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TRANSIENT EXPRESSION OF *uidA* AND *gfp* GENES IN *PHYSALIS PERUVIANA* L.

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Physalis peruviana is a plant species which finds its application in agriculture and food industry due to the synthesis of numerous compounds such as fisagulins, fisalingulinides, visangulatine, fisalins. In recent years the properties of *Physalis* were improved with genetic engineering methods. We studied the possibility of transient expression of genes in *P. peruviana* plants using the *Agrobacterium rhizogenes* and *A. tumefaciens*. The intact plants were cultivated in greenhouse (22—26 °C, 14-hour light photoperiod, 3000—4500 lx) before transformation. *A. rhizogenes* A4 carrying pICH5290 or pCB131 vector and *A. tumefaciens* with pICBV19 vector were used for obtaining plants with transiently expressed reporter *uid*A and *gfp* genes. Also we additionally used *A. tumefaciens* GV3101 strain with pICH6692 genetic vector with gene coding the P19 protein (the suppressor of gene silencing). The pICH5290 genetic vector contained *bar* and *gfp* genes; the pCB131 genetic vector contained the *bar, gfp* and *fbp*B (ΔTMD) — gene, coding Ag85B of *Mycobacterium tuberculosis*. The pCBV19 genetic vector contained *uidA* and *bar* genes. Transient expression of *uidA* and *gfp* genes was confirmed in adult plants after conduction of vacuum infiltration with the bacteria. Maximum of *gfp* expression was observed between the 5th and 12th days after the transformation. The most intensive expression of the *gfp* and *uidA* genes was detected in the upper leaves (2nd—3rd) of young plants. There was no significant difference in gene expression levels in case of using agrobacteria with or without the genetic vector which carried out the gene silencing suppressor.

Key words: *Physalis peruviana* L.*, Agrobacterium*, transformation, transient expression, *uidA*, *gfp* gene.

Physalis peruviana is a species of Solanaceae family. Plant biomass obtained from steams, leaves and fruits of *P. peruviana* finds its application in agriculture and food industry. Using the fruits is of great interest because they contain A, C, B vitamins and polyphenols, which have anti-inflammatory and antioxidant properties [1—4]. Medical studies show that the use of *Physalis* can reduce the risk of developing of chronic degenerative diseases [5].

Both leaves and stems are used for medicinal purposes. The following bioactive steroids: fisagulins A—Q, fisalingulinides A—C, visangulatine A—I,

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fisalins B, D, F, G, H were detected in *Physalis* plants [6—8]. Physalins B, F showed significant antitumor activity. Alcohol extracts are most often prepared drug from this raw material. These extracts possessed antitumor, antimycobacterial (against *Mycobacterium tuberculosis*, *M. avium*, *M. kansii*, *M. malmoense*, *M. intacellulare*) [9], antiparasitic (for malaria treatment), antipyretic, immunomodulatory properties. Tinctures are used to treat asthma, hepatitis, dermatitis, and also as a diuretic drug [10]. Treatment of cancer cells with leaf extracts induced cell cycle arrest and apoptosis of human lung cancer cells *in vitro* culture [11].

Betulinic acid was isolated from the vegetative organs of *P. peruviana*. The antitumor activity of this acid has been studied against various types of human cancer, including neuroblastoma, glioblastoma, medulloblastoma, and carcinoma (head, neck, colon, breast, liver, lung, prostate, kidney, ovary) [12, 13]. In addition, there is evidence that betulinic acid is cytotoxic to metastatic cell lines compared to non-metastatic melanoma. This compound can be used in combination with other methods including ionizing radiation [14] and chemotherapeutic drugs for tumor disease treatments [15, 16].

The properties of *P. peruviana* can be improved *via* genetic engineering methods. Experiments on the transformation of plants of the *Physalis* genus have been already performed. Transgenic plants were obtained for some species of *Physalis*, such as *P. ixocarpa* [17], *P. ixocarpa* cv. *rendidora* [18]. *A. tumefaciens* was used for the stable transformation with selective and reporter genes such as *npt* II, *uidA*, *gfp* [18, 19]. However, until now, there are no reports dedicated to transformation of *P. peruviana*. It must be noted that study of the possibility of transient expression of reporter genes in plants of *Physalis* genus after agrobacterial transformation is of special interest. The usage of reporter genes makes it possible to optimize the transformation process and demonstrate the possibility of transferring foreign genes and the synthesis of the reporter proteins.

The aim of our work was genetic transformation of *P. peruviana* using *Agrobacterium* spp. and obtaining of plants which transiently expressed reporter genes.

Materials and methods

Plant material. Firstly, we tested the germination, energy of seed germination in non-sterile conditions to determine the initial seed quality according the methods proposed by several groups of researchers [20—22]. For this purpose, the seeds were sown in the pots with soil and cultivated during 7 days at 22—26 °C at 14-hour photoperiod, and illumination of 3000—4500 lx.

Surface sterilization of seeds was used for obtaining *in vitro* plants. The germination and energy of seed germination under sterile conditions were checked. We compared these parameters under non-sterile and sterile conditions, as concentrations and residence times in sterilizing agents can also inhibit seed germination.

The seeds were treated with 70 % ethanol solution for 2 min. Then they were transferred to 15 % bleach («Bilizna») for 10 minutes. After surface sterilization the seeds were washed three times with sterile distilled

water (for 15 min) and cultivated in Petri dishes on $MS_{(30)}$ medium [23]. Plants were grown *in vitro* for 3 weeks at a temperature of 22—24 °C. Then the plants were transplanted into greenhouse conditions in pots with soil. In the greenhouse, the plants were cultivated for 2 weeks at 22—26 °C (14-hour photoperiod, illumination of 3000—4500 lx).

Agrobacterium cultivation and vectors used for the transformation. In order to determine the possibility of genetic transformation, we used *A. rhizogenes* A4 strain with genetic vectors pCB131 (vector carrying *gfp*, *bar*, *fbp*B (ΔTMD) — gene, coding Ag85B of *Mycobacterium tuberculosis* under control of 35S promoter) [24] or pICH5290 (vector carrying *gfp*, *bar* genes under control of 35S promoter), *A. tumefaciens* pICBV19 (vector carrying *uidA* and *bar* genes under control of 35S promoter) [25, 26]. Also, we used *A. tumefaciens* GV3101 strain with genetic vectors pICH6692 (vector carrying *npt*II and *p19* genes). The *p19* gene is gene, coding the P19 protein (suppressor of gene silencing under control of 35S promoter).

50 mg/l of rifampicin and 25 mg/l of gentamicin were used for cultivation of *A. tumefaciens* (pCBV19) and 100 mg/l of ampicillin were used for cultivation of *A. rhizogenes*. 1 ml of a suspension of each agrobacterial strain was added into 50 ml of Luria-Bertani (LB) medium [27, 28] with 1.96 mg of acetosyringone. Bacteria were grown in liquid LB medium for 24 hours with stirring on a shaker Gio Gyrotory(®) shaker (New Brunswick Scientific Co., Inc. Edison, USA). Then *Agrobacterium* was precipitated for 12 min, 5000 rpm (Eppendorf Centrifuge 5430 R). Then bacteria were resuspended in liquid medium 1/2 $MS_{(15)}$ with sucrose (15 g/l).

Transformation assay. To study the possibility of *Physalis* transformation, whole potted plants were immersed in a flask containing LB medium with daily suspension of *A. rhizogenes* or/and *A. tumefaciens* strains (for 5—10 min, 22—24 °C) in a vacuum chamber at a pressure of 0.1 mPa.

Bacterial strains were used alone or in combination. The variants of the used strains and genetic vectors are presented in the Table.

The variants of the used strains, genetic vectors and genes

Variants	Bacterial strains	Genetic vectors	Genes which were integrated in the genetic vector
1	*A. rhizogenes* A4	pICH5290	*bar, gfp*
2	*A. rhizogenes* A4	pCB131	*bar, gfp, fbp*B (ΔTMD)
3	*A. tumefaciens* GV3101	pICBV19	*bar, uidA*
4	*A. rhizogenes* A4 + *A. tumefaciens* GV3101	pICH5290 + pICH6692	*bar, gfp* + *p19*
5	*A. rhizogenes* A4 + *A. tumefaciens* GV3101	pCB131 + pICH6692	*bar, gfp, fbp*B (ΔTMD) + *p19*
6	*A. tumefaciens* GV3101 + + *A. tumefaciens* GV3101	pICBV19+ pICH6692	*bar, uidA* + *p19*
7	Negative control 1 (without usage of agrobacterial suspensions)	—	—
8	Negative control 2 (without infiltration)	—	—

After infiltration, the plants were returned to greenhouse conditions and continued to grown there for 18 days (under conditions 22—26 °C, 14-hour photoperiod, lighting 3000—4500 lx).

Two groups of plants were used as negative controls. One group of the control plants were infiltrated under the conditions described above but without the addition of agrobacterial suspension to the medium, and the second group of the control plants wasn't infiltrated. The leaves of transformed *Nicotiana tabacum* with stable expression of the *uidA* gene (for histochemical reaction) were used as a positive control.

Analysis of transient expression of uidA gene in Physalis plants. For this study, the leaves of different ages (which were at different tiers on each plant), from the upper to lower one were cut in 12 days after infiltration. The histochemical reaction was performed at 37 °C according to the method of Jefferson [29]. For conduction of histochemical reaction, the leaves were stained using X-gluc substrate in order to detect the product of the *uidA* gene. Then, the leaves were washed 5 times in 70 % ethanol at intervals of 1 hour. After that, the leaves were placed on slides for microscopic examination (at magnification ×64). Specific activity was detected visually with the appearance of blue color of plant tissues.

Analysis of transient expression of gfp gene in Physalis plants. The leaves of *Physalis*, in 1, 2, 3 ... 18 days after infiltration with a suspension of *A. rhizogenes* A4 strain with genetic vectors pICH5290, pCB131 (both constructs contained the *gfp* gene) were studied visually under 365—400 nm ultraviolet light (lamp Blak-ray®, model B 100 AP). The results were considered positive with the appearance of green tissue fluorescence.

Statistical methods. Fifteen experimental plants were used to obtain transient expression of genes in plant tissues (15 plants for each variant of infiltration with *Agrobacterium*). The experiment was carried out in triplicate. We calculated the standard error (SE) and the arithmetic mean (M) in the program Excel 2007 in order to determine the accuracy of the obtained results.

Results and discussion

The germination of Physalis seeds in non sterile and in sterile conditions. The laboratory germination of *Physalis* seeds in nonsterile conditions was 94±0.2 % and germination energy (on the 4th day) was 92±0.5 %. Laboratory germination of seeds in sterile conditions was 90±0.4 %, and germination energy (6th day) — 87±0.5 %.

The results of seed germination under non-sterile and sterile conditions do not differ greatly. The germination rate was quite high, which indicates an initially high quality of seed material. A high percentage of seed germination after treatment with sterilizing agents indicates that the combinations, concentrations of sterilizing agents and the time of exposure of the seeds in these agents were selected correctly.

Transient uidA gene expression in Physalis plants. Positive results were obtained for all infiltrated *Physalis* plants. Appearance of blue areas on the tested leaves in histochemical reactions demonstrated the presence of *uidA* gene (Fig. 1).

Differences were found in the levels of transient gene expression in the leaves of different tiers. The darkest blue areas were revealed in the leaves of the upper tiers (2nd—3rd upper leaves), while on the lowest 6th leaf areas with transient expression were blue or pale blue. Also, areas with blue tones on the leaves of the upper tier were larger than on the leaves of the lower tiers.

In this way, the area with transient expression of *uidA* gene (which was in the pCBV19 genetic vector) on the 1st leaf occupied 55.22 % from the entire surface of the leaf blade, on the 2nd — 58.06 %, on the 3rd — ~ 56.13 %, on the 4th — ~ 41.26 %, on the 5th — ~ 32.68 %, on the 6th — ~ 21.4 %. The increasing of transient expression wasn't observed when used pICBV19 and pICH6692 genetic vectors in combination.

Fig. 1. Transient expression of the *uidA* gene in *Physalis peruviana* leaf after infiltration with *A. tumefaciens* suspension (pICBV19)

The obtained results show that the expression of the *uidA* gene and the synthesis of the GUS protein, respectively, occurred most intensively in the 2nd and 3rd upper leaves. Probably, the activity of the gene *uidA* decreases from the 10th day after infiltration, which is visually detected by a decrease of the stained areas. We recommend to use the 2nd—3rd upper leaves for further experiments, aim of which is obtaining the expression of other genes and protein synthesis.

The difference in gene expression levels with or without gene silencing suppressor (if compare the results of transient expression after infiltration of plants with *A. tumefaciens* (pICBV19) or *A. tumefaciens* (pICBV19) + + *A. tumefaciens* GV3101 (pICH6692) were not found.

Transient gfp gene expression in Physalis plants. Maximum of *gfp* gene expression was observed between 5—12th days after infiltration with *A. rhizogenes* A4 (pICH5290, pCB131) and *A. rhizogenes* A4 (pICH5290) + *A. tumefaciens* GV3101 (pICH6692) or *A. rhizogenes* (pCB131) + *A. tumefaciens* GV3101 (pICH6692). According to the results of *gfp* activity, the most suitable for infiltration were young leaves with intensive growth (2nd, 3rd and 4th leaf from the apex of the shoot) (Fig. 2).

The points of fluorescence were observed on a plant leaf infiltrated with *A. rhizogenes* GV3101 containing the pCB131 vector on the 3rd day after infiltration. At the same time, the necrosis zones that appeared on 5th day on a plant leaf infiltrated with *A. rhizogenes* (vector pCB131), indicated that this vector was not very suitable for *Physalis* infiltration, whereas obtained results indicate that the vectors pICH5290 and pICBV19 are suitable for further use in transformation experiments for *P. peruviana* plants.

There was no significant difference in gene expression levels with or without gene silencing suppressor (if compare the results of transient expression after infiltration of plants with *A. rhizogenes* A4 (pICH5290) and

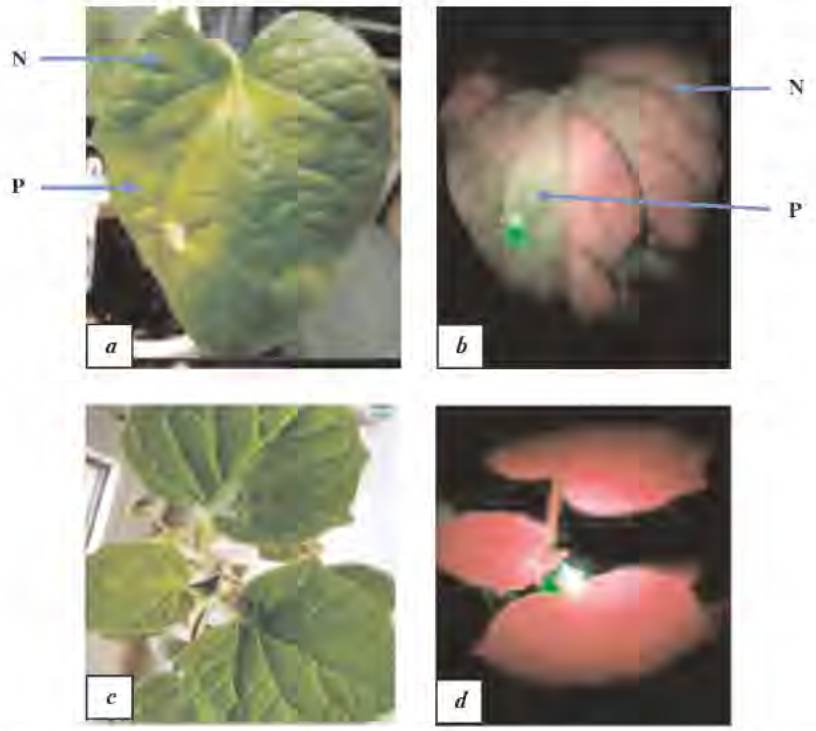

Fig. 2. Transient expression of the *gfp* gene (vector pICH5290) in leaves of *Physalis peruviana* on the 12th day after infiltration: *a* — 2nd leaf from the apex of the shoot under the daylight, *b* — the same leaf under the UV rays (N — zone without *gfp* expression, P — zone with *gfp* expression), *c* — control plants of *P. peruviana*, which were infiltrated with water, in daylight and under light with a wavelength of 365 nm (*d*); green fluorescence is due to GFP protein, red fluorescence is due to chlorophyll

A. rhizogenes A4 (pICH5290) + *A. tumefaciens* GV3101 (pICH6692), *A. rhizogenes* (pCB131) or *A. rhizogenes* (pCB131) + *A. tumefaciens* GV3101 (pICH6692)).

There are currently no reports of transient expression of *uidA* and *gfp* genes for species of the *Physalis* genus. It is obvious that the dynamics of transient expression of *uidA* and *gfp* genes can be specific. For example, the results from the transient expression of the *uidA* and *gfp* genes were obtained by several groups of researchers for tobacco (*N. tabacum*). One group of researchers observed a maximum of *gfp* gene expression between the 6th and 10th days after infiltration of plants with agrobacterial suspension, and on the 3rd day the expression was not high [30]. Another group of researchers headed by Wydro [31] argued that usually the highest levels of *gfp* gene expression can be obtained in the upper leaves of *N. tabacum*. In their experiment the highest levels of *gfp* gene expression was detected regardless of which genetic vector was used in the work and regardless of the presence or absence of the suppressor of gene silencing in the the genetic vector.

A number of experiments (the main goal of which was to obtain transient expression of various genes, including transient expression of the *gfp* gene) were performed by Sheludko, Sindarovskaya and colleagues [32, 33]. They used 7 different species of tobacco in their experiments. According to

their results, the content of the target protein during the transient expression depended on the age of the plant that was infiltrated, and the age of each of the leaf. The highest expression levels for different species of tobacco were observed between the 3rd and 8th day after infiltration and in 2nd–3rd leaves from the apex of the shoot of young plants. Two other groups of researchers also claimed that the highest levels of expression were obtained in the upper leaves of young tobacco plants [34, 35].

Transient expression of the *uidA* and *gfp* genes was obtained by Shah et al. [36]. In their work, they used genetic vectors with and without the suppressor of gene silencing. It was noted that suppressor of gene silencing significantly enhanced the expression of target proteins. In our experiments, the most intensive expression of the *gfp* gene occurred in the upper leaves of young plants between the 5th and 12th days after infiltration. The usage of genetic vector pICH6692 with gene of silencing suppressor didn't enhance the transient expression of *gfp* gene.

Conclusions. Transient expression of *gfp* and *uidA* genes in *P. peruviana* plants was evaluated. The *A. rhizogenes* (with pCB131 and pICH5290 genetic vectors), *A. tumefaciens* (with pCBV19 genetic vector) are suitable for transient expression of *gfp* and *uidA* genes. Optimal conditions for temporal expression of transferred genes in *P. peruviana* plants have been identified. The intensity of *gfp* and *uidA* transient expression was different in the younger and older leaves. Namely, the most intensive transient expression was observed in the 2nd—3rd from the apex of the shoot leaves (young leaves).

The intensity of transient expression of reporter genes changed during the time passed after infiltration. The maximum of *gfp* and *uidA* expression was detected between the 5th and 12th days after infiltration.

Thus, the proposed no only *A. tumefaciens* but also *A. rhizogenes* can be used to obtain transient expression of reporter genes in *P. peruviana* plants. So, we can suggest that the same bacteria can be used to transfer also target genes into the *P. peruviana* plants.

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ТРАНЗІЄНТНА ЕКСПРЕСІЯ *uidA* І *gfp* ГЕНІВ У *PHYSALIS PERUVIANA* L.

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Physalis peruviana — це вид рослин, який знаходить своє застосування в сільському господарстві та харчовій промисловості завдяки синтезу численних сполук, таких як фісагуліни, фісалінгуліниди, вісангулатин, фісаліни. В останні роки властивості фізалісу були покращені методами генної інженерії. Ми вивчили можливість тимчасової експресії генів у рослинах *P. peruviana* при використанні *Agrobacterium rhizogenes* і *Agrobacterium tumefaciens*. Інтактні рослини перед трансформацією культивували в теплиці (22—26 °С, 14-годинний фотоперіод, 3000—4500 лк). *A. rhizogenes* A4 з вектором pICH5290 або pCB131 та *A. tumefaciens* з вектором pICBV19 використовували для отримання рослин з репортерними генами *uidA* і *gfp*, які тимчасово експресувалися. Також додатково використовували штам *A. tumefaciens* GV3101 з вектором pICH6692 із геном, що кодує білок P19 (супресор мовчання генів). Генетичний вектор pICH5290 містив гени *bar* і *gfp*. Генетичний вектор pCB131 містив гени *bar*, *gfp* і *fbpB* (ΔTMD), що кодує Ag85B *Mycobacterium tuberculosis*. Генетичний вектор pCBV19 містив гени *uidA* і *bar*. Тимчасова експресія генів *uidA* і *gfp* була підтверджена в рослинах після проведення вакуумної інфільтрації з використанням бактерій. Максимум експресії *gfp* гена спостерігався між п'ятим і дванадцятим днями після інфільтрації. Найінтенсивніша експресія генів *gfp* і *uidA* виявлена у верхніх листках (2—3-й) молодих рослин. Не було значної різниці в рівнях експресії генів у разі використання агробактерій з генетичним вектором, який містив ген-супресор мовчання генів, або без використання цього вектора.

Ключові слова: *Physalis peruviana* L., *Agrobacterium*, трансформація, транзієнтна експресія, *uidA*, *gfp* гени.