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Y.R. SINDAROVSKA [1], I.M. GERASYMENKO [1],
Y.V. SHELUDKO [1], Z.M. OLEVINSKAYA [2],
N.Y. SPIVAK [2], N.V. KUCHUK [1]

[1] Institute of Cell Biology and Genetic Engineering NAS of Ukraine, Kiev 03680, Ukraine,
E-mail: ysheludko@ukr.net

[2] Institute of Microbiology and Virology NAS of Ukraine, Kiev

PRODUCTION OF HUMAN INTERFERON ALFA 2b IN PLANTS OF *NICOTIANA EXCELSIOR* BY *AGROBACTERIUM*-MEDIATED TRANSIENT EXPRESSION

Human interferon α2b gene was transiently expressed in Nicotiana excelsior plants. Fusion with N. plumbaginifolia calreticulin signal peptide for improved apoplast targeting and carrying out the expression under optimized conditions resulted in maximal interferon activity of 3.2 · 10^3 IU/g fresh weight (FW) with an average of 2.1 ± 0.8 · 10^3 IU/g FW. It proves that N. excelsior is a suitable host for Agrobacterium-mediated transient expression of genes encoding physiologically active human proteins. The transient expression conditions optimized for GFP marker protein were confirmed to be preferable for hIFN α2b.

Introduction. Interferons are a large family of multifunctional secreted proteins involved in animal antiviral defence, cell growth regulation and immune system activation [1]. Interferons are used to treat several diseases including some types of cancer and hepatitis C [1]. Recombinant human interferons have been produced in bacteria, yeast, insect and mammalian cells, and several plant species [2–11]. Plants as a source of foreign proteins have a number of advantages over microbial or animal cell systems. In contrast to bacteria correct posttranslational modifications of recombinant proteins take place in plant cells as well as folding and assembling of multimeric proteins, e.g. antibodies [12–14]. Plants do not contain bacterial toxins and human pathogens like viruses and prions, that makes the recombinant proteins of plant origin safer [15]. In some cases they can be used without prior purification as edible vaccines that lowers production costs considerably [15]. The main drawback of plants with stably transformed nuclear genome is the moderate level of target protein accumulation. The recombinant protein production usually does not exceed of 1 % of total soluble proteins (TSP) [16] due to the low transgene expression level and/or the protein product instability (for strategies to enhance stable transgene expression and product accumulation in plants, see [17]). Plastid transformation often allows selecting of transplastomic plants with high level of recombinant protein accumulation [18]. However, it is a time-consuming task, up to date feasible for a restricted number of plant species. Modern approaches to transient gene expression in plants lead to accumulation of large amount of recombinant proteins within a very short time [19]. This method was successfully applied for production of a number of recombinant proteins, e.g. tumor-specific antibodies [20] and vaccines [21] and human growth hormone [22]. The efficiency of the transient expression may be increased by vector system modification [23, 24] and/or by optimization of the expression conditions and choosing of an appropriate plant host species [25].

Here we report the production of active human interferon alfa 2b in plants of *Nicotiana excelsior*, previously selected as a promising host species for *Agrobacterium*-mediated transient expression using GFP marker protein [25].

Materials and methods. *Plant material.* Seeds of *N. benthamiana* and *N. excelsior* were obtained from the National Germplasm Bank of World Flora of

the Institute of Cell Biology and Genetic Engineering (Kiev, Ukraine). In greenhouse plants were grown at 20–25 °C and 14 h light period (3000–4000 lux).

Bacterial strains and genetic constructs. Genetic constructs pICH10881, pICH10570, pICH13301 (with the native hIFN-α2b gene), pICH17311 (with the recombinant hIFN-α2b gene) and pICH7410 (with reporter GFP gene) represent a viral-based module vector system described in details in [23]. The plasmid pICH6692 contained the gene of the p19 protein of tomato bushy stunt virus, a suppressor of post-transcriptional gene silencing [26] driven by 35S CaMV promoter. All the mentioned plasmids were obtained for scientific purposes from Icon Genetics GmbH (Halle/Saale, Germany).

Agrobacterium tumefaciens strain GV3101 transformed with individual constructs was grown overnight in LB medium supplemented with 50 mg/l of rifampicin and 50 mg/l of carbenicillin or kanamycin, and 100 μM of acetosyringone.

*Transient expression assay***.** Plant infiltration was performed as described in [27] with several modifications [23]: *A. tumefaciens* cells of overnight culture were centrifuged and resuspended with the infiltration buffer (10 mM MES, pH 5.5; 10 mM $MgSO_4$; 100 μM acetosyringone). The *Agrobacterium* suspensions harboring different plasmid vectors were mixed in the equal volumes so that the final optical density (OD 600) of each suspension in the infiltration buffer amounted 0.5. The leaves of greenhouse grown plants were infiltrated with *Agrobacterium* mixture (50 mkl/leaf) by using a syringe without a needle. The *Agrobacterium* mixture was injected into mesophile tissue of the 2^{nd} – 4^{th} upper leaves. After infiltration, the plants were further grown under greenhouse conditions and harvested at 14–18 days post infiltration. All experiments were carried out in 4–6 replications.

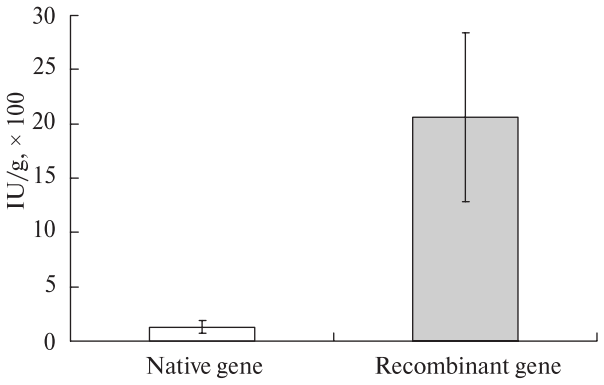
Interferon activity assay. Extracts from plant leaves were prepared in equal volume of 100 mM Tris/HCl buffer, pH 8.0, containing 5 mM Na_2EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol, and 2.5 % PVP. The total protein content was measured by the method of Bradford [28].

The assays were performed in sterile 96-well microtiter plates. Each well was filled with 100 μl of transmissible neonatal pig testis cell suspension (10^5 cell/ml) in RPMI-1640 medium [31]. The cells were cultivated for 18 hours at 37 °C. Samples to be titrated for interferon activity were diluted 20-fold with RPMI-1640 medium and placed in the first row of wells of an empty plate. Two-fold dilutions of the samples with RPMI-1640 medium were made serially to the end of the well columns. The RPMI-1640 medium in the wells with the cells was replaced with sample dilution rows and the cells were further incubated for 18 hours at 37 °C. Thereafter 100 μl of vesicular stomatitis virus (100 CPE_{50}/0.1 ml) was added to each well (except of cell control wells, where 100 μl of RPMI-1640 medium were applied). The cells were cultivated at 37 °C for 24 hours (until the cytopathic effect was fully developed in the virus control wells). The medium from the wells was evaporated and the cells were stained with crystal violet. The wells where 50 % of the cells were protected from the virus cytopathic effect were detected by microscopic examination. Standard interferon solution was included in all assays to determine the absolute titer.

Results and discussion. Transient expression is often carried out in *N. benthamiana* [e.g., 14, 22], but this plant species has a rather small biomass yield that hinders its application for large-scale production of recombinant proteins. In our previous studies we have selected *N. excelsior* as a better host for transient expression. This species displayed the best characteristics in regard to biomass yield as well as GFP accumulation level [25].

The target hIFN-α2b gene was introduced into *N. excelsior* plants as a part of a viral-based module vector system. This system consists of three elements delivered in plant by simultaneous infiltration of *Agrobacterium* strains carrying corresponding plasmids. Two modules containing viral genes and regulatory elements and the target gene are combined inside of the plant cell by the *Streptomyces* phage PhiC31 site-specific recombinase encoded in the third plasmid. The resulting DNA molecule contains the viral genes of the RNA-dependent RNA polymerase, movement protein and the target gene driven by the subgenomic promoter of a viral coat protein. DNA is able to move from cell to cell due to the movement protein [23].

In our experiments the greenhouse grown *N. excelsior* plants were infiltrated with a mixture of four *Agrobacterium* strains carrying the three modules of the viral-based expression system and a vector with the gene encoding p19 protein of tomato bushy stunt virus, a suppressor of gene silencing



Activity of interferon in leaf extracts of *N. excelsior* transiently expressing native or recombinant (fused to *N. plumbaginifolia* calreticulin signal peptide) human interferon α2b gene

[26]. For monitoring of the transient expression process, one of the leaves on each plant was infiltrated with an analogous *Agrobacterium* mixture but carrying the marker GFP gene instead of hIFN-α2b. The activity of interferon in the leaf extracts was measured by its ability to protect animal cells *in vitro* against viral replication [29, 30]. For the construct with native hIFN-α2b gene we determined interferon activity in the extracts and found that it reached the maximum level of $8 \cdot 10^2$ IU/g FW with an average of $1.3 \pm 0.59 \cdot 10^2$ IU/g FW. This value corresponds to 1.3–2 ng/g of leaf fresh weight.

We checked several parameters which were shown to influence on recombinant protein production via transient expression using GFP as a reporter [25]. It was found that the activity of interferon was approximately 3–4 times higher in the upper leaves of the plant than in lower ones ($2.5 \cdot 10^2$ IU/g and $0.65 \cdot 10^2$ IU/g, respectively). Co-expression of the p19 protein, a suppressor of gene silencing [17], improved the interferon yield at least 15-fold ($8 \cdot 10^2$ IU/g compared with $0.5 \cdot 10^2$ IU/g in the experiments without the p19 suppressor of silencing). These data corresponds well to the results obtained for GFP reporter protein [25].

The accumulation level of interferon was considerably lower than that of GFP obtained under the same conditions [25]. These results can be explained by lower stability of interferon in the plant cells as compared with GFP. The action of cell proteases is known to be a limiting factor for recombinant protein accumulation in plants [32]. One of possible ways to overcome this problem is subcellular protein targeting to organelles (e.g. chloroplasts or endoplasmic reticulum (ER)) or into apoplast [17, 22]. It is accomplished by fusion of the protein of interest with specific aminoacid sequences that direct it to the corresponding cell compartment. To be excreted into apoplast, a protein should contain on its N-terminus a signal sequence that is usually cleaved during protein translocation through the ER membrane. Native hIFN-α2b gene encodes signal sequence that ensures its secretion from leukocytes. This sequence supports analogous targeting in plant cells, but possibly at lower rate.

To amend the process of interferon excretion into apoplast, we have used a recombinant hIFN-α2b gene attached to *N. plumbaginifolia* calreticulin signal peptide. It was reported previously that fusion with *N. plumbaginifolia* calreticulin signal peptide led to high level of transient expression of hIFN-α2b gene in *N. benthamiana* [22]. In our experiments the transient expression of the recombinant hIFN-α2b gene resulted in approximately 15-fold higher interferon activity than in case of using the native gene ($2.06 \pm 7.8 \cdot 10^3$ IU/g of leaf extract corresponding to 20–30 ng/g FW) (Figure). The maximum activity amounted to $3{,}2 \cdot 10^3$ IU/g of leaf extract (30–50 ng/g FW).

Physiologically active human interferons have been produced earlier in several plant species by stable nuclear transformation [2–7], chloroplast transformation [8] and transient expression [9–11]. The highest level of hIFN-α2b (up to 20 % TSP, or 3 mg/g FW) was reported for tobacco transplastomic plants [8]. The interferon activity in stable nuclear transformants reached approximately $5 \cdot 10^2$ IU/g FW [5, 11]. Transient expression resulted in approximately 10-fold higher activities ($3.1 \cdot 10^4$ IU/ml for interferon β in lettuce [10] and $2.1 \cdot 10^4$ IU/g FW for interferon α2a in cucurbits [9]), although in some cases the interferon content was lower (0.3 ng/g FW for chicken interferon α in lettuce [11]). Our results with $2.1 \pm 0.8 \cdot 10^3$ IU/g FW of human interferon α2b in *N. excelsior* prove the effectiveness of the transient expression method.

We can conclude that *N. excelsior* is a suitable host for transient expression of genes encoding physiologically active human proteins, e.g. interferon α2b. The transient expression conditions optimized for GFP marker protein were confirmed to be preferable for hIFN α2b.

Я.Р. Синдаровская, И.М. Герасименко, Ю.В. Шелудько, З.М. Олевинская, Н.Я. Спивак, Н.В. Кучук

ПОЛУЧЕНИЕ ИНТЕРФЕРОНА АЛЬФА-2b ЧЕЛОВЕКА МЕТОДОМ *AGROBACTERIUM*-ОПОСРЕДОВАННОЙ ТРАНЗИЕНТНОЙ ЭКСПРЕССИИ В *NICOTIANA EXCELSIOR*

Ген интерферона α2b был транзиентно экспрессирован в растениях *Nicotiana excelsior*. Слияние целевого гена с последовательностью калретикулинового сигнального пептида из *N. plumbaginifolia* для улучшения транспорта продукта в апопласт и проведения транзиентной экспрессии в оптимальных условиях позволило добиться максимальной активности интерферона в листьях $3.2 \cdot 10^3$ МЕ/г сырой массы при среднем значении $2.1 \pm 0.8 \cdot 10^3$ МЕ/г. Полученные результаты свидетельствуют о возможности использования *N. excelsior* для *Agrobacterium*-опосредованной транзиентной экспрессии фармацевтически активных белков человека. Показано, что условия транзиентной экспрессии, оптимизированные для получения репортерного белка GFP, подходят также для экспрессии гена интерферона α2b человека.

Я.Р. Сіндаровська, І.М. Герасименко, Ю.В. Шелудько, З. М. Олевинська, М.Я. Співак, М.В. Кучук

ОТРИМАННЯ ІНТЕРФЕРОНУ АЛЬФА-2b ЛЮДИНИ МЕТОДОМ *AGROBACTERIUM*-ОПОСЕРЕДКОВАНОЇ ТРАНЗІЄНТНОЇ ЕКСПРЕСІЇ В *NICOTIANA EXCELSIOR*

Ген інтерферону α2b було транзієнтно експресовано у рослинах *Nicotiana excelsior*. Злиття цільового гена з послідовністю калретикулинового сигнального пептиду з *N. plumbaginifolia* для поліпшення транспорту продукту в апопласт і проведення транзієнтної експресії в оптимальних умовах дозволило добитися максимальної активності інтерферону в листях $3.2 \cdot 10^3$ МО/г сирої маси при середньому значенні $2.1 \pm 0.8 \cdot 10^3$ МО/г с.в. Отримані результати свідчать про можливість використання *N. excelsior* для *Agrobacterium*-опосередкованої транзієнтної експресії фармацевтично активних білків людини. Було показано, що умови транзієнтної експресії, оптимізовані для отримання репортерного білка GFP, підходять також для експресії гена інтерферону α2b людини.

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