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***Nicotiana cavicola* as a host for production of recombinant proteins by *Agrobacterium*-mediated transient gene expression.**

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Aim. To analyze a novel plant species as a host for obtaining recombinant proteins via transient gene expression. **Methods.** *Agrobacterium*-mediated transient gene expression, protein analysis, statistical data processing. **Results.** *N. cavicola* plants demonstrate good biotechnological characteristics; they are susceptible to agrobacterial infection and plant viruses. Green fluorescent protein (GFP) and human interferon alpha were produced in *N. cavicola* after transient gene expression using two different vector systems. The level of recombinant proteins depended on the gene and the system used. GFP content reached 6.0 % and 12.6 % TSP (0.44 mg/g FW). The interferon antiviral activity of the leaf extracts was 840 IU/g FW and 1710 IU/g FW. **Conclusion.** Here we propose *N. cavicola* species as a novel host for obtaining recombinant proteins which can be used as an alternative to the *N. benthamiana* host.

Keywords: *Nicotiana cavicola*, *Agrobacterium*-mediated transient expression, recombinant proteins, human interferon alpha, GFP

Introduction

The plant transient expression (TE) of transgenes is a fast, reliable and simple method that extensively used for fundamental physiological research and applied biotechnological purposes [1–3]. Potentially TE can be used for rapid production of pharmaceutically valuable proteins, including antibodies [4–7]. Rapid production of antibodies or vaccine proteins is preferable in the treatment of advanced cancer

diseases or in case of season epidemic diseases. In case of TE, the transgenes are not integrated into plant genome thus avoiding the position effects and providing considerably increased levels of recombinant proteins [8].

The protocols for *Agrobacterium*-mediated TE of transgenes in *Nicotiana benthamiana* plants are well developed and widely applicable. *N. benthamiana* demonstrates high sus-

ceptibility to agrobacterial and viral infections [9, 10], and is considered as a model host for TE. However, common utilization of one species gives some limitations and hides the advantages of other species, e.g. increased recombinant protein levels or better biotechnological characteristics. Earlier, it was proved the important role of host species for successful production of recombinant proteins [11].

Here we describe a new host for TE of transgenes, namely *Nicotiana cavicola*. This species can be easily infiltrated, it is susceptible to agrobacterial transfection, assures transgene expression from various expression cassettes (different vector systems), and produces recombinant proteins in large amounts. The final levels of green fluorescent protein (GFP) and human interferon alpha 2b obtained in *N. cavicola* plants were comparable to or surpassing the corresponding levels obtained in *N. benthamiana* plants.

Materials and Methods

Plant growth conditions. *Nicotiana* spp. seeds were obtained from “National Germplasm Bank of World Flora of the Institute of Cell Biology and Genetic Engineering” (Kyiv, Ukraine). Seeds were germinated either in unsterile conditions (commercial soil) or in aseptic conditions on MS agar medium [12] after surface sterilization [13]. Plants grew in greenhouse conditions: 16-hour light day at 22–28°C, 3000–4000 lux. Leaves of 6–8-week old plants were used for the experiments.

Agrobacterial strains and genetic constructions. Vector constructions were harbored into *Agrobacterium tumefaciens* GV3101 strain. The used plasmids (pICH5290, pICH7410, pICH10570, pICH10881,

pICH17311, pICH6692) were obtained for scientific purposes from Icon Genetics GmbH (Halle, Germany) and described in [14, 15]. The plasmid pCB125 was described in [16]. The sequence coding for the human interferon alpha 2b was fused with calreticulin signal from *N. plumbaginifolia* for the protein targeting to intracellular space [15].

Two different vector systems used in the experiments were described in the earlier publication [11]. One of them represents a simple vector construction where a gene of interest was driven by the 35S CaMV promoter (named herein as 35S-system).

Another vector system (named herein as recombinase vector (Rec) system) includes combination of viral genome elements and binary plasmid of *A. tumefaciens*. This vector system requires simultaneous introduction of three vector modules into the same cell for *in planta* assembly of the RNA replicon [11, 14].

Bacteria growing and infiltration procedure. Growth and preparation of agrobacteria for the infiltration were performed as described [11, 15]. Infiltration was carried out according to Schob *et al.* [17] with minor modifications [15]. Mixed bacterial suspension (100 µl/sample) was injected at the same leaf position to exclude sample variability [18]. There were two control groups of plants: the first group was infiltrated with the buffer only; the second one was infiltrated just with the agrobacterium clone which harbored the vector construction with the *p19* gene (a suppressor of post-transcriptional gene silencing). All experiments were done in 7–10 replications.

Extraction of total soluble proteins. Plant material was collected on the 4th day post infiltration (dpi) if 35S expression system was

used or on the 16th dpi if the recombinase expression system was used.

GFP extraction procedure: 0.1–0.2 g of fresh plant material was ground on ice in 1.5 ml tubes in 500 µl of cold sodium phosphate buffer, and debris was precipitated by centrifugation at the top speed. The supernatant was transferred into clean tube. A new portion of buffer (500 µl) was added to the sediment and procedure was repeated. The total supernatant was used for the next analysis.

Interferon extraction procedure: plant leaf tissue was collected, weighted and ground on ice with a mortar and a pestle in one volume of cold Tris-base buffer. Grinded leaves were transferred in 1.5 ml tubes and the debris was precipitated by two-round top speed centrifugation to clarify a supernatant. The supernatant was used for the next analysis.

GFP extraction buffer: 50 mM Na₂HPO₄/NaH₂PO₄, pH 7.0. Interferon extraction Tris-base buffer was described in [15].

Measurement of the concentration of total soluble proteins. The quantity of total soluble proteins (TSP) was measured according to Bradford's method [19] using bovine serum albumin as a standard.

GFP assay. The presence of recombinant GFP in leaf tissues was determined and its quantity was calculated as described earlier [11]. The percentage of GFP per TSP was calculated as well.

Assessment of antiviral activity of interferon. The protective antiviral activity of experimental extracts was demonstrated in titration assay described in [15]. The dilution of sample where 50 % of the cells survived after vesicular stomatitis virus infection was determined as the titer of interferon. Based on titers

of standard interferon alpha 2b the activity of extracts was estimated in International Units (IU).

Statistical data processing. For statistical data manipulations, standard deviation (SD) and Student's test were used. Bars on diagrams demonstrate standard deviation [20].

Results and Discussion

Nicotiana cavicola, an Australian species of tobacco is classified together with *N. benthamiana* in the same subgenus. Several studies revealed a high sensitivity of *N. cavicola* to a wide range of plant viruses [21, 22]. This feature is distinctive also for *N. benthamiana*, which suggests that *N. cavicola* along with *N. benthamiana* may be used for the heterologous protein production via transient expression (TE) if the gene is located in a viral based vector system.

To investigate a biological potential of *N. cavicola*, two mentioned species were compared for several parameters: growth in greenhouse conditions, plant biomass, easiness of infiltration procedure, possibility of *Agrobacterium*-mediated TE of transgenes and the production of desired proteins.

Growth in greenhouse conditions. The mature seeds of *N. cavicola* sprouted on the 4th–6th days similarly to *N. benthamiana* ones, and we did not observe any difference in the plant germination rate between the surface sterilized or non-sterilized seeds. Subsequently, the non-sterilized seeds were germinated directly in a commercial soil mixture. We found that *N. cavicola* can be used for TE experiments at the age of 6–8 weeks but before appearance of flowers [11]. The 6–8 weeks old *N. benthamiana* plants were often used for TE as well [23,

24]. The *N. cavicola* plants demonstrate efficient growth in greenhouse and their fully expanded leaves are bigger than those of *N. benthamiana* ones grown under the same conditions (Fig. 1).

Plant biomass and infiltration procedure. Total biomass weights of *N. cavicola* and *N. benthamiana* plants available for the protein production were compared. As shown in the Table 1, one fully expanded leaf of *N. cavicola* weighs more than that of *N. benthamiana*. The difference was significant ($P < 0.001$). *N. benthamiana* demonstrates the best production of recombinant proteins in five upper mature leaves [11], whereas only three upper mature leaves can be used in *N. cavicola*. Thus, there was no difference between the species in the total leaf biomass useful for protein production.

N. cavicola leaves are nicely infiltrated by bacterial suspension without lateral vein restriction. Simplicity of leaf infiltration is typical for young *N. benthamiana* plants.



Fig. 1. Plants of *N. cavicola* (right) and *N. benthamiana* (left) grown in greenhouse (8 weeks old). Paper scale is 5 cm.

Table 1. Comparison of biomass characteristics of two *Nicotiana* species grown under greenhouse conditions

Plant species	Mean leaf weight, g \pm standard deviation (SD)	Number of leaves used for TE	Sum biomass of leaves used for TE, g \pm SD
<i>N. cavicola</i>	1.182 \pm 0.359	3	3.5 \pm 0.88
<i>N. benthamiana</i>	0.534 \pm 0.137	5	2.67 \pm 0.3

Recombinant protein production: Green Fluorescent Protein (GFP). Vital reporter protein, GFP, was used for the assessment of recombinant protein production in *N. cavicola*. Two vector systems carrying the *gfp* gene were used for the experiments. One vector includes the *gfp* gene driven by 35S CaMV promoter, whereas another system, a recombinase vector (Rec) system [8, 14], includes two pro-vectors and the *gfp* gene appears under the viral subgenomic promoter after the recombination event.

Green fluorescence was detected under short-wave UV-light within infiltrated areas that confirmed the presence of heterologous GFP

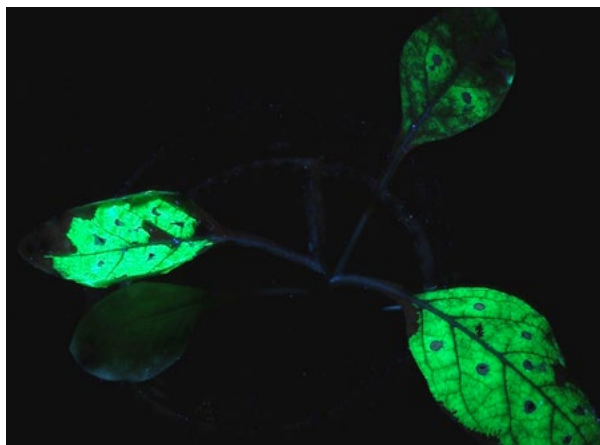


Fig. 2. *N. cavicola* plants producing recombinant protein GFP, view under UV light. Green colour leaf areas demonstrate GFP accumulation (clear areas in black-white variant).

(Fig. 2). The viral suppressor of gene silencing p19 was used to increase the heterologous protein production [25, 26].

Total soluble proteins (TSP) were extracted from the infiltrated leaf areas and the contents of GFP and TSP were calculated as described.

The GFP content in *N. cavicola* leaves reached 6.0 ± 1.51 % TSP when 35S system was used. In *N. benthamiana* leaves the GFP level reached 3.84 ± 1.31 % TSP (Fig. 3). The difference between the means was significant ($P < 0.05$). The data were compared with the data obtained earlier for other *Nicotiana* spp. [11] and it was noted that the percentage of GFP in *N. cavicola* was the highest. In general, a higher percentage of heterologous protein will invest in facilitation of the next purification procedure.

The GFP content calculated per a plant biomass unit was 0.440 ± 0.140 mg/g fresh weight (FW) in *N. cavicola* and 0.312 ± 0.075 mg/g FW in *N. benthamiana*. The difference was not

statistically significant. The average amount of TSP in *N. benthamiana* tissues was higher than that in *N. cavicola*: 9.43 ± 3.07 mg/g FW and 7.36 ± 2.01 mg/g FW, respectively.

Then GFP content was measured after applying the Rec system. Usage of this system resulted in an increase of GFP percentage compared to 35S system but the amount of GFP per gram of fresh weight was similar to 35S system: 12.59 ± 6.48 % TSP (or 0.438 ± 0.218 mg/g FW) in *N. cavicola* and 16.2 ± 5.7 % TSP (or 0.365 ± 0.122 mg/g FW) in *N. benthamiana* (Fig. 4). Further analysis revealed that the TSP level in experimental leaves reduced: 3.86 ± 1.32 mg/g FW in *N. cavicola* and 2.26 ± 1.04 mg/g FW in *N. benthamiana*. The possible explanation is senescence of leaves and diminution of proteins during the experimental period: the maximal level of recombinant protein accumulation after using the Rec system is reached on the 14th-16th days post infiltration (dpi), whereas the 35S system al-

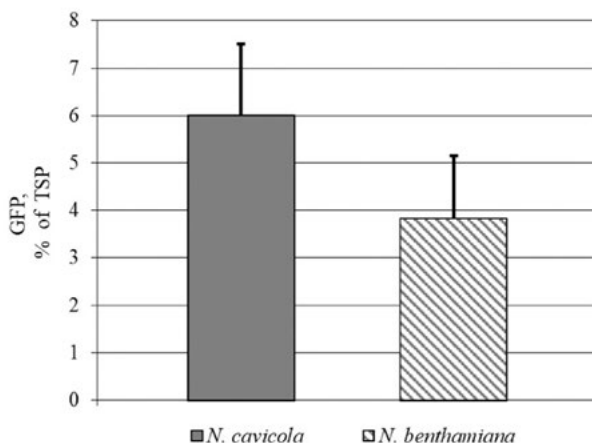


Fig. 3. Comparison of GFP content in plant extracts (35S system). The difference between means was significant ($P < 0.05$). Bars mean SD.

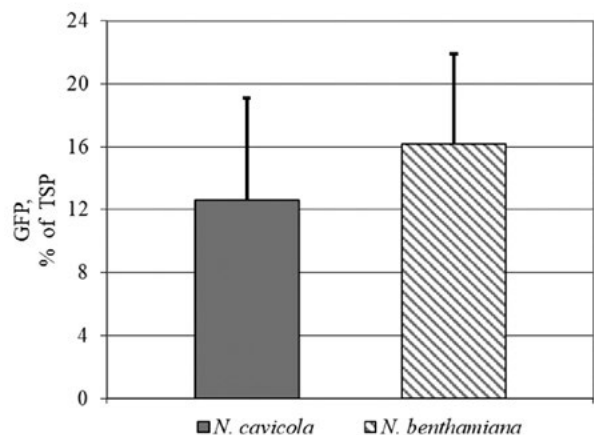


Fig. 4. Comparison of GFP content in plant extracts (the Rec system). The difference between means was not significant ($P > 0.05$). Bars mean SD.

lows collection of leaves on the 4th dpi. An increased GFP percentage after applying the Rec system may ensure advantages for the following protein purification steps. In the previous paper [11] it was shown that not all *Nicotiana* species were sensitive to the Rec system and some of them did not accumulate GFP in measurable quantities.

The obtained data proved that *N. cavicola* is susceptible to agrobacterial infection, and T-DNA is transferred efficiently in the plant cells. Additionally, *N. cavicola* demonstrates visible sensitivity to different expression systems. This confirms that *N. cavicola* is a prospective host for obtaining heterologous proteins via the *Agrobacterium*-mediated transient gene expression.

Human interferon (INF) alpha 2b production. To check if the tested host can produce the proteins not as stable as GFP, human interferon $\alpha 2b$ was chosen for the next experiments. To reach the aim, the identical expression systems (the 35S system and Rec system) were used but the *gfp* gene was replaced by the *inf* gene. The experimental conditions were the same except for the extraction procedure. In the course of experiment, it was confirmed the presence of antiviral activity in the extracts prepared from the infiltrated leaves. This can be regarded as a proof of the bioactive heterologous interferon production in *N. cavicola* cells.

If the 35S system was used, the average antiviral activity detected in the *N. cavicola* leaf extracts was 844 ± 302 International Units (IU)/g FW (variation range 770–1570 IU/g FW), and the average antiviral activity detected in the *N. benthamiana* leaf extracts was 281 ± 105 IU/g FW (variation range 170–370 IU/g FW) (Fig. 5). The INF production in *N. cavicola* was about

3 times higher than in *N. benthamiana*. The difference between the means was significant ($P < 0.01$). These data agree with the data shown for the GFP production.

Further, antiviral activity was measured in the leaf extracts obtained after using the Rec system. In this case antiviral activity of extracts increased if compared with the data shown for the 35S system: the average activity was 1714 ± 855 IU/g FW (variation range 800–2400 IU/g FW).

In general, the total amount of INF was less than the total amount of GFP obtained in the same manner. The possible explanation of such results is a different degradation rate of the various proteins in plant cells. GFP conformation provides its high proteolytic stability [27] whereas native human INF is attacked by plant proteases more frequently. Moreover, optimal conditions for accumulation of recombinant proteins can vary significantly.

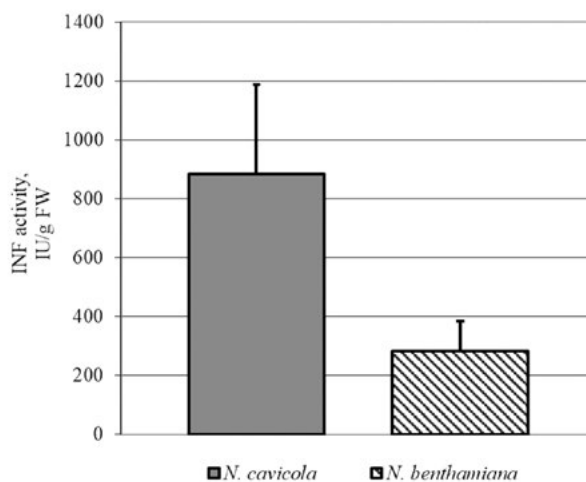


Fig. 5. Comparison of interferon activity in plant extracts from two *Nicotiana* species (the 35S system). The difference between means was significant ($P < 0.01$). Bars mean SD.

Conclusions

A detailed inspection of characteristics essential for efficient production of recombinant proteins showed that the *Nicotiana cavicola* leaves are suitable for infiltration, the species is susceptible to agrobacterial transfection and assures the transgene expression from various expression cassettes. Two recombinant proteins, namely reporter protein GFP and human interferon alpha 2b, were successfully produced in the *N. cavicola* plants after transient gene expression. The final GFP and INF levels differed, mainly because of different protein properties. The INF level was not as high as the GFP level and some other plant system can produce higher quantities of recombinant INF, therefore *Nicotiana cavicola* still looks promising species for the recombinant protein production via transient gene expression.

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- Nicotiana cavicola як господар для виробництва біофармацевтичних препаратів у рослинах шляхом *Agrobacterium*-опосередкованої транзйентної експресії генів**
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- Мета.** Аналізувати новий вид рослин як господаря для отримання рекомбінантних білків шляхом транзйентної експресії генів. **Методи.** *Agrobacterium*-опосередкована транзйентна експресія генів, аналіз білків, статистична обробка даних. **Результати.** Рослини *N. cavicola* демонструють гарні біотехнологічні характеристики, і вони сприйнятливі до агробактеріальної інфекції та до рослинних вірусів. В результаті транзйентної експресії

генів з використанням двох різних векторних систем в *N. cavicola* напрацьовувався зелений флуоресцентний білок (GFP) і людський інтерферон альфа. Рівень рекомбінантних білків залежав від використаного гена і системи. Вміст GFP сягав 6,0 % і 12,6 % СРБ (0,44 мг/г СВ). Протівірусна активність інтерферону з екстрактів листя становила 840 МО/г СВ і 1710 МО/г СВ.

Висновки. У даній статті ми пропонуємо вид *N. cavicola* в якості нового господаря для отримання рекомбінантних білків, який може бути використаний як альтернатива господарю *N. benthamiana*.

Ключові слова: *Nicotiana cavicola*, *Agrobacterium*-опосередкована транзйентна експресія, рекомбінантні білки, людський інтерферон альфа, GFP.

***Nicotiana cavicola* как хозяин для производства биофармацевтических препаратов в растениях путём *Agrobacterium*-опосредованной транзйентной экспрессии генов**

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Цель. Анализировать новый вид растений в качестве хозяина для получения рекомбинантных белков посредством транзйентной экспрессии генов. **Методы.**

Agrobacterium-опосредованная транзйентная экспресия генов, анализ белков, статистическая обработка данных. **Результаты.** Растения *N. cavicola* демонстрируют хорошие биотехнологические характеристики, и они восприимчивы к агробактериальной инфекции и к растительным вирусам. В результате транзйентной экспресии генов с использованием двух различных векторных систем в *N. cavicola* нарабатывался зеленый флуоресцентный белок (GFP) и человеческий интерферон альфа. Уровень рекомбинантных белков зависел от используемого гена и системы. Содержание GFP достигало 6,0 % и 12,6 % СРБ (0,44 мг/г СВ). Противовирусная активность интерферона из экстрактов листьев составляла 840 МЕ/г СВ и 1710 МЕ/г СВ.

Выводы. В данной статье мы предлагаем вид *N. cavicola* в качестве нового хозяина для получения рекомбинантных белков, который может быть использован как альтернатива хозяину *N. benthamiana*.

Ключевые слова: *Nicotiana cavicola*, *Agrobacterium*-опосредованная транзйентная экспресия, рекомбинантные белки, человеческий интерферон альфа, GFP.

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