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MATCHING OF THE GFP GENE EXPRESSION LEVELS BY DIFFERENT TERMINATOR SEQUENCES REGULATION

Oksana Varchenko[1,2], Mykola Kuchuk[1], Myroslav Parii[2,3], Yuriy Symonenko[1,2]

[1]Institute of Cell Biology and Genetic Engineering, NAS of Ukraine,
148 Acad. Zabolotny Str., Kyiv, 03143, Ukraine
[2]Ltd "Ukrainian Scientific Institute of Plant Breeding" (VNIS),
30 Vasylkivska Str., Kyiv, 03022, Ukraine
[3]National University of Life and Environmental Sciences of Ukraine,
15 Heroyiv Oborony Str., Kyiv, 03041, Ukraine
e-mail: okvarchenko@gmail.com

The ability to express foreign genes in plant cells provides a powerful tool for studying the function of specific genes. In addition, the creation of genetically modified plants may provide new important features that are useful for industrial production or pharmaceutical applications. One of the key parameters for the development of a high level of heterologous genes expression is the efficiency of terminators used in genetic engineering, since the level of gene expression depends on its choice. ***Aim.*** *Study of the gfp gene expression regulation in Nicotiana rustica L. (N. rustica L.) tissues by different terminators.* ***Methods****. The Golden Gate method of molecular cloning was used for genetic constructs creation. The tissues of N. rustica L. plants were infiltrated by the created genetic vectors for transient gene expression. The expression level was determined by spectrofluorometric (level of green fluorescent protein (GFP) fluorescence) and protein analysis: determination of water-soluble proteins concentration and its electrophoresis separation in polyacrylamide gel (PAGE).* ***Results****. Five different terminators with polyadenylation signal/3'-untranslated region (3'UTR) were selected for the study: the 7th gene isolated from Agrobacterium tumefaciens L. (A. tumefaciens L.) (Atug7), the terminator of the gene that encode mannopinsyntase from A. tumefaciens (mas), the terminator of tomato (Solanum lycopersum L.) adenosine 5'-triphosphatase (ATPase), the potato histone H4 terminator (Solanum tuberosum L.) and the 35S Cauliflower Mosaic Virus (35S CaMV) terminator. All transcriptional units additionally contained a 5'-untranslated region out of the 2B gene from the family of genes encoding the small subunit of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (5'UTR RbcS2B), the coding sequence of the gfp gene and double 35S Cauliflower Mosaic Virus promoter (D35S CaMV). Thus, we created 5 genetic constructs with different terminator sequences. The presence of recombinant GFP protein in total protein extracts and its identity to standard protein was proved by the spectrofluorometric and PAGE analyzes. For the first time was shown the difference of GFP reporter protein accumulation in N. rustica L. tissues by terminator regulation of transient gfp gene expression.* ***Conclusions****. We detected the highest expression of the gfp gene when the Atug7 terminator was used and the lowest level with the histone H4 terminator. The difference between protein accumulations using these terminators was in 2.89 times. It showed that the terminator sequence has a high influence on the gene expression. It choice is an important step in genetic constructs creation, since terminator can be used for regulating the level of gene expression depending on the goals.*

Keywords: Nicotiana rustica L., molecular cloning, genetic constructs, terminators, green fluorescent protein, transient expression, spectrofluorometric analysis, protein analysis.

Plant systems are often used to accumulate various pharmaceutically valuable recombinant proteins. Plants are versatile factories which have several advantages over microbial and mammalian synthesis. The use of plants reduces the likelihood of contamination with animal pathogens, abates the cost of the final product and it has a high scalability [1].

Agrobacterium-mediated transient heterologous genes expression is an alternative for creating stable transformants because it significantly reduces production time.

The output of recombinant proteins is a critical factor in the practical application of product storage systems, so one of the strategies is to use such regulatory elements which increase the level of

gene expression in plants [3].

Control of gene expression both endogenous and heterologous is a key component of genetic engineering. Although a large number of papers, characterizing promoters to achieve it, have been published, much less effort has been made to clarify the role of terminators [3].

Terminators are important components of transcriptional units in genetic cassettes and can affect to the net protein yield by controlling the half-life of messenger ribonucleic acid (mRNA). Eukaryotic terminators bind and recruit enzymes responsible for transcription termination, mRNA cleavage, and polyadenylation. In addition, the terminator is a genetically encoded element that determines the sequence and structure of the 3'UTR, and thus promotes mRNA stability [4, 5]. Terminators also serve as a reference point for gene expression in eukaryotes due to the stability of the 3'end of the mRNA. Unlike promoters, the cataloging of terminators has not been so large, until recently [6].

Genetic constructs which are used in plant genetic engineering include terminators which have different sources: bacterial, viral, plant [7].

Terminators, which are frequently used in studies, have not been the most effective, such as the bacteriophage T7 terminator demonstrates low termination efficiency [8–10]. Moreover, the collection of terminators which is available for researchers has traditionally been much smaller [11].

Frequently used terminators need to be diversified according to the usage specifications, by opening new and changing already known by genetic engineering methods [12].

Thus, the search and attraction of new terminators for genetic engineering is very important. Therefore, the aim of our work was to create genetic constructs with different terminator sequences and to compare their work on the example of transient expression of the GFP reporter protein in *N. rustica* L. plants.

Materials and methods. All genetic vectors were created using the Golden Gate Modular Molecular Cloning (MoClo) method [13, 14]. The MoClo system uses standardized genetic elements (modules in L0 level vectors) to assemble them into transcriptional units (L1 level vectors). Multiple transcription units of the L1 level vectors can then be assembled into the L2 vectors. The L0 base vectors and vector modules were taken from the MoClo Plant Parts Kit kindly provided by Nicola Patron (Addgene kit # 1000000047), and the MoClo Toolkit kindly provided by Sylvestre Marillonnet (Addgene kit # 1000000044) [14–16].

The study used the coding sequence of the *gfp* reporter gene, which was isolated from *A. victoria*, and encodes a green fluorescent protein (Green Fluorescent Protein, GFP) [17, 18]; double 35S Cauliflower Mosaic Virus (D35S CaMV promoter) [19], a 5'-untranslated region of the 2B gene from the family of genes that encode the small subunit of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (5'UTR RbcS2B) isolated from *Arabidopsis thaliana* L. (*A. thaliana* L.) and five terminators, together with a polyadenylation signal and a 3'-untranslated region: a terminator of 7th gene isolated from *Agrobacterium tumefaciens* L. (*A. tumefaciens* L.) (Atug7) [20] , the terminator of the mannopinsyntase gene from *A. tumefaciens* L. (mas) [21], the terminator of tomato (*Solanum lycopersicum* L., *S. lycopersicum* L.) adenosine 5'-triphosphatase (ATPase), H4 histone terminator of potatoes (*Solanum tuberosum* L., *S. tuberosum* L.) and 35S terminator of Cauliflower Mosaic Virus (Cauliflower Mosaic Virus) (35S CaMV terminator) [19].

Cloning was performed in the final vector of the second level pAGM4673 (Table 1), which is resistant to kanamycin and has color CRed selection. Transformed bacterial colonies of *Escherichia coli* (*E.coli*) XL-blue strain [22] were checked by *polymerase chain reaction* (PCR) and made restriction analysis of isolated plasmids [23]. The cloning mixture was incubated for 3 hours at 37°C with subsequent restriction inactivation 5 minutes(') at 50 °C, ligases 5' at 80° C [14].

Restriction and amplification products were separated in agarose gel. Computer simulation *in silico* was performed in SerialCloner program. For restriction analysis Bgl II FastDigest™ restriction enzyme was used. PCR analysis was performed to detect the *gfp* gene in genetic vectors (amplification product size 717 bp); the following primers were used for this purpose: forward: GTG AGC AAG GGC GAG GA; reverse: TTA CTT GTA CAG CTC GTC [24].

After plasmids verification competent *A. tumefaciens* L. cells, strain GV3101, were transformed by the freeze/thaw method [25]. Next, PCR analysis with primers for the *gfp* gene was performed on selected colonies using antibiotics in the medium (rifampicin 50 mg/l, gentamycin 25 mg/l, kanamycin 100 mg/l).

Table 1

Reagents of the cloning reaction mixture

Buffer Ligase 10x (ThermoFisher Scientific™)	1.5 µl
Bsa I (Bpi I) (restriction enzyme) 10 u/µl (ThermoFisher Scientific™)	0.2 µl
T4 DNA ligase 10 u/µl (ThermoFisher Scientific™)	0.5 µl
Bovine Serum Albumin (BSA) 10 mg/ml (ThermoFisher Scientific™)	0.15 µl
All module elements 100 ng/µl each	1 µl each
MQ water	7.65 µl
Total volume	15 µl

The overnight bacterial culture with created genetic constructs were grown in LB medium [26] supplemented with 50 mg/l rifampicin, 25 mg/l gentamicin and 100 mg/l kanamycin.

N. rustica L. plants were grown under greenhouse conditions at 25/18 °C and 16/8 hour photoperiod (day/night, respectively). Leaves of the middle tier of 4-week plants were used for infiltration. The seeds were kindly provided by Ph.D. Belokurova V.B. from National collection of extracts and germplasm bank of world flora (ICBGE NAS of Ukraine).

To study the level of *gfp* gene expression regulated by different terminator sequences *N. rustica* L. plants were transformed transiently. For this purpose, agrobacteria overnight culture was grown to an optical density 1 (OD_{600}=1) of suspension and resuspended in infiltration buffer (10 mM MgSO4, pH 5.6–5.8) with a final optical density OD_{600}=1. Plant infiltration was performed using a syringe [27]. Plant tissues infiltrated with GV3101 *A. tumefaciens* L. strain without vector which includes *gfp* gene and tissues were used as a negative control.

The expression of the *gfp* gene was detected on the 7th day after infiltration by visual and spectrofluorometric analysis on a fluorescence spectrofluorometer "Fluorate-02-Panorama" (excitation at a wavelength of 395 nm, emission at 509 nm).

Plant infiltrated tissues of *N. rustica* L. were triturated with extraction buffer (80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl, pH 7–7,5) in a pre-chilled mortar (+ 4° C) [25] at a dilution 3:1 (300 µl of buffer per 100 mg of tissue), after which the suspension was precipitated (+4° C, 14000 rpm, 30 minutes). The supernatant was used to determine the quantitative concentration of water-soluble proteins [28].

To identify the GFP reporter protein, we further performed polyacrylamide gel electrophoresis to separate water-soluble proteins in the presence of sodium dodecyl sulfate under denaturing (with mercaptoethanol) and non-denaturing separation conditions. Protein extracts were mixed with sample buffer and applied to the wells with and without boiling denaturation. The presence of the GFP reporter protein was observed immediately after electrophoresis by illuminating the gel with ultraviolet light using a hand lamp. Other proteins were visualized by staining the gel with a Coomassie Brilliant Blue reagent [29].

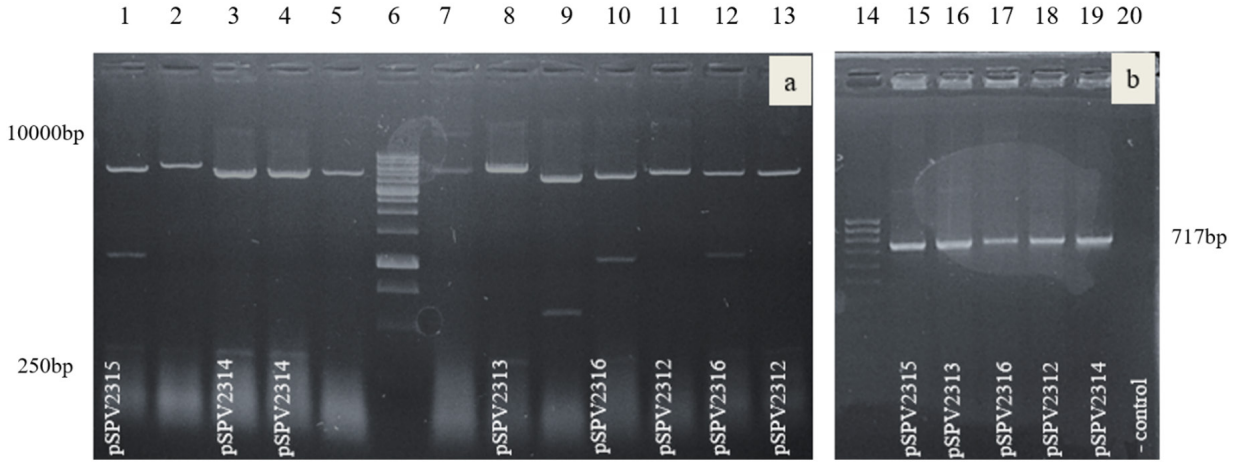
All statistical analyzes were performed in Microsoft Office Excel, determining the mean and standard deviation for each experiment. For the statistical processing of the spectrofluorometric analysis, the average value was determined through the logarithmic transformation of the spectrofluorometer parameters. The limits of marginal random deviations of the obtained results (L*east Significant Difference, LSD)* calculated as the product of the Student's *t*-distribution and the standard deviation. Processing of proteins separation in polyacrylamide gel data were performed in the GelAnalyzer 19.1 program.

Results. The MoClo method that we used for genetic constructs creating based on the use of type II restriction enzyme and T4 DNA ligase [12, 14].

Consequently five genetic constructs (pSPV2312, pSPV2313, pSPV2314, pSPV2315, and pSPV2316) were created (Fig. 1) and the only difference was in the terminator sequences/ polyadenylation signals and the 3'UTR.

PCR and restriction analysis were used to check constructs (Figs. 2a, 2b). For this purpose, restriction enzyme Bgl II (ThermoFisher ™) was used, with the following restriction fragment lengths: pSPV2312 – 5432, 327; pSPV2313 – 6312, 327; pSPV2314 – 5848, 327; pSPV2315 – 5023, 1053, 327; pSPV2316 – 4783, 1031, 327 bp. The *gfp* gene was detected using specific primers, the size of the amplicon 717 bp.

F i g. 1. Schematic representation of T-DNA region of genetic constructs: pSPV2312 – 35S CaMV terminator, pSPV2313 – Atug7 terminator pSPV2314 – mas terminator, pSPV2315 – ATPase terminator, pSPV2316 – Histone H4 terminator



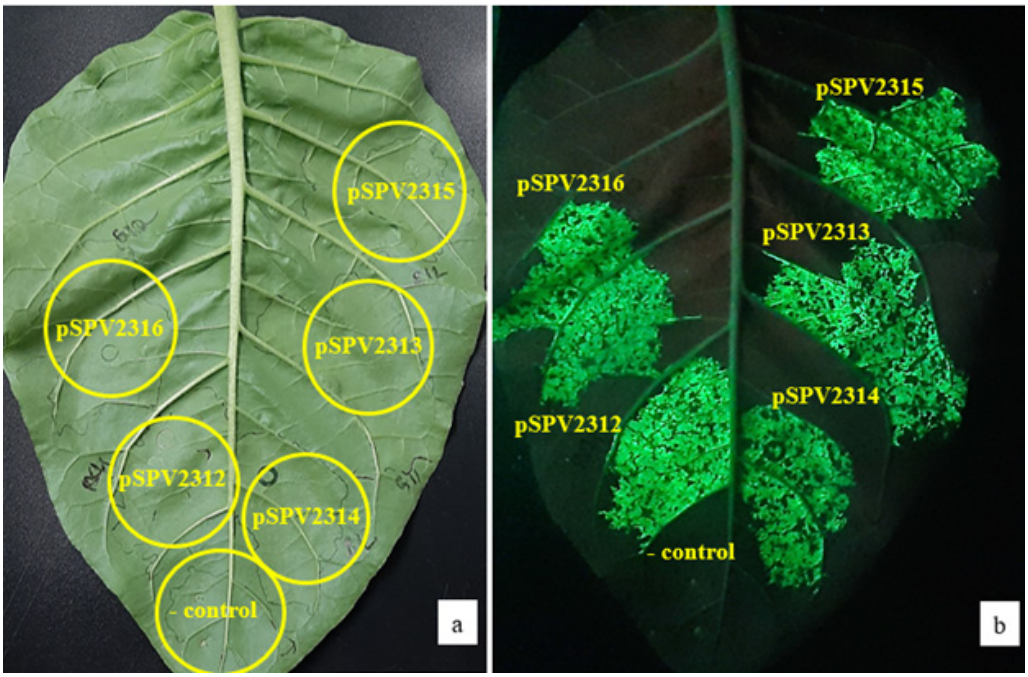
F i g. 2. Molecular biology analysis of created constructs: a) agarose gel electrophoregram of restriction products: 1 – pSPV2315; 2, 8 – pSPV2313; 3, 4 – pSPV2314; 6 – DNA Gene Ruler 250-10000bp; 10, 12 – pSPV2316; 11, 13 – pSPV2312; b) agarose gel electrophoregram of *gfp* PCR products: 14 – DNA Gene Ruller 100–1000bp; 15 – pSPV2315; 16 – pSPV2313; 17 – pSPV2316; 18 – pSPV2312; 19 – pSPV2314; 20 – negative control

Visual detection of tissues actualized on the 7th day after infiltration. Tissues of *N. rustica* L. leaves had not difference in infiltrated and non-infiltrated regions (fig. 3a) at daylight. Infiltrated regions (Fig. 3b) with vectors that included *gfp* gene had GFP fluorescence at ultraviolet light. As a negative control (-control) was used *A. tumefaciens* strain GV3101 without vector with *gfp* gene. Spectrofluorometric analysis showed (Fig. 4) that the highest fluorescence rate, 1.79 relative units, was observed in *N. rustica* L. tissues infiltrated by pSPV2313 (Table 2) which includes Atug7 terminator. The lowest fluorescence rate, 0.63 relative units, detected when the pSPV2314 construct with H4 histone terminator was used.

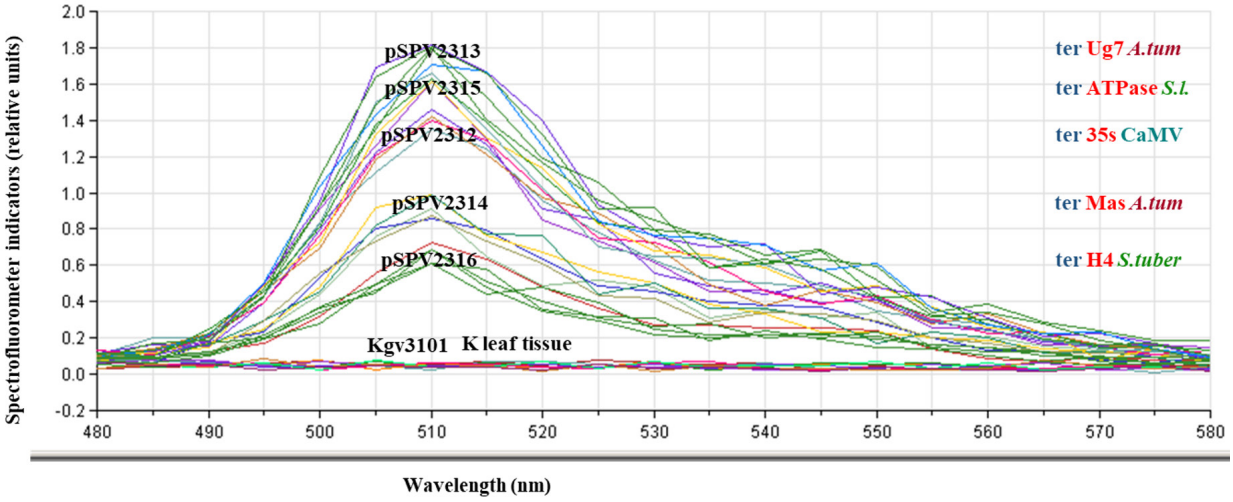
The level of GFP fluorescence in *N. rustica* L. tissues by spectrofluorometric analysis using 35S CaMV terminator was 1.40±0.03 relative units, what in 1.28 times less than when Atug7 was used. When pSPV2315 construct was used for transient transformation, which includes ATPase terminator, GFP fluorescence level was in 1.15 times higher than with 35S CaMV and in 1.12 times less than with Atug7. Tissues infiltrated with genetic vector that included mas terminator had 0.92±0.04 relative units of fluorescence, that in 1.92 times less than after using Atug 7. All means were statistically different (Table 2).

Level of GFP fluorescence by spectrofluorometric analysis coincides with the increasing of total water-soluble proteins concentration (Fig. 5). During transient expression of the *gfp* gene accumulation of total water-soluble proteins showed next indicators depending on terminators: the 35S CaMV terminator 2444.33±4.04 μg/ml, the ATPase gene terminator – 2636.67±3.51 μg/ml, the Atug7 gene terminator – 2843.67±4.16 μg/ml, while the mas gene terminator is 1901.67±3.21 μg/ml and the histone H4 terminator is 1695.67±2.89 μg/ml. The concentration of total proteins in controls (K strain GV3101 and K leaf tissue) was 545.67±2.08 and 546.33±4.04 μg/ml, respectively (Fig. 4). In contrast to previous researchers, we showed that the expression level of GFP in *N. rustica L.* tissues using Atug7 and ATPase terminators is higher than with using 35SCaMV terminator, what wasn't described before.

Detection of fluorescence, under ultraviolet light, after non-denaturing protein electrophoresis separation in polyacrylamide gel showed the functional activity of the GFP reporter protein and coincides with it molecular weight (Fig. 6b). Negative controls did not show GFP fluorescence.



F i g. 3. Visual detection of transient *gfp* gene expression: a) leaf on the 7th day after infiltration (daylight); b) leaf on the 7th day after infiltration (ultraviolet light): pSPV2315 (ATPase), pSPV2315 (Atug7), pSPV2314 (mas), pSPV2312 (35S CaMV), pSPV2316 (H4 Histone), – control – *A. tumefaciens* stain GV3101

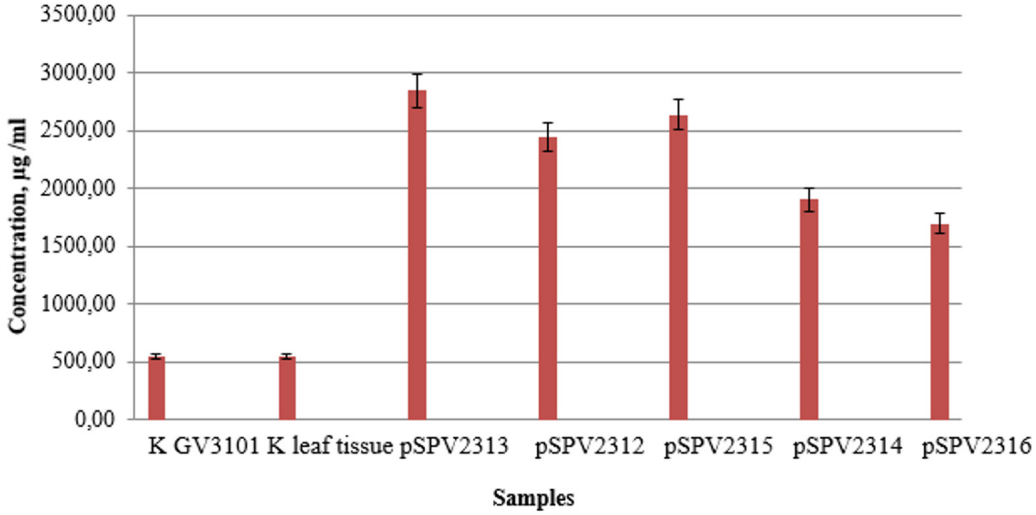


F i g. 4. Spectrofluorometric analysis of the fluorescence level in transient transformed *N. rustica* L. tissues

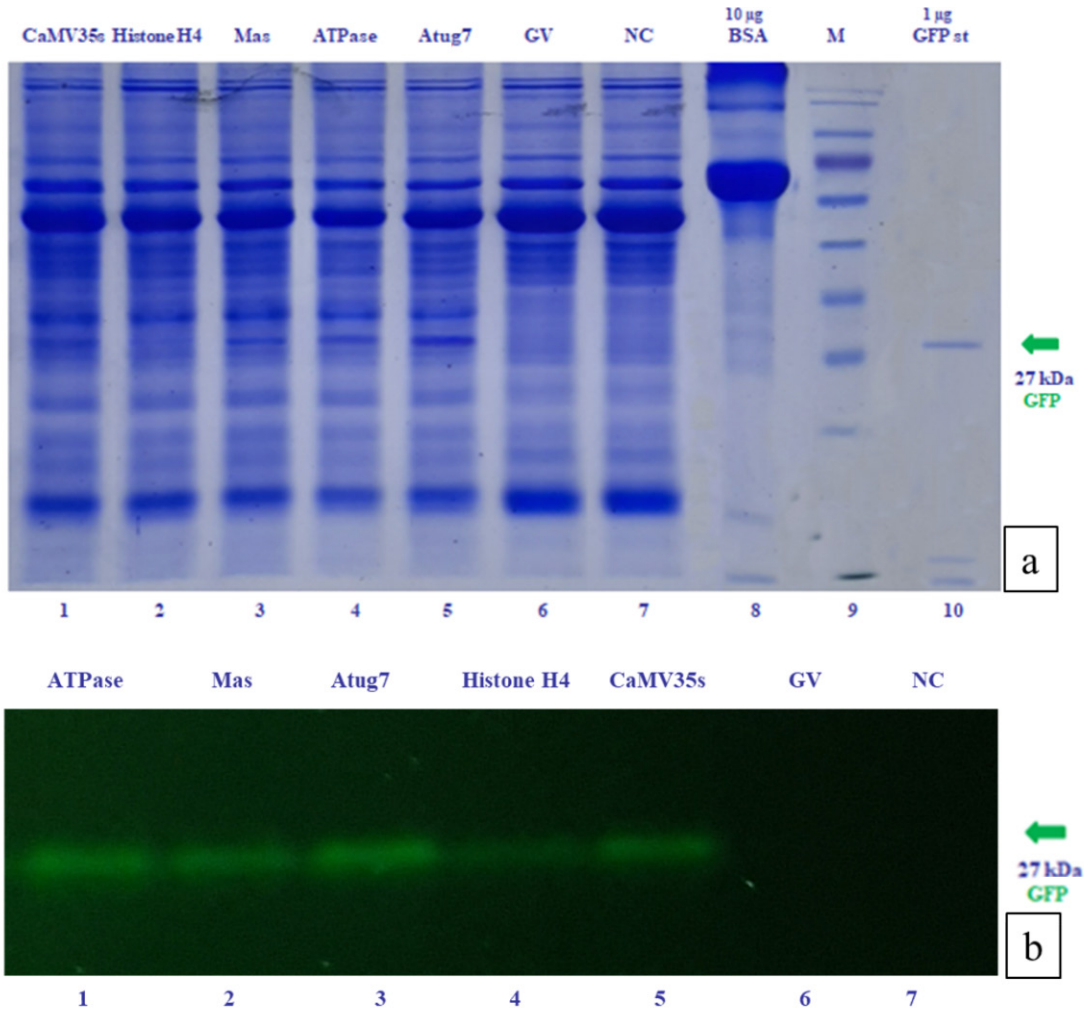
Table 2

Fluorescence level of transient transformed *N. rustica* L. tissue (relative units)

Genetic construct	Mean	– (Deviation)	+ (Deviation)
pSPV2312	1.40	0.03	0.03
pSPV2315	1.61	0.02	0.02
pSPV2313	1.79	0.03	0.03
pSPV2314	0.92	0.04	0.04
pSPV2316	0.63	0.04	0.04
K leaf tissue	0.02	0.00	0.01
K GV3101	0.01	0.00	0.01
$LSD_{0.05}$	0.16		



F i g. 5. Concentration of total water-soluble proteins in transient transformed tissues of *N. rustica* L.



F i g. 6. Electrophoretic separation of *N. rustica* L. proteins in polyacrylamide gel: a) denaturing conditions; b) non- denaturing conditions (detection of GFP in ultraviolet light): 1–5 – protein extracts of *N. rustica* L. tissues, in which the *gfp* gene is transiently expressed under the control of different terminators; 6, 7 – extracts of *N. rustica* L. control plants infiltrated with strain GV3101 of *A. tumefaciens* L. and uninfiltrated; 8 – molecular weight marker; 9 – standard Bovine Serum Albumin (BSA) **(concentration 10 µg); 10 – standard GFP (concentration 1 µg). The GFP protein is indicated by an arrow.**

The concentration of GFP in terms of fresh leaf weight (table 3) in reference to the mass of the GFP (1μg) and BSA (10μg) standards was determined by processing the data of polyacrylamide gel electrophoresis (Fig. 6b) in the GelAnalyzer 19.1 program. We showed that the highest accumulation of recombinant GFP, 0.5585±0.0085 g/kg of fresh leaf weight, was obtained when the terminator Atug7 was used in genetic construct. The lowest GFP accumulation, 0.1930±0.0042, was detected when the *gfp* gene expression was regulated by H4 histone terminator. For 35SCaMV, mas and ATPase terminators indicator of GFP accumulation was 0.4060±0.0085; 0.3555±0.0078; 0.4670±0.0099 respectively.

Increasing the level of gene expression coincides in both spectrofluorometric and protein assays. The highest accumulation of recombinant GFP was obtained when terminator Atug7 was used in the genetic construct and the lowest when the terminator of histone H4 gene from potato was used.

Table 3

GFP accumulation in fresh tissues of *N. rustica* L. plants transient transformed with genetic constructions

Construct	Mass (g/kg)	Deviation
pSPV2312	0.4060	0.0085
pSPV2313	0.5585	0.0120
pSPV2314	0.3555	0.0078
pSPV2315	0.4670	0.0099
pSPV2316	0.1930	0.0042
$LSD_{0.05}$	0.0556	

Discussion. The level of fluorescence directly correlates with the level of gene expression and recombinant protein accumulation, as evidenced before [30, 31].

Previously was described that Atug7 and 35S terminators have positively affected on stable and transient *cry2Ab*, *cry1Ac* gene expression for pest resistance [32].

35S CaMV terminator is a commonly used element in T-DNA cassettes in genetic engineering [33–36].

Ordon et al. showed the expression level of the *gus* gene targeted by the Cas9 system. The expression cassettes with the Cas9 gene had different terminators, thus determined the level of editing which depends on the terminator selection. Transient expression in *Nicotiana benthamiana* L. (*N. benthamiana* L.) plants showed that the *gus* gene expression was not significantly different when the Atug7 and ATPase terminators were used, while for the H4 histone terminator the level of *gus* activity was significantly lower. 35S CaMV terminator using showed the highest level of editing. The reporter *gus* gene while using the mas terminator in the experiment had significantly higher expression than when Atug7 was used [37], which is not confirmed by our research.

The effect of the selected terminators on the *gfp* reporter gene expression was first performed using transient transformation of *N. rustica L.* plants leaf tissues, and the difference in the GFP expression and accumulation level were determined.

We have shown that terminators have a significant effect on the level of gene expression. Moreover, the level of gene expression can be increased or decreased by terminator choosing, which is important in plant genetic transformation.

The highest level of the *gfp* gene expression was observed when the terminator Atug7 was used, and the lowest when the potato histone H4 terminator was used, which proves by fluorescence and protein assays.

We showed for the first time that the *gfp* gene expression level in *N. rustica L.* tissues was lower when 35SCaMV terminator was used in comparison with Atug7 and ATPase terminators.

The presence of recombinant GFP in total protein extracts was proved and its identity was confirmed by standard spectrofluorometric method and electrophoretic analyzes. The presence of biological activity of GFP in *N. rustica L.* plants extracts samples were shown by protein electrophoretic separation in non-denaturation conditions.

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ПОРІВНЯННЯ РІВНІВ ЕКСПРЕСІЇ ГЕНА *GFP* ЧЕРЕЗ РЕГУЛЯЦІЮ РІЗНИМИ ТЕРМІНАТОРНИМИ ПОСЛІДОВНОСТЯМИ

О.І. Варченко[1,2], М.В. Кучук[1], М.Ф. Парій[2,3], Ю.В. Симоненко[1,2]

[1]Інститут клітинної біології та генетичної інженерії НАН України, вул. Академіка Заболотного, 148, Київ, 03143, Україна
[2]Всеукраїнський науковий інститут селекції, вул. Васильківська, 30, Київ, 03022, Україна
[3]Національний університет біоресурсів і природокористування України, вул. Героїв Оборони, 15, Київ, 03041, Україна

Резюме

Здатність рослинних клітин експресувати чужорідні гени забезпечує можливість для вивчення функцій конкретних генів. Крім того, створення генетично модифікованих рослин з новими важливими ознаками актуальне для промислового виробництва або фармацевтичних застосувань. Для високого рівня експресії гетерологічних генів одним із ключових параметрів є ефективність термінаторів, що застосовуються в генетичній інженерії, оскільки рівень експресії гена залежить від його вибору. **Мета**. Дослідження впливу різних термінаторів на регуляцію експресії гена *gfp* (зеленого флуоресцентного білка) в тканинах *Nicotiana rustica* L. *(N. rustica* L.*)*. **Методи**. Для створення генетичних конструкцій використовували метод модульного молекулярного клонування Golden Gate, що базується на використанні ендонуклеаз рестрикції IIS типу та Т4 ДНК-лігази. Тканини *N. rustica* L. інфільтрували мануально за допомогою шприца суспензією агробактерій, що містили створені генетичні вектори для транзієнтної експресії гена *gfp*. Рівень експресії гена *gfp* визначали спектрофлуорометрично, за рівнем флуоресценції зеленого флуоресцентного білка та за білковим вмістом. Визначали концентрацію водорозчинних білків за Бредфордом та проводили їх електрофоретичне розділення у поліакриламідному гелі. Статистичну обробку даних проводили в програмі Microsoft Office Excel. Визначали середнє значення та його стандартне відхилення для кожного експерименту. Для статистичної обробки спектрофлуорометричного аналізу середнє значення визначали шляхом логарифмічного перетворення значень спектрофлуорометра. Обробку значень для визначення концентрації білків за даними поліакриламідного гелю проводили в програмі GelAnalyzer 19.1. **Результати**. Для дослідження створено п’ять генетичних конструкцій, що містять 5 різних термінаторів з сигналами поліаденілювання/3’-нетрансляційними послідовностями (3’UTR). Було обрано термінатори: 7-го гена, виділеного з *Agrobacterium tumefaciens* L. (*A. tumefaciens* L.) (Atug7), гена манопін синтази – з *A. tumefaciens* (mas), аденозин 5’-трифосфатази томатів (*Solanum lycopersum* L.) (ATPase), гістону H4 картоплі (*Solanum tuberosum* L.) та вірусу мозаїки цвітної капусти (35S CaMV). Всі транскрипційні одиниці додатково містили: 5’-нетрансляційну послідовність гена 2B сімейства генів, що кодують малу субодиницю рибулозо-1,5-бісфосфат карбоксилази/оксигенази (Rubisco) (5’UTR RbcS2B), кодуючу послідовність гена *gfp* та подвійний 35S промотор вірусу мозаїки цвітної капусти (D35S CaMV). Присутність рекомбінантного білка GFP у загальних білкових екстрактах та його ідентичність стандартному білку було доведено спектрофлуорометрично та методом поліакриламідного гель-електрофорезу. Вперше була показана різниця накопичення репортерного білка GFP в тканинах *N. rustica* L. шляхом термінаторної регуляції експресії гена *gfp*. **Висновки**. Найвищий рівень експресії гена *gfp* детектували при використанні в генетичній конструкції термінатора Atug7, а найнижчий – з термінатором гістону H4. Різниця між накопиченням GFP за білковим аналізом, використовуючи Atug7 та H4 термінатори, складала 2,89 разів. Ми показали, що послідовність термінатора має високий вплив на експресію гена. Вибір термінаторів є важливим кроком у створенні генетичних конструкцій, оскільки термінатор може бути використаний для регулювання рівня експресії генів залежно від цілей.

Ключові слова: Nicotiana rustica L., молекулярне клонування, генетичні конструкції, термінатори, зелений флуоресцентний білок, транзієнтна експресія, спектрофлуорометричний аналіз, білковий аналіз.

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