УДК 57.085.2 + 582.926.2 + 577.21

Y.R. SINDAROVSKA, I.M. GERASYMENKO,
Y.V. SHELUDKO, I.K. KOMARNYTSKYY,
M.A. BANNIKOVA, N.V. KUCHUK

Institute of Cell Biology and Genetic Engineering,
Zabolotnogo str. 148, Kyiv 03143, Ukraine
E-mail : sheludko@iicb.kiev.ua; ysheludko@fromru.com

TRANSGENIC PLANTS REGENERATED FROM HAIRY ROOTS OF *NICOTIANA BENTHAMIANA*: A PROMISING HOST FOR TRANSIENT EXPRESSION OF FOREIGN PROTEINS

Hairy root cultures of Nicotiana benthamiana have been obtained by co-cultivation of leaf explants with Agrobacterium rhizogenes strain A4 harboring a binary vector plasmid, and transgenic nature of the obtained cultures was confirmed by PCR analysis. Transgenic plants were regenerated from hairy roots. The biomass yield of transgenic plants grown in vitro was almost two-fold higher than those of wild-type N. benthamiana plants. They differed from untransformed plants by short internodes, reinforced stem, thick and wrinkled leaves and more developed root system. The level of Agrobacterium-mediated transient expression of green fluorescent protein (GFP) in the regenerated plants was similar to that of untransformed plants.

Introduction. Transient expression of foreign genes in plants attracted considerable attention during recent decade. In addition to other potential applications, this approach may be regarded as a promising method for high-scale inexpensive production of recombinant proteins. In contrast to bacterial expression systems, plant cells are able to synthesize proteins with proper post-translation modifications, as well as correctly folded and assembled multimeric proteins [1]. Plant-made biopharmaceuticals are free of bacterial toxins and human pathogens like viruses and prions. From the economical point of view, production of raw plant material is significantly less expensive compared with the animal or even bacterial cells grown in bioreactors [2]. The main obstacle on the way of using transgenic plants as a source of recombinant proteins is the low level of foreign gene expression in case of stable integration into plant nuclear genome [3], often due to the transcriptional or post-transcriptional gene silencing of the nuclear transgenes [4, 5]. Although plastid transformation allows to achieve considerably higher level of recombinant protein accumulation and to avoid gene silencing [6], it usually takes many months or even years to obtain the plants with transgenic plastids. The factor of time may be important for production of some recombinant proteins, e.g. patient-specific cancer vaccines, which should be generated during several weeks. Transient expression of foreign genes allows production of large amount of recombinant proteins within a very short (days) time. The reporter protein may be detected on the 2nd day after infiltration with *Agrobacterium* carrying the corresponding gene [7]. *Agrobacterium*-mediated transient gene expression system was successfully applied for production of tumor-specific antibodies in tobacco leaves within a week [8]. The level of recombinant protein accumulation in case of transient expression of foreign genes sometimes is comparable with that reported for chloroplast transformants [9, 10].

Transient expression is often carried out in *Nicotiana benthamiana* [9, 11, 12]. This plant is characterized by high level of transient expression (up to 5 mg of reporter protein/g FW [10]), but has a rather small biomass yield which hinders its application for large-scale production of recombinant proteins. It has been reported that genetic transformation with *Agrobacterium rhizogenes* followed by spontaneous or phytohormone-induced regeneration results in obtaining of plants with an

altered phenotype which may sometimes have increased leaf number [13] and/or higher biomass yield [14]. In order to study possibilities to improve biotechnological characteristics of *N. benthamiana* (biomass yield, level of transient expression) we have transformed it with *A. rhizogenes* and regenerated plants from the transgenic hairy root cultures. As far as we know this is the first report on production of hairy roots and plant regeneration in *N. benthamiana*. We found that *in vitro* grown transgenic regenerated plants produce approximately twofold higher biomass compared to intact *N. benthamiana* plants while demonstrating similar level of reporter gene transient expression, thus making them a promising host for high-scale production of recombinant proteins.

Materials and methods. *Plant materials.* Seeds of *N. benthamiana* were obtained from the National Germplasm Bank of World Flora of the Institute of Cell Biology and Genetic Engineering (Kyiv, Ukraine). Seeds were sterilized with 70 % (v/v) ethanol during 45 sec, then commercial solution of hypochlorite (1 : 1 water dilution) during 2 min and, after washing with sterile distilled water, germinated on solid hormone-free MS medium [15] at 25 °C and 16 h light period (3000 lux). Aseptic *N. benthamiana* plants were maintained on the above mentioned medium under the same conditions with the subcultivation period of 30—35 days. In greenhouse *N. benthamiana* plants were grown at 20—25 °C and 16 h light period (3000—4000 lux).

Bacterial strains and genetic constructs. A. rhizogenes strain A4, harboring a binary vector on the basis of pBI121 (Clontech, USA) with marker genes of neomycin phosphotransferase II (*nptII*) under nopalin synthase promoter and β-glucuronidase (*gusA*) under 35S CaMV promoter [16], was used for genetic transformation. Bacterial suspension was grown in liquid LB medium [17] supplemented with 50 mg/L kanamycin and 100 μM acetosyringone at 25 °C during 36 h on rotary shaker (70 rpm). A fresh portion of acetosyringone was added to *A. rhizogenes* suspension 1—2 h before transformation so that the final concentration came to 200 μM.

A. tumefaciens strain GV3101 was used for transient expression of the reporter gene coding for green fluorescent protein (GFP). The plasmid pIC5290 carried the reporter synthetic GFP gene [18] driven by the CaMV 35S promoter. The plasmid pIC6692 contained the gene of p19 protein of tomato bushy stunt virus, a suppressor of post-transcriptional gene silencing [9]. Bacteria harboring either pIC5290 or pIC6692 were grown in LB medium supplemented with 50 mg/L rifampicin, 50 mg/L carbenicilin and 100 μM acetosyringone at 25 °C during 36 h on rotary shaker (70 rpm) to an optical density (OD 600) of around 1.0. The plasmids pIC5290 and pIC6692 were generously donated by Icon Genetics GmbH (Halle/Saale, Germany).

Obtaining of hairy root cultures. Leaf explants (appr. 1 cm × 1 cm) of aseptic *N. benthamiana* plants were placed into the *A. rhizogenes* suspension for 10—20 min, transferred onto solid MS medium and incubated during 24 h at 25 °C. The explants were rinsed with sterile water and cultivated on solid MS medium without phytohormones supplemented with carbenicillin (300 mg/L) and cefotaxime (400 mg/L) during 2—4 passages of 14 days. After *Agrobacterium* elimination the emerged roots were transferred onto solid MS medium without phytohormones supplemented with 50 mg/L kanamycin. Selected hairy root cultures were maintained on solid MS medium without phytohormones and antibiotics at 25 °C in the dark with subcultivation period of 21—30 days.

Regeneration of transgenic plants. Transgenic roots were transferred onto solid MS medium supplemented with 50 mg/L kanamycin without phytohormones or containing benzylaminopurine (BAP, 1 mg/L) and either indoleacetic acid (IAA, 0.1 mg/L) or naphthaleneacetic acid (NAA, 0.1 mg/L) or IAA (0.1 mg/L) and thidiazuron (TDZ, 1 mg/L). Regeneration was carried out at 25 °C and 16 h light period (3000 lux). Regenerated plants were maintained on solid MS medium without phytohormones supplemented with 50 mg/L kanamycin at 25 °C and 16 h light period (3000 lux) with subcultivation period of 30—40 days.

Polymerase chain reaction. DNA was isolated from plant material according to Cheung et al. [19]. PCR was carried out using Tercyc IMO2 thermocycler («DNA Technology», Russia). The reaction mixture contained either 0.2 ng of plasmid DNA as positive control or 20—40 ng of plant DNA (DNA from intact plants as negative control or DNA from putative transgenic root cultures or plants), 0.25 μM of each primer, 1x reaction buffer

(10 mM Tris/HCl, pH 9.0, 50 mM KCl, 0.1 % Triton X-100), 2 mM $MgCl_2$, 800 μM deoxyribonucleoside 5′-triphosphate mixture (200 μM each) and 1 U of *Taq* DNA polymerase. Amplification of 646 bp fragment of *nptII* gene was carried out using primer pair *nptII*-1 (5′-GAGGCTATTCGGCTATGACT-3′) and *nptII*-2 (5′-CAAGCTCTTCAGCAATATCACG-3′) under the following conditions: 95 °C 4 min 30 sec, 56 °C 1 min, 72 °C 45 sec → 94 °C 1min, 56 °C 1 min, 72 °C 45 sec (35 cycles) → 72 °C 8 min. Fragment of *rolB* gene 780 bp in length was amplified with primer pair *rolB*-1 (5′-ATGGATCCCAAATTGCTATTCCTTCCACGA-3′) and *rolB*-2 (5′-TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3′) under following conditions: 95 °C 4 min 30 sec, 65 °C 1 min, 72 °C 45 sec → 94 °C 1min, 65 °C 1 min, 72 °C 45 sec (35 cycles) → 72 °C 8 min. Amplification products were analyzed by electrophoresis in 1 % agarose gel.

Transient expression assay. Plant infiltration was performed as it was described by Marillonnet [10] with minor modifications: *A. tumefaciens* suspension was centrifuged and resuspended in the equal volume of 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 equal volumes before infiltration. For typical assay the leaves of greenhouse grown *N. benthamiana* plants were infiltrated with *Agrobacterium* mixture (50 mkl/leaf) by using a syringe without a needle. The plant material was harvested on the 4th day after inoculation. Accumulation of GFP was monitored with a hand-held black ray lamp (UVP, Upland, USA) and the infiltrated area was cut out. The leaf tissue was extracted with 50 mM phosphate buffer (pH 7.0). The supernatant after centrifugation was used for protein analysis. The content of GFP was calculated by measurements of fluorescence intensity in leaf extracts using fluorescence spectrophotometer Hitachi 850 (Hitachi, Tokyo, Japan) (excitation at 395 nm, emission at 509 nm) on the basis of standard values (GFP standard was generously granted by Icon Genetics GmbH (Halle/Saale, Germany). The background fluorescence of control extracts (from leaves infiltrated with bacteria carrying pIC6692 only) was subtracted from values of GFP containing extracts. The identity of GFP in the extracts to the standard was proved by

Fig. 1. Hairy root culture of *N. benthamiana* (S1 strain)

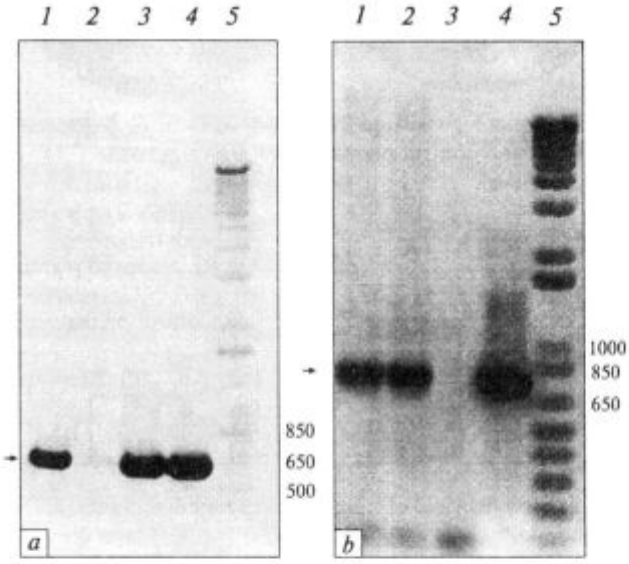

Fig. 2. Results of PCR amplification of DNA isolated from *N. benthamiana* hairy roots: *a*) *1* — plasmid on the basis of pBI121 amplificated with primer pair *nptII* (positive control); *2* — DNA of untransformed *N. benthamiana* roots amplificated with primer pair *nptII* (negative control); *3*—*4* — DNA of hairy roots amplificated with primer pair *nptII* (S1 and S5 strains, respectively); *5* — DNA size marker; *b*) *1*—*2* — DNA of hairy roots amplificated with primer pair *rolB* (S1 and S5 strains, respectively); *3* — DNA of untransformed *N. benthamiana* roots amplificated with primer pair *rolB* (negative control), *4* — Ri plasmid of *A. rhizogenes* amplificated with primer pair *rolB* (positive control); *5* — DNA size marker. The amplificated 646 bp and 780 bp DNA fragments are indicated by arrow

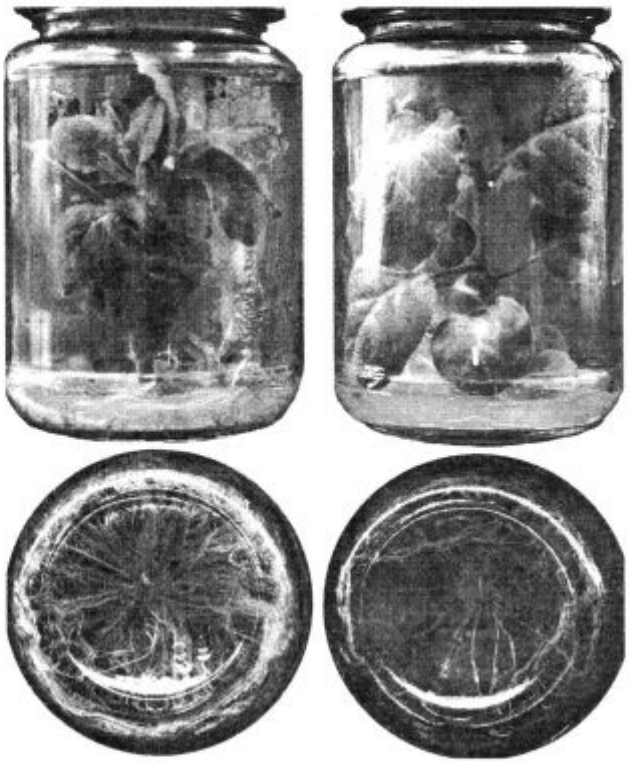

Fig. 3. Plants regenerated from hairy roots of *N. benthamiana* (left) and control plants (right) grown *in vitro*

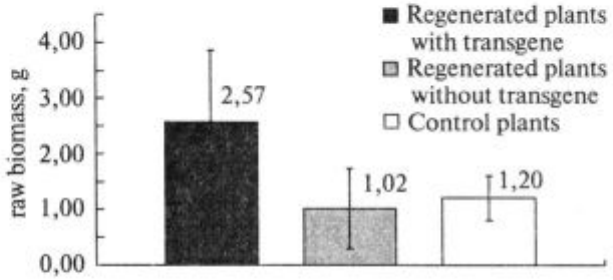

Fig. 4. Biomass yield of transgenic regenerated plants (left), regenerated plants without transgene (center), and control plants of *N. benthamiana* (right) grown *in vitro*. Bars represent confident intervals

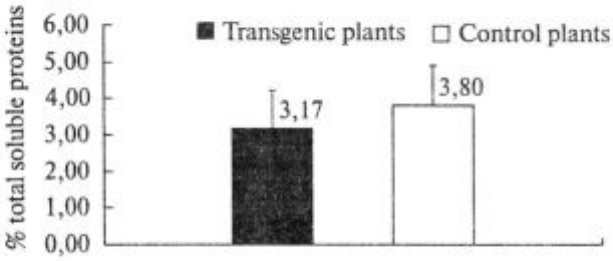

Fig. 5. GFP transient expression level in transgenic regenerated plants (left) and control plants of *N. benthamiana* (right). Bars represent confident intervals

recording their fluorescence spectra. The concentration of total soluble protein was determined by the method of Bradford [20].

Results and discussion. *Induction of N. benthamiana hairy root cultures.* Roots appeared on *N. benthamiana* leaf explants on the 10th—12th day after co-cultivation with *A. rhizogenes*. In total, 44 emerging roots were detected on 12 explants. After 7—8 days the roots were detached and transferred onto hormone-free MS medium containing kanamycin. Ability to grow on media without phytohormones is a characteristic feature of transgenic hairy roots [21], while the resistance to kanamycin is conferred by *nptII* gene present in the T-DNA of the binary vector. Two kanamycin-resistant root cultures with the best growth characteristics were chosen for further analysis. The selected root cultures grew vigorously and showed the typical hairy root phenotype: profuse branching, high density of root hairs and plagiotropism (Fig. 1), that suggests the transfer and expression of the genes of Ri-plasmid T-DNA [21, 22]. PCR amplification of *rolB* (gene, located in TL-DNA region of Ri-plasmid and playing an important role in hairy root induction [22]) and *nptII* gene fragments confirmed the transfer and integration of both T-DNA of Ri-plasmid as well as T-DNA of binary vector in the genome of the obtained hairy root cultures (Fig. 2).

Regeneration of transgenic N. benthamiana plants. Obtaining of plants from transgenic hairy roots has been reported for numerous plant species. Regeneration may occur spontaneously or be induced by phytohormones. Transgenic plants usually have a characteristic Ri-phenotype with wrinkled leaves, shortened internodes, branched stems and developed root system [23]. Transgenic roots of *N. benthamiana* were cultivated under the light on MS media without phytohormones or with several phytohormone combinations. The best results were obtained with MS medium containing 1 mg/L BAP and 0.1 mg/L IAA. Regeneration was detected after 40—45 days; 20—30 days later regenerated plantlets were detached and transferred onto phytohormone-free medium. Plants regenerated from transgenic roots grew on medium supplemented with 50 mg/L kanamycin and differed from intact *N. benthamiana* plants by short internodes, reinforced stem, thick and wrinkled leaves and well-developed root system (Fig. 3). After 3 subcultures several regenerated plants flowered *in vitro* and

formed viable seeds. For comparison of biomass yield, the seeds of intact *N. benthamiana* and seeds of the plants regenerated from transgenic roots were germinated *in vitro*. Progeny of the regenerated plants was examined by PCR amplification of *rolB* gene fragment. It was shown that 8 R1 plants carried the *rolB* gene, while the other 10 ones did not. The biomass yield of plants bearing the *rolB* transgene grown *in vitro* surpassed about twofold the corresponding value of intact *N. benthamiana* plants as well as regenerated plants without the *rolB* gene (Fig. 4). The difference is significant ($P = 0.95$). It is noteworthy, that the biomass yield of the regenerated offspring without the *rolB* gene did not differ significantly from the value of control plants.

Transient expression of GFP in transgenic N. benthamiana plants. Seeds of the plants regenerated from transgenic roots were germinated *in vitro* and after 1—2 weeks the plants were successfully established in the greenhouse. After acclimatization, the greenhouse grown R1 plants carrying the *rolB* transgene (as confirmed by PCR) were used for transient expression of the GFP gene under control of the 35S CaMV promoter. The p19 protein of tomato bushy stunt virus, a suppressor of post-translational gene silencing, was co-expressed with the GFP gene to increase the reporter protein accumulation [9]. The content of GFP in the leaf tissue of the transgenic plants amounted to 3.17 ± 1.06 % of the total soluble protein, that does not differ significantly from the level of the greenhouse grown intact *N. benthamiana* plants (Fig. 5).

Based on the obtained results, we can outline two major strategies to enhance production of transiently expressed recombinant proteins in plants: modification of the expression system, which may include co-expression of viral suppressors of post-transcriptional gene silencing [9] and/or construction of viral-based vectors that could be delivered by *Agrobacterium* infiltration and allow to achieve the extremely high level of recombinant protein accumulation (up to 80 % TSP [10]). On the other hand, the optimization of transient expression conditions and plant host characteristics allows further improving the effectiveness of a given genetic construct [24]). The described here approach allowed obtaining transgenic *N. benthamiana* plants with considerably higher biomass while maintaining the level of reporter protein accumulation similar to that of wild-type plants. This material represents a promising host for the large-scale production of recombinant proteins by means of transient expression. Additional selection and/or screening of hairy root clones may further increase the level of transiently expressed proteins in regenerated plants.

The authors are grateful to the Ministry for Education and Science of Ukraine (grant DNTP 3.2. DP/148-2003) for financial support.

РЕЗЮМЕ. Кореневі культури «hairy roots» *Nicotiana benthamiana* було отримано кокультивуванням із штамом *Agrobacterium rhizogenes* A4, який містив бінарну векторну систему, і трансгенну природу отриманих ліній коренів було доведено методом ПЛР. З трансгенних коренів було регенеровано рослини. Біомаса регенерованих рослин, які культивували *in vitro*, перевершувала біомасу нетрансформованих рослин *N. benthamiana* майже вдвічі. Вони відрізнялися від інтактних рослин короткими міжвузлями, потовщеним стеблом, товстими та скривленими листями та більш розвиненою кореневою системою. Рівень *Agrobacterium*-опосередкованої транзієнтної експресії репортерного білка GFP в регенерованих трансгенних рослинах був подібним до такого у нетрансформованих рослин.

РЕЗЮМЕ. Культуры корней «hairy roots» *Nicotiana benthamiana* были получены кокультивированием листовых эксплантов со штаммом *Agrobacterium rhizogenes* A4, содержащим бинарную векторную систему, и трансгенная природа полученных линий корней была доказана методом ПЦР. Из трансгенных корней были регенерированы растения. Биомасса регенерированных растений, культивировавшихся in vitro, превышала биомассу нетрансформированных растений *N. benthamiana* приблизительно в два раза. Они отличались от интактных растений укороченными междоузлиями, утолщенным стеблем, толстыми искривленными листьями и более развитой корневой системой. Уровень *Agrobacterium*-опосредованной транзиентной экспрессии репортерного белка GFP в регенерированных растениях не отличался от такового у нетрансформированных растений.

REFERENCES

1. *Stoger E., Sack M., Fischer R., Christou P.* Plantibodies: applications, advantages and bottlenecks // Curr. Opin. Biotechnol. — 2002. — **13**. — P. 161—166.
2. *Larrick J.W., Thomas D.W.* Producing proteins in transgenic plants and animals // Curr. Opin. Biotechnol. — 2001. — **12**. — P. 411—418.
3. *Daniell H., Streatfield S.J., Wycoff K.* Medical molecu-

lar farming: production of antibodies, biopharmaceuticals and edible vaccines in plants // Trends Plant Sci. — 2001. — **6**. — P. 219—226.
4. *Fagard M., Vaucheret H.* (Trans)gene silencing in plants: how many mechanisms? // Annu. Rev. Plant Physiol. Plant Mol. Biol. — 2000. — **51**. — P. 167—194.
5. *Yu H., Kumar P.P.* Post-transcriptional gene silencing in plants by RNA // Plant Cell Rep. — 2003. — **22**. — P. 167—174.
6. *Heifetz P.B., Tuttle A.M.* Protein expression in plastids // Curr. Opin. Plant Biol. — 2001. — **4**. — P. 157—161.
7. *Kapila J., Rycke R. De, Montagu M. Van, Angenon G.* An *Agrobacterium*-mediated transient gene expression system for intact leaves // Plant Sci. — 1997. — **122**. — P. 101—108.
8. *Vaquero C., Sack M., Chandler J., Drossard J., Schuster F., Monecke M., Schillberg S., Fischer R.* Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves // Proc. Nat. Acad. Sci. USA. — 1999. — **96**. — P. 11128—11133.
9. *Voinnet O., Rivas S., Mestre P., Baulcombe D.* An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus // Plant J. — 2003. — **33**. — P. 949—956.
10. *Marillonnet S., Giritch A., Gils M., Kanzia R., Klimyuk V., Gleba Y. In planta* engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium* // Proc. Nat. Acad. Sci. USA. — 2004. — **101**. — P. 6852—6857.
11. *McCormick A.A., Kumagai M.H., Hanley K., Turpen T.H., Hakim T., Grill L.K., Tuse D., Levy S., Levy R.* Rapid production of specific vaccines for lymphoma by expression of the tumor-derived single-chain Fv epitopes in tobacco plants // Proc. Nat. Acad. Sci. USA. — 1999. — **96**. — P. 703—708.
12. *Thomas C.L., Leh V., Lederer C., Maule A.J.* Turnip crinkle virus coat protein mediates suppression of RNA silencing in *Nicotiana benthamiana* // Virology. — 2003. — **306**. — P. 33—41.
13. *Tanaka N., Matsumoto T.* Regenerants from Ajuga hairy roots with high productivity of 20-hydroxyecdysone // Plant Cell Rep. — 1993. — **13**. — P. 87—90.
14. *Benjamin B.D., Roja G., Heble M.R. Agrobacterium rhizogenes* mediated transformation of *Rauwolfia serpentina*: Regeneration and alkaloid synthesis // Plant Cell Tissue and Organ Culture. — 1993. — **35**. — P. 253—257.
15. *Murashige T., Skoog F.* A revised medium for rapid growth and bioassays with tobacco tissue cultures // Physiol. Plant. — 1962. — **15**. — P. 473—497.
16. *Jefferson R.A., Kavanagh T.A., Bevan M.W.* GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants // EMBO J. — 1987. — **6**. — P. 3901—3907.
17. *Sambrook J., Fritsch E.F., Maniatis T.* Molecular cloning: a laboratory manual, 2nd ed. — New York : Cold Spring Harbor Laboratory Press, 1989.
18. *Chiu W., Niwa Y., Zeng W., Hirano T., Kobayashi H., Sheen J.* Engineered GFP as a vital reporter in plants // Curr. Biol. — 1996. — **6**. — P. 325—330.
19. *Cheung W.Y., Hubert N., Landry B.S.* A simple and rapid DNA microextraction method for plant animal and insect suitable for RAPD and other PCR analyses // PCR Meths. Applies. — 1993. — **3**. — P. 69—70.
20. *Bradford M.M.* A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding // Anal. Biochem. — 1976. — **72**. — P. 248—254.
21. *Sevon N., Oksman-Caldentey K.M. Agrobacterium rhizogenes*-mediated transformation: root cultures as a source of alkaloids // Planta Med. — 2002. — **68**. — P. 859—868.
22. *Nilsson O., Olsson O.* Getting to the root: the role of the *Agrobacterium rhizogenes* rol genes in the formation of hairy roots // Physiol. Plant. — 1997. — **100**. — P. 463—473.
23. *Tepfer D.* Genetic transformation using *Agrobacterium rhizogenes* // Physiol. Plant. — 1990. — **79**. — P. 140—146.
24. *Sindarovska Y., Sheludko Y., Gerasymenko I., Kuchuk N.* Optimization of *Agrobacterium* mediated transient expression in *Nicotiana species* // Acta Physiol. Plant. — 2004. — **26**(S). — P. 150.

Received 24.06.05