Agarose gels with different concentrations of agarose (1 and 2%) were used in the study to improve

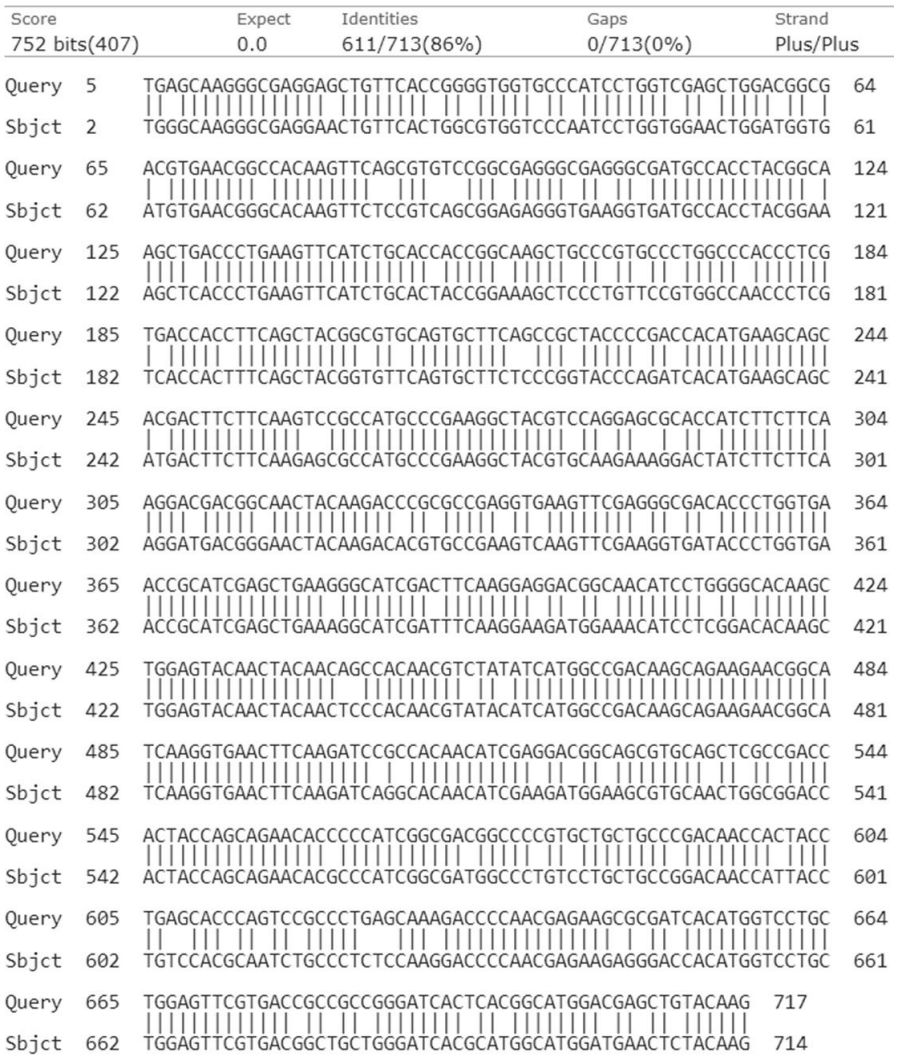

Fig. 3. **BLASTN pairwise alignment of the different versions of *gfp* nucleotide sequence**
The *gfp* sequence in pICH5290 (Query) is aligned against the sequence of *S65Tpgfp* in pCB271 (Sbjct).

the visualization of small-sized amplicons. Increasing the concentration of the gel to 2% enabeled us to visualize small amplicons much better.

The alignment let us clarify identity of the nucleotide sequences from different *gfp* versions. The identity between them comprised 86% (Fig. 3). It was clear why the primer pair gfp1F-gfp1R did not perfectly interacts with the synthetic mutant gene *S65Tpgfp*. The forward primer hybridized to 11 nucleotides of 18 only while the reverse one to 7 of 18. It explains appearance of faint signal at increased annealing temperature in tobacco samples containing the mutant gene. The primer pair gfp2F-gfp2R had perfect complementarity with 100% coverage to each of the gene versions. At the same time gfp3F-gfp3R hybridized to the gene *S65Tgfp* only but not to gene *gfp* wild-type. It was specific to the sites that differed between gene sequences. The bioinformatic data perfectly correlated with the obtained PCR results.

Therefore, each of the investigated pairs of primers interacted differently with the *gfp* gene, which was located in tobacco or maize DNA. Using a pair of primers gfp1F-gfp1R made it possible to detect only the wild-type *gfp* gene in the plant genome. Using the gfp3F-gfp3R primer pair, it was possible to identify only the mutant synthetic gene *S65Tpgfp* in tobacco DNA, but not in maize samples. The primer pair gfp2F-gfp2R proved to be universal, with which it was possible to detect both modifications of the *gfp* gene in both tobacco and maize samples. The acquired knowledge can be useful in screening large arrays of plant material for the *gfp* transgene

presence that is often employed in plant biotechnology as a reporter gene. Universal primers allow the researchers to detect quickly all gene modifications in plant material while the others pinpoint gene specify. It was found the primers show specificity in relation to the plant species. So, with the help of primers gfp3F-gfp3R, it was possible to detect the *S65Tpgfp* gene in the DNA of tobacco but not maize. The acquired knowledge will help researchers to avoid false conclusions regarding the absence of a transgene in plant material, when in fact it may be present there.

Author Contributions: Conceptualization, I.O.N.; methodology, I.O.N. and V. M.B.; validation, I.O.N.; formal analysis, I.O.N., D.Yu.P., and B.V.M.; investigation, I.O.N., D.Yu.P., and B.V.M.; resources, I.O.N., and B.V.M.; writing original draft preparation, I.O.N. and Yu.P. D.; writing review and editing, B.V.M.; visualization, Yu.P.D., and B.V.M.; supervision, I.O.N.; project administration, I.O.N. and B.V.M.; funding acquisition, B.V.M. All authors have read and agreed to the published version of the manuscript.

Acknowledgment

This work was financially supported by the projects "Study of Vital Activity Peculiarities of Biotechnological Plants after Genomic Modifications" (State registration number 0123U100462) and "Molecular Marker Systems for Selection of Favorable Genotypes of Cultivated Plants" (State registration number 0122U001512) provided by the National Academy of Sciences of Ukraine. The authors express their sincere gratitude to Prof. Tatiana Satarova from the Institute of Grain Crops of National Academy of Agrarian Sciences of Ukraine for the shared plant material and helpful guidelines on plant cultivation.

REFERENCES

1. ISAAA. Global status of commercialized biotech/GM crops in 2019. *Ithaca, NY: The International Service for the Acquisition of Agri-biotech Applications*. 2020. [online], Available at: https://www.isaaa.org/resources/publications/briefs/55/default.asp
2. USDA. Grain: world markets and trade. September 2023 [Internet]. Available at: https://apps.fas.usda.gov/psdonline/circulars/grain.pdf.
3. Eurostat statistics explained. Agricultural production — crops. 2022, https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Agricultural_production_-_crops
4. *Lu A., Diehn S., Cigan M.* (2015). Maize Protein Expression. In: Azhakanandam, K., Silverstone, A., Daniell, H., Davey, M. (eds) Recent Advancements in Gene Expression and Enabling Technologies in Crop Plants. Springer, New York, NY. https://doi.org/10.1007/978-1-4939-2202-4_1
5. *Chalfie M., Tu Y., Euskirchen G., Ward W. W., Prasher D.C.* Green fluorescent protein as a marker for gene expression. *Science*. 1994, 263, 802–805. https://doi.org/10.1126/science.8303295
6. *Varchenko O. I., Kuchuk M. V., Parii M. F., Symonenko Yu. V.* Comparison of *gfp* gene expression levels after *Agrobacterium*-mediated transient transformation of *Nicotiana rustica* L. by constructs with different promoter sequences. *Cytol Genet*. 2020, 54, (6), 531–538. https://doi.org/10.3103/S0095452720060110
7. *Sindarovska Y., Kuchuk M.* Long-term potato virus X (PVX)-based transient expression of recombinant GFP protein in *Nicotiana benthamiana* culture *in vitro*. *Plants*. 2021, 10, 2187–2207. https://doi.org/10.3390/plants10102187
8. *Pang S.-Z., DeBoer D. L., Wan Y., Ye G., Layton J. G., Neher M. K., Armstrong C. L., Fry J. E., Hinchee M. A., Fromm M. E.* An improved green fluorescent protein gene as a vital marker in plants. *Plant Physiol.* 1996, 112(3), 893–900. https://doi.org/10.1104/pp.112.3.893
9. *Nitovska I. O., Vasylenko M. Yu., Morgun B. V.* Effect of monocot introns on transgene expression in genus *Nicotiana* plants. *Biotechnol. Acta*. 2018, 11(4), 69–79. https://doi.org/10.15407/biotech11.04.073
10. *Stewart N. C. Jr., Via L. E.* A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR application. *BioTechnique*. 1993, 14(5), 748–749.
11. QIAGEN CLC Main Workbench 6.9.2, Aarhus, Denmark.
12. *Benson D. A., Cavanaugh M., Clark K., Karsch-Mizrachi I., Lipman D. J., Ostell J., Sayers E. W.* GenBank. *Nucleic Acids Res*. 2013, 41(Database issue), D36-42. https://doi.org/10.1093%2Fnar%2Fgks1195
13. *Lambert T. J.* FPbase: a community-editable fluorescent protein database. *Nature Methods*. 2019, 16, 277–278. https://www.fpbase.org/protein/egfp/
14. *Lipp Joao K. H., Brown T. A.* Enhanced transformation of tomato co-cultivated with *Agrobacterium tumefaciens* C58C1Rif(r)::pGSFR1161 in the presence of acetosyringone. *Plant Cell Rep*. 1993, 12, 422–425. https://doi.org/10.1007/BF00234705
15. *Brody J. R., Kern S. E.* History and principles of conductive media for standard DNA electrophoresis. *Anal. Biochem*. 2004, 333, 1–13. https://doi.org/10.1016/j.ab.2004.05.054
16. *Kalle E., Kubista M., Rensing Ch.* Multi-template polymerase chain reaction. *Biomol. Detect. Quantif*. 2014, 2, 11–29. https://doi.org/10.1016/j.bdq.2014.11.002

ОСОБЛИВОСТІ ВИЯВЛЕННЯ ТРАНСГЕНА ЗЕЛЕНОГО ФЛУОРЕСЦЕНТНОГО ПРОТЕЇНУ МЕТОДОМ ПЛР У РОСЛИНАХ ТЮТЮНУ ТА КУКУРУДЗИ

І. О. Нітовська[1], *Д. Ю. Палеха* [2], *Б. В. Моргун* [1]

[1]Інститут клітинної біології та генетичної інженерії НАН України, Київ
[2]ННЦ «Інститут біології та медицини», КНУ ім. Тараса Шевченка, Україна

E-mail: iranit@ukr.net

Метою роботи було виявити ген зеленого флуоресцентного протеїну (*gfp*) різних модифікацій у трансгенних рослинах тютюну та кукурудзи методом полімеразної ланцюгової реакції (ПЛР).

Методи. Виділення сумарної ДНК, ПЛР, електрофорез ДНК в агарозному гелі, біоінформатичні методи.

Результати. При аналізі методом ПЛР ДНК тютюну і кукурудзи на присутність гена *gfp* дикого типу або мутантного *S65Tpgfp* використовували три пари праймерів. Пара праймерів gfp1F-gfp1R взаємодіяла лише з геном *gfp* дикого типу. За допомогою праймерів gfp2F-gfp2R можна було виявляти ген *gfp* різних модифікацій як у геномі тютюну, так і кукурудзи. Пара праймерів gfp3F-gfp3R взаємодіяла з модифікованим геном *S65Tpgfp*, що знаходився в ДНК тютюну, але не кукурудзи.

Висновки. Верифіковано праймери для детекції гетерологічного гена *gfp*, які є як вузько специфічними (можна виявити лише одну модифікацію гена), так і універсальними (можна виявити більш однієї модифікації гена). Показано, що пара праймерів gfp2F-gfp2R є універсальною для виявлення гена *gfp* методом ПЛР як у рослин тютюну, так і кукурудзи. Результати, отримані за допомогою gfp2F-gfp2R надійно відтворюються, тому ця пара праймерів рекомендується для загального застосування.

Ключові слова: *gfp*; *S65Tpgfp*; *Zea mays* L.; *Nicotiana tabacum* L.; ПЛР; виявлення трансгена; молекулярні маркери.