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Morphological and Molecular Analysis of Isolated Cultures of Tobacco Adventitious Roots Obtained by the Methods of Biolistic Bombardment and *Agrobacterium*-Mediated Transformation

G. R. Gumerova[a], *, A. V. Chemeris[a], Yu. M. Nikonorov[a], and B. R. Kuluev[a]

[a] *Institute of Biochemistry and Genetics, Subdivision of the Ufa Federal Research Centre of the Russian Academy of Sciences, Ufa, 450054 Russia*

**e-mail: gulnar.yas@mail.ru*

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Abstract—Plant infection with *Agrobacterium rhizogenes* leads to the development of a hairy root disease notable for the rapid agravitropic growth of roots on hormone-free nutrient media. In order to look into the interaction of *A. rhizogenes* with plants and assess opportunities of practical application of hairy root culture, new approaches to their production are elaborated. A method of bacterium-free and plasmid-free production of genetically modified roots (hairy roots) by means of biolistic transformation of leaf explants with a DNA fragment (size of 5461 bp) consisting of genes *rolA*, *rolB*, *rolC*, and *rolD* are proposed. In most cases, such transformation resulted in the emergence of only adventitious roots with transient expression of *rol*-genes, and the growth of such roots on hormone-free media ceased in 2–3 months in contrast to genuine hairy roots capable of unrestricted growth. Molecular analysis of different systems of target genes' expression showed an important role of transgene *rolC* and host gene of cyclin-dependent protein kinase *CDKB1-1* in the maintenance of rapid growth of hairy roots in vitro (in isolated cultures).

Keywords: Nicotiana tabacum, *rolB*, *rolC*, adventitious roots, auxins, biolistic transformation, cell division, hairy roots, cell growth and elongation, cyclin-dependent protein kinases, expansins

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INTRODUCTION

Upon infection of dicotyledons, gram-negative soil bacterium *Agrobacterium rhizogenes* causes a hairy root disease resulting in the development of pRi-transformed roots or so-called hairy roots that can be cultured in vitro [1, 2]. This transformation of plant tissues is possible owing to the presence in the bacteria of a large Ri-plasmid and their ability to transfer and integrate a fragment of this plasmid called T-DNA into the plant genome [3]. T-DNA of Ri-plasmid contains 18 open reading frames (ORF), and the development of hairy root disease entirely depends on the presence of four *rol*-genes (from "**ro**ot **l**ocus"): *rolA* (ORF10), *rolB* (ORF11), *rolC* (ORF12), and *rolD* (ORF13) [4]. Expression of these genes impairs normal operation of a plant organism affecting hormonal balance and modifying the level of expression of numerous genes involved in regulation of growth and development. This leads to the growth of hairy roots that consist of differentiated root cells. Literature reports suggest that the most important role in rhizogenesis in plants infected with agrobacteria belongs to a product of gene *rolB*, since transformation with this gene alone brings about the formation of hairy roots [5]. There are indications that gene *rolB* encodes a protein with tyrosine phosphatase [6] and β-glycosidase activity [7]. It is assumed that the major function of the rolB protein is to release indoleacetic acid (IAA) from its conjugates, which results in activation of auxins in the cell [7]. The product of gene *rolC* also shows β-glycolytic activity; however, its constitutive expression brings about a rise in free cytokinins [4, 7]. Thus, activity of rolB and rolC proteins is realized for the most part via signal systems of auxins and cytokinins, but it remains unknown which genes and products are responsible for induction of hairy roots.

Auxins and cytokinins are the most important regulators of plant growth and development; their interaction with various promoter elements is shown in a beneficial influence on the processes of cell division and elongation in the shoots and underground parts of plants. Auxins are known to induce cell divisions in callus culture in the presence of cytokinins and activate division of cambium cells [8]. It is assumed that the 5'-untranslated region of different nuclear genes participating in the cell cycle contains auxin-responsive elements (AuxREs) [9]. For instance, accumulation of auxin in the cells of pericycle governs the development of lateral roots via induction of cell divisions [10]. Auxin regulation of the mitotic cycle first of all depends on

protein complexes consisting of cyclin-dependent protein kinase (CDK) and cyclin (CYC). For example, upon root treatment with exogenous auxin and auxin-activated formation of lateral roots, the levels of expression of genes *CYCA2;4*, *CYCB1;1*, *CYCD3;1*, *CYCD6;1*, *CDKB2;1*, and *CDKB2;2* rise [10]. Cytokinins also play an important role in regulation of cell divisions since they stimulate DNA replication and regulate passage from phase G_1 to phase S and from phase G_2 to mitosis. The same as auxins, cytokinins display their activity via the influence on the synthesis and activity of cyclins and cyclin-dependent protein kinases [10].

Auxins and cytokinins are major regulators of growth by elongation. Cell elongation is regulated by auxins and depends on the dose of phytohormone: high concentrations inhibit growth by elongation in suspension culture of tobacco [11, 12]. It was shown that exogenous 10^{-6} M auxin stimulates cell elongation in the stem and in almost all the shoot tissues, whereas a reduction in the phytohormone level below 10^{-9} M inhibits growth by elongation in the roots of *Arabidopsis thaliana* [12]. The experiments on *A. thaliana* also showed a sharp acceleration of growth by elongation in the stems and hypocotyl within a short interval after auxin treatment [13], which is accounted for by the activation of expansins and subsequent loosening of the cell wall. As to cytokinins, these phytohormones participate in the regulation of expression of the genes of expansins and xyloglucan endotransglycosylases [14] that, in turn, ensure the growth by elongation. Among currently examined genes of tobacco expansins, a relatively high level of expression in the roots was observed for the gene *NtEXPA5* [15]. Thus, one can assume that elevated expression of the genes *rolB* and *rolC* in the roots may improve expression of the genes encoding both cyclin-dependent protein kinases and expansins; however, such information in the literature is not available.

Cultures of hairy roots are more and more widely used in today's biotechnology as producers of primary metabolites (for instance, of recombinant proteins) and root-specific secondary plant metabolites. Thus, new methods of transformation of different plant organs and tissues mediated by *A. rhizogenes* are currently elaborated with the purpose of hairy roots' production [16]. Besides, absolutely different approaches were proposed for production of hairy roots using individual *rol*-genes or their combinations placed in binary vectors that subsequently transform the strains of *A. tumefaciens* used for inoculation of plant material [17, 18]. One of the drawbacks of the abovementioned techniques is a tedious and time-consuming process of elimination from agrobacteria and dependence of the efficiency of transformation on bacterial strain and the species of an infected plant. Proceeding from these considerations, we worked out a method of bacterium-free and plasmid-free transformation of tobacco leaf discs via bombardment with gold particles carrying an amplicon of 5461 bp merely composed of genes *rolA*, *rolB*, *rolC*, and *rolD* [19]. As a result, we obtained 20 lines of adventitious roots able to grow on hormone-free nutrient media; however, only two of them showed a reliable integration of *rol*-genes into the genome. Since some lines of adventitious roots could grow on hormone-free nutrient media for a rather long time even without *rol*-genes in their DNA, their comparison with normal roots and pRi-transformed roots in isolated cultures was of profound interest.

The aim of this work was to make morphological and molecular analysis of earlier produced and newly obtained (by the method of bombardment of cotyledon leaves) lines of tobacco adventitious roots as compared with control material (wild type) and genetically transformed and auxin-induced adventitious roots as well as to look into correlations between the levels of expression of *A. rhizogenes* genes *rolB* and *rolC* and tobacco genes of cyclin-dependent protein kinase *NtCDKB1-1* and expansin *NtEXPA5* in all the types of roots.

MATERIALS AND METHODS

Test subjects. All the experiments were performed with in vitro cultures of adventitious (obtained after bombardment or induced by exogenous auxin), pRi-transformed (hairy roots), and growing in isolated culture (wild type) control roots of tobacco *Nicotiana tabacum* L., cv. Petit Havana SR1. Explants for production of the first two types of roots were taken from cotyledon and first true leaves, since it is known that particularly these organs of tobacco plant are the most suitable for *Agrobacterium*-mediated transformation with subsequent efficient organogenesis. Tobacco hairy roots were induced with *A. rhizogenes,* strain A4. Control roots of wild type seedlings were obtained after germination of tobacco seeds surface-sterilized using standard agents (70% ethanol for 1 min, 3% sodium hypochlorite supplemented with 0.1% Tween 20 for 10 min, and fivefold rinse with sterile distilled water). The seedlings were grown in Petri dishes on hormone-free Murashige-Skoog medium (MS). Two-day-old seedlings were accommodated in polycarbonate Magenta pots allowing free root growth.

Production of auxin-induced adventitious roots of tobacco. The experiments were conducted on the leaves of a robust 2-month-old tobacco plant grown under standard watering, temperature, and illumination (5 klx, 16/8 h, day/night) conditions. The leaves were rinsed in 70% ethanol, then surface-sterilized in 1.5% sodium hypochlorite supplemented with Tween 20 (0.1%) with subsequent fivefold rinse in sterile distilled water. We then transferred the leaves to sterile Petri dishes, removed white edges, and cut into explants (approximately 1 cm^2 in area) with a sterile scalpel. In order to induce adventitious roots, sterile leaf explants were cultured on MS medium with 2 mg/L IAA. In two weeks after the emergence, adventitious roots were

excised from the leaf explants and transferred to hormone-free MS medium. In early stages of development, auxin-induced roots were phenotypically similar to hairy roots, but they lost the ability to rapidly grow on hormone-free media in 2–3 weeks of culturing after separation from the leaf explants.

Production of tobacco hairy roots. For production of hairy roots, we used leaf explants of 6-week-old *N. tabacum* that were disinfected as described above. Hairy roots were induced using a wild type strain A4 of *A. rhizogenes* according to a standard method [1]. After two days of cocultivation, agrobacteria were washed off and the explants were placed on MS medium supplemented with augmentin and cefotaxime (by 250 mg/L).

Production of adventitious roots of tobacco by the method of biolistic bombardment. Total DNA from *A. rhizogenes,* strain A4, was isolated using a DNA-Sorb kit (InterLabService, Russia). A fragment of agrobacterial DNA (size of 5401 bp) containing genes *rolA*, *rolB*, *rolC*, and *rolD* was amplified from plasmid DNA of *A. rhizogenes* by means of LR Plus polymerase (Sileks, Russia). In order to add right- and left-border sequences of *A. rhizogenes* T-DNA to the amplicon, we performed reamplification using modified forward and reverse primers. Biolistic transformation was conducted using a Biolistic PDS-1000/He particle delivery system (Bio-Rad, United States) with Bio-Rad microscopic gold particles (average size of 0.6 μm) as microcarrier. DNA was precipitated using a calcium/spermidine method. In order to improve survival of the transformed tissue, we employed osmotic pretreatment (culturing of cotyledon and first true leaves on the medium with 90 g/L sucrose). In the course of biolistic process, the distance between the source of particles and target tissue was 6 cm. After the bombardment, leaf explants were placed on agar hormone-free MS medium. All the root cultures produced as described above were maintained on a liquid (for fresh weight determination) or solid (for growth rate assessment) MS medium, pH 5.7, at a temperature of 25°C in the light.

Fresh weight determination and growth rate assessment. Wild type roots of 2-week-old seedlings were separated from the shoots and placed on hormone-free solid (for root length measurement) and liquid (for fresh weight determination) MS medium. Auxin-induced roots were also detached from the leaf explants. For the determination of growth parameters, we selected the roots of approximately the same length (10 mm). For the assessment of root growth in dynamics, the measurements were taken every three days during one month (10 measurements during 27 days). Root inocula (50 mg) were cultured on a shaker at 80 rpm in 100-mL flasks with 2 mL of liquid MS medium. One cycle of culturing in liquid medium lasted three days (the medium was renewed after each fresh weight determination) and that on solid medium lasted one month. Root cultures were weighed on an OHAUS Adventurer precision balance (China) in increments of 0.0001 g every three days during one month. Before weighing, root cultures were blotted on sterile filter paper. All the manipulations required sterility. For all the measurements (root length and fresh weight), we found the means for six root lines (48 lines altogether).

PCR analysis. Out of various methods we used for isolation of DNA, the most appropriate for tobacco roots turned out to be the cetyltrimethylammonium bromide (CTAB) method [20]. Polymerase chain reaction (PCR analysis) for detection of genes *rolB* and *rolC* was carried out using primers that were also used for real-time reverse transcription PCR (RT-PCR) (Table 1). As a positive control, we took hairy roots of tobacco produced using strain A4 of *A. rhizogenes*. In order to rule out bacterial contamination, we made PCR analysis for gene *virC*. For all the three genes, the amplification procedure was the following: preliminary denaturation at 95°C for 2 min, 30 cycles with denaturation at 95°C for 40 s, annealing at 54°C for 40 s and elongation at 72°C for 40 s, followed by final chain extension at 72°C for 2 min. Electrophoresis in 1.5% agarose gel showed that the size of products of genes *rolB* (266 bp), *rolC* (250 bp), and *virC* (730 bp) corresponded to the expected values.

Isolation of RNA. Our preliminary investigations showed that total RNA from tobacco roots isolated with standard TRIzol Reagent is unsuitable for subsequent construction of cDNA and RT-PCR analysis. Therefore, total RNA from tobacco roots of different origin was isolated using the CTAB method. For this purpose, we ground 100 mg of the roots in liquid nitrogen using a ceramic mortar, added 1.2 mL of extraction buffer (2% CTAB, 2% polyvinyl pyrrolidone K 40, 25 mM EDTA, 100 mM Tris-HCl (pH 8.0), 2 M NaCl, 0.5 g/L spermidine, and 3% β-mercaptoethanol) heated to 65°C, and incubated during 30 min with stirring every 5 min. Then the samples were centrifuged for 10 min at 12000 rpm. Supernatant was poured into different microcentrifuge test-tubes, mixed with an equivalent volume of the mixture chloroform: isoamyl alcohol (24 : 1) during 2 min, and centrifuged for 10 min at 12000 rpm and 4°C. After repeated purification step, aqueous phase was decanted into different microcentrifuge test-tubes, supplemented with 1/3 volume of 8 M LiCl, stirred by pipetting, and incubated overnight at 4°C. After centrifugation for 60 min at 12000 rpm and 4°C, the pellet was washed with cold 70% ethanol and dissolved in 50 μL of sterile distilled water. Isolated total RNA was treated with DNAase (SibEnzyme, Russia), and purified with standard kits for RNA isolation in accordance with manufacturer's protocols (CleanRNA Standard, Evrogen, Russia). Quality of isolated RNA was checked by means of agarose gel electrophoresis and its quantity determined using a SmartSpec Plus spectrophotometer (Bio-Rad).

Table 1. Primer sequences for PCR analysis

	Gene name	Primer sequences, 5'→ 3'		Size of product, bp
1	*EF1α*	F	GAATTGGTACTGTCCCTGTT	289
		R	TTGCCAATCTGTCCTGAAT	
2	*NtEXPA5*	F	TGGTGCAATCCCCCTCTC	245
		R	GACATTGTTTGCCATCCAGTATTA	
3	*NtCDKB1-1*	F	GGCTTTCACTGTCCCAATAA	297
		R	GAGGCCAAGTTCTGAGGTTC	
4	*rolB*	F	TTCAGATTTACTATAGCAGGC	266
		R	GCAAGTACCTTGTTCATTCA	
5	*rolC*	F	GATGATGCGATGCTTTTATG	250
		R	CAGAGACTTTCCCTTTGTTGA	
6	*virC*	F	ATCATTTGTAGCGA	730
		R	AGCTCAAACCTGCTTC	

Real time qRT-PCR. The first strand of cDNA was constructed by means of an oligo(dT) primer and M-MuLV reverse transcriptase (NEB, United States). mRNA (after conversion into cDNA) of the genes under investigation was determined by means of real time qRT-PCR in the presence of SYBR Green I intercalating dye using a Rotor-Gene™ 6000 thermal cycler (Corbett Research, Australia). The transcripts of the examined genes were quantified using the primers shown in Table 1. Amplification proceeded in 0.2-mL test-tubes (AXYGEN Inc., United States) in 25 μL of M-427 reaction mixture for real time PCR (Syntol, Russia).

Statistical treatment. Real time qRT-PCR was repeated three times with two replicates. Expression of the examined genes was calculated using the $2^{-\Delta\Delta C_T}$ method [21]. mRNA of elongation factor *EF-1α* (AF120093.1) was used as a standard and its expression was taken as 100% since this gene showed the steadiest level of mRNA under any changes in environment [22]. The results of measurements of the root weight and length are shown as the means ± confidence interval for the specified number of experiments.

RESULTS

Bacteria-Free and Plasmid-Free Production of Tobacco Adventitious Roots via Biolistic Transformation

A DNA fragment of *A. rhizogenes,* strain A4, size 5401 bp, containing genes *rolA*, *rolB*, *rolC,* and *rolD* was amplified by means of LR-Plus polymerase. During subsequent reamplification, we added to the amplicon sequences corresponding to right and left borders of *A. rhizogenes* T-DNA, which participate in recombination with plant genome and are necessary for improvement of this method. Electrophoresis showed that the size of the amplicon composed of *rol*-genes with T-borders corresponds to the expected value (5461 bp). Sequencing of this region corroborated its identity to the studied T-DNA region and the absence of nucleotide substitutions. We then performed biolistic transformation of cotyledon and the first true leaves of tobacco using the amplicon with T-borders (5461 bp). In 7–15 days after bombardment, some leaf explants showed the onset of rhizogenesis. In this work, we used 80 cotyledon and the first true leaves; out of them, six (7.5%) and 15 (18.75%) explants, respectively, showed the beginning of root development (Figs. 1a and 1b). In control dishes (30 cotyledon leaves) where bombardment was performed with gold particles lacking DNA, the roots did not develop; however, spontaneous rhizogenesis occurred in a stage of the first true leaves (PCR analysis for genes *rolB* and *rolC* showed negative results) with the probability 16% (four of 25 control explants).

In order to verify integration of *rol*-genes into adventitious roots, we made PCR analysis for the presence of gene *rolB,* since this gene is believed to play a key role in formation of hairy roots [3]. For PCR analysis, we selected 13 lines of putative hairy roots, since the remaining eight lines ceased growing during the first week. Electrophoresis showed that all 13 studied lines of adventitious roots lack the gene *rolB*. Moreover, we observed a strong suppression of growth and emergence of necrotic zones in these 13 lines of adventitious roots after one month of culturing. In this relation, one can suppose that active growth of adventitious roots induced by bombardment in early stages of development depended on a transient expres-

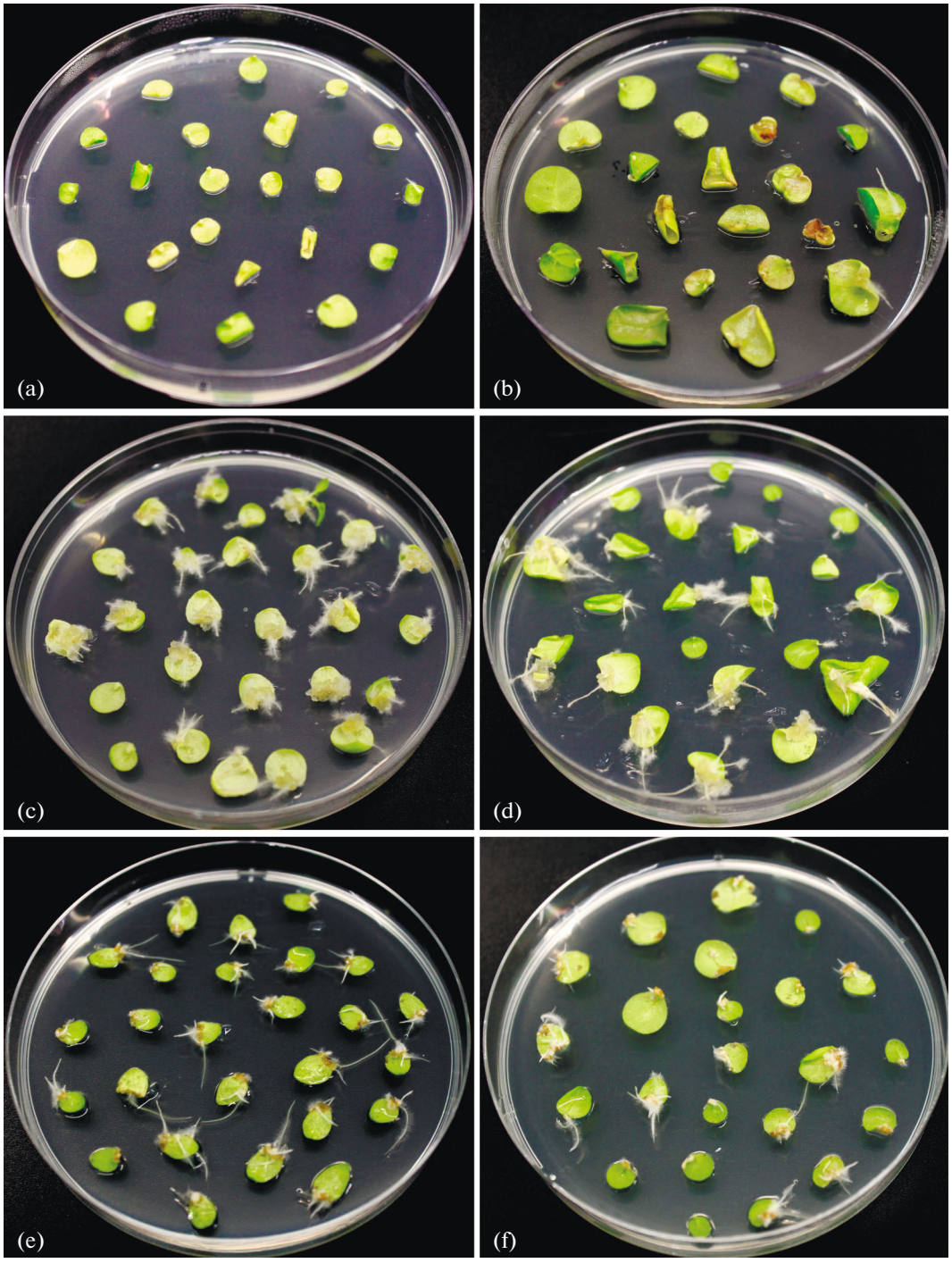

Fig. 1. Rhizogenesis on cotyledon (a, c, e) and the first true leaves (b, d, f) induced by a transient expression after biolistic transformation by an amplicon with four *rol*-genes (a, b—1 week after bombardment); by exogenous auxin (c, d—3 weeks after the beginning to IAA treatment); by *A. rhizogenes*-mediated genetic transformation (e, f—3 weeks after coculturing).

sion of *rol*-genes without reliable integration into the plant genome. This assumption is corroborated by the fact that, upon biolistic transformation with DNA-free gold particles, only one sample of 40 leaf explants (2.5%) showed spontaneous root development, which is probably a response to mechanical stress.

Production of Auxin-Induced Adventitious Roots and Genetically Transformed (Hairy) Roots of Tobacco

Auxins are known to induce formation of adventitious roots on the leaf explants [14, 23] owing to stimulation of cell divisions. This is accounted for by activation of expression of some cyclins and cyclin-depen-

dent protein kinases after auxin treatment long before the first cell division, which was shown in the experiments on *A. thaliana* cell cultures [24]. Guided by these reports, we obtained auxin-induced adventitious roots on tobacco leaf explants and intended to use them in subsequent molecular and morphological comparative analysis. In 7–10 days after culturing on MS medium with 2 mg/L IAA, individual leaf explants showed the onset of rhizogenesis and adventitious roots emerged on 65% of leaf explants in three weeks. The remaining explants did not form any adventitious roots and then became necrotic or produced numerous calli. Tobacco adventitious roots induced with exogenous auxin were also obtained on cotyledon and the first true leaves. On the sixth day of the experiment, individual explants showed the onset of rhizogenesis, and we counted leaf explants with the points of root development in three weeks. On 46 of 50 (92%) cotyledon leaves (Fig. 1c), we observed root formation; as to the first true leaves (Fig. 1d), the frequency of adventitious roots' emergence was 78% (39 of 50). In early stages of development, auxin-induced roots were phenotypically similar to hairy roots that are known to produce numerous lateral roots (owing to lack of apical dominance) and show rapid plagiotropic growth on hormone-free medium [17, 25–27]. Auxin-induced roots also displayed active formation of lateral roots and rapid growth, but their growth noticeably slowed down after the transfer to hormone-free medium and stopped in some cases as a result of tissue necrosis.

Tobacco hairy roots were produced using strain A4 on leaf explants, cotyledon and the first true leaves. Altogether, via inoculation with a suspension of *A. rhizogenes,* strain A4, we transformed 50 samples of each plant material. In ten days after 2-day-long cocultivation of explants with the soil agrobacteria, hairy roots developed in all the Petri dishes. Efficiency of transformation was assessed three weeks after inoculation: hairy roots emerged on 49 of 50 cotyledon leaves (Fig. 1e). Thus, efficiency of *Agrobacterium*-mediated transformation of cotyledon leaves was the highest: 98%. On the first true leaves (Fig. 1f), hairy roots emerged with a probability of 92% (on 46 of 50 leaves). It is interesting that the use of cotyledon leaves for production of hairy roots was also most efficient in other plant species, such as *Capsicum annuum*, *C. frutescens* [27], and *Chenopodium murale* [28].

Morphological Comparison of Tobacco Isolated Adventitious Roots Obtained by Different Methods

Average weight of isolated roots produced by intact seedlings in early stages of culturing in liquid medium increased slightly (Fig. 2a). Only on the 18th day, the weight of wild type roots became the largest and exceeded the initial level 3.6 times; subsequently, root growth ceased and their average weight started decreasing (Fig. 2a). At the same time, when isolated roots of a wild type were cultured on solid hormone-free MS medium, the roots slowly elongated during one month by 22.65 ± 4.49 mm from the initial length (Fig. 2b). As to auxin-induced roots cultured on liquid medium, their weight gradually increased 2.2 times during a month as compared with the initial root weight (Fig. 2a). On the solid medium, an increment in the length of auxin-induced roots was uniform and amounted to 37.29 ± 3.01 mm as compared with initial length (Fig. 2b). In hairy roots produced using *A. rhizogenes*, strain A4, growth parameters were quite different. Initial average weight of hairy root culture maintained on liquid hormone-free medium was 382 ± 59.3 mg; on the 27th day, it rose to 1750.2 ± 44.17 mg; i.e., it increased 4.6 times (Fig. 2a). Therefore, true hairy roots of tobacco in liquid medium showed exponential growth. On the solid medium, the growth of hairy roots was also rapid: initial length of the roots increased by 42.7 ± 2.93 mm (Fig. 2b). It is interesting that it was very difficult to measure the length of hairy roots because of lack of apical dominance therein. Adventitious roots obtained as a result of biolistic transformation did not differ from auxin-induced roots by growth parameters; for this reason, we consolidated these groups. On the whole, the roots excised from tobacco seedlings during the first ten days adapted to isolated root culture conditions; however, their growth completely ceased during the first month of culturing. Auxin-induced roots grew in vitro more rapidly than the roots of wild type without adaptation, and their growth did not stop during the first month. In contrast to other types of root culture, hairy roots grew more rapidly and their growth did not tend to slow down for a long time (during more than ten passages).

Molecular Analysis of Differently Produced Adventitious Tobacco Roots Growing in Isolated Culture

In order to analyze the level of expression of *A. rhizogenes* genes *rolB* and *rolC* and genes of cyclin-dependent protein kinase *NtCDKB1-1* and expansin *NtEXPA5* in two different systems of target gene expression as compared with control samples (wild type and auxin-induced adventitious roots), we performed real time PCR where mRNA of elongation factor *EF-1α* was used as a reference and its level of expression was taken as 100%. In 7-day-old isolated roots of wild type tobacco, the levels of expression of the genes of cyclin-dependent protein kinase *NtCDKB1-1* and expansin *NtEXPA5* were rather low (55 and 52%, respectively) (Fig. 3), which correlates with the results of morphological analysis (Fig. 2a). However, in the course of adaptation of the roots to isolated growth, the content of mRNA of the genes *NtCDKB1-1* and *NtEXPA5* in 2-week-old roots of wild type considerably rose (to 587 and 803%, respectively), which correlated with active accumulation of root biomass (Fig. 2a). In early stages of development of auxin-induced adventitious roots, the level of mRNA of gene *NtCDKB1-1* turned out to be higher than the expression of gene

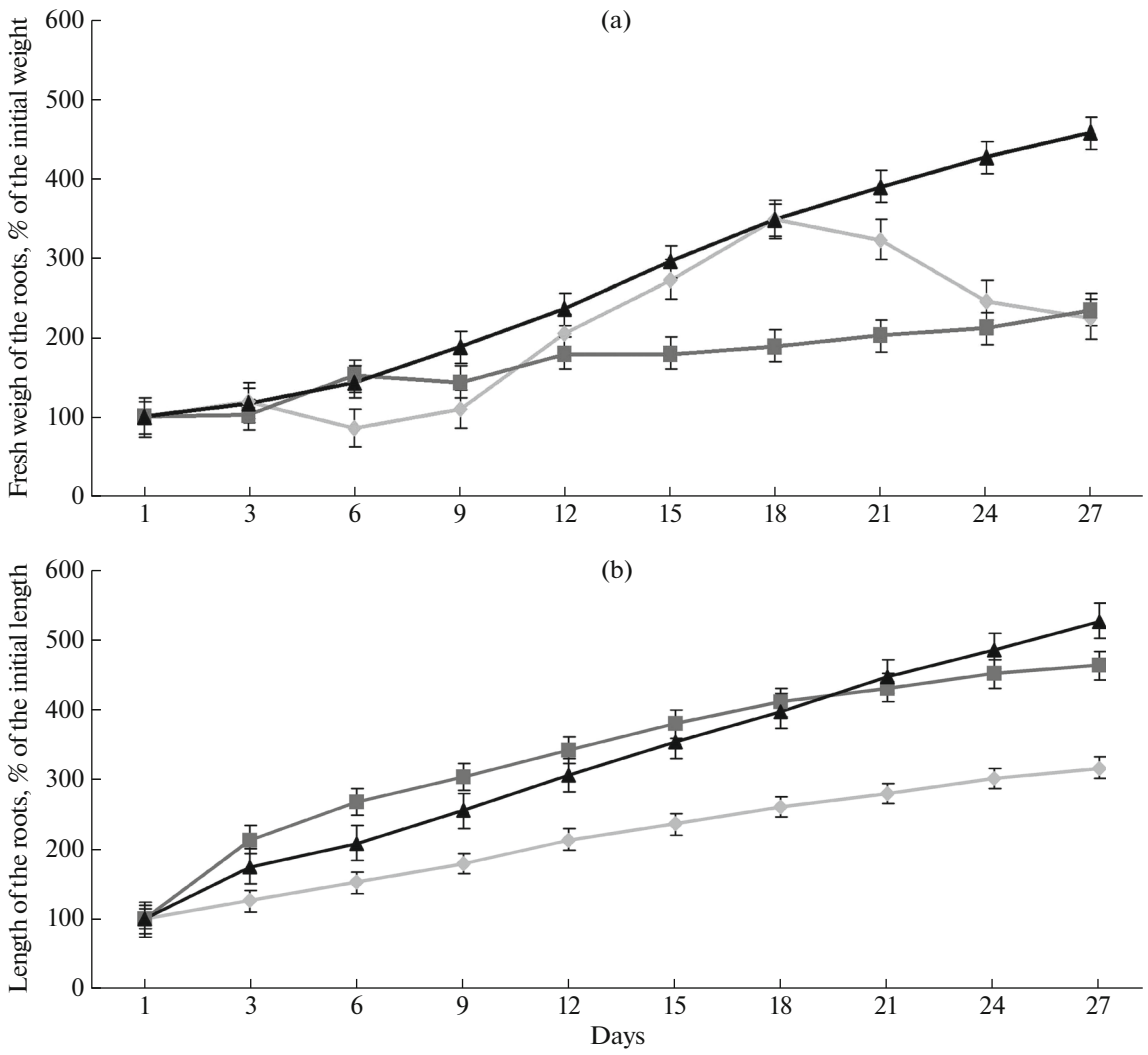

Fig. 2. Results of morphological analysis of the seedling roots in isolated culture (●), auxin-induced roots (■), and hairy roots (▲): (a) average fresh weight and (b) average length.

NtEXPA5 (155 and 66%, respectively). In 2-week-old adventitious roots, we observed an increase in the content of mRNA of the examined genes (to 201 and 783%, respectively), which correlated with accelerated growth of auxin-induced roots (Fig. 3).

In hairy roots, the level of expression of the studied genes was determined only after one month of culturing. In these roots, the level of expression of the gene of cyclin-dependent protein kinase *NtCDKB1-1* was very high (1742%). Expression of expansin gene *NtEXPA5* was also rather high (233%), but hairy roots less differed from other types of isolated roots by this parameter. Besides, hairy roots showed an elevated content of mRNA of *rolB* and *rolC* genes of *A. rhizogenes* as compared with mRNA level of reference gene *EF-1α* (30 and 3827%, respectively). This means that the level of expression of gene *rolC* in hairy roots was 128 times higher than expression of gene *rolB*. It is interesting that a minute expression of gene *rolC* also occurred in three lines of auxin-induced nontransformed adventitious roots (0.37, 0.15, and 8.9%). The line of auxin-induced roots with the greatest content of mRNA of gene *rolC* (8.9%) also showed higher levels of mRNA of the gene of cyclin-dependent protein kinase *NtCDKB1-1* (509%) and the gene of expansin *NtEXPA5* (1412%) as compared with average figures of expression of the examined genes in this group of roots (201 and 783%, respectively). Additional check-up experiments on isolation of RNA and determination of expression of gene *rolC* corroborated its expression in the same lines of auxin-induced adventitious roots.

As to two lines of adventitious roots growing well on a hormone-free medium and produced as a result of biolistic transformation, we obtained the following results: the levels of expression of gene *NtCDKB1-1* were 260 and 13.5%; expression of expansin gene *NtEXPA5* was 126 and 67.5% and expression of gene *rolC* was 1.2 and 0.0861% (lines 3 and 10, respec-

tively). It is interesting that we also observed expression of gene *rolB* in line 3, although it was extremely low (0.0788%). A series of experiments demonstrated that this result cannot be accounted for by contamination, since PCR analysis of total DNA showed a lack of genes *rolB*, *rolC,* and *virC* in the genome of these adventitious roots. These results suggest that bombardment with gold particles failed to ensure reliable integration of these genes, and minor levels of expression of gene *rolB* can be explained by a transient expression. Thus, examined lines of adventitious roots produced via bombardment did not have the same high content of mRNA of *A. rhizogenes* genes *rolB* and *rolC* and did not show unrestricted growth as did hairy roots' cultures obtained by a classic method. For instance, we already observed after three passages a considerable retardation of growth and emergence of numerous necrotic areas in all the lines of adventitious roots produced by the biolistic method. It is necessary to note that, on hormone-free media, ordinary and auxin-induced tobacco roots stopped growing as early as during the first month of isolated culturing.

DISCUSSION

By the method of bombardment of tobacco cotyledon leaves with gold particles and using only an amplicon composed of four *rol*-genes as a DNA carrier, we produced 13 lines of adventitious roots able to grow on hormone-free nutrient media for two months. However, we failed to corroborate the presence of *rol*-genes in the genome of these adventitious roots by means of PCR. The method of biolistic transformation with only an amplicon of *rol*-genes we worked out is for the time being unsuitable for production of hairy roots, since reliable integration of transgenes into the genome was not ensured. We used this approach earlier and produced only two lines of hairy roots with reliable integration of *rol*-genes mainly owing to an increase in the number of experiments [19]. However, these investigations should continue since elaboration of universal bacterium-free methods of hairy roots' production is a priority task in contemporary genetic engineering of plants.

Isolated roots of wild type tobacco, which were cultured on a hormone-free medium after their excision from juvenile plants, died within the first month. During the first week on liquid media, these roots did not grow and even reduced in size, which correlated with rather low levels of expression of genes *NtCDKB1-1* and *NtEXPA5*. However, later wild type tobacco roots switched on adaptation mechanisms that ensured elevation of expression of the examined genes of cyclin-dependent protein kinase and expansin. This was phenotypically shown in a sharp activation of roots' growth that lasted for no longer than ten days; after that, they stopped accumulating biomass. The levels of expression of genes *NtCDKB1-1* and *NtEXPA5* correlated with parameters of root growth; therefore, these genes can be used as molecular markers of the rate of biomass accumulation in root crops.

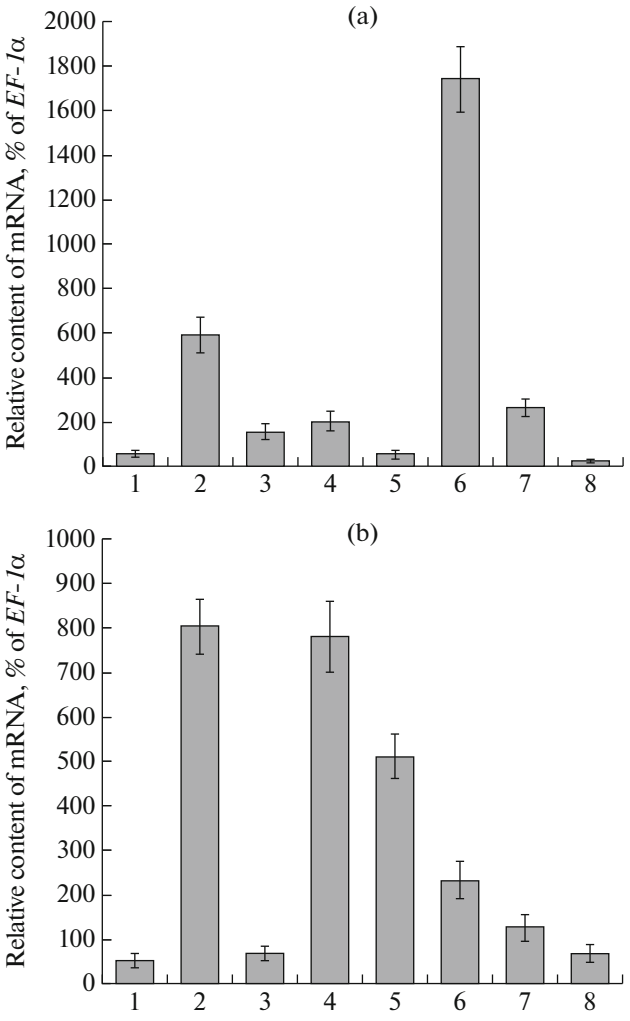

Fig. 3. Expression of the gene of cyclin-dependent protein kinase *NtCDKB1-1* (a) and expansin *NtEXPA5* (b) in the roots of different origin: *1*—7-day-old and *2*—2-week-old roots of seedlings; *3*—7-day-old, *4*—2-week-old, and *5*—1-month-old auxin-induced roots; *6*—1-month-old genetically transformed roots; *7*—line 3 and *8*—line 10 of 1-month-old adventitious roots produced by means of biolistic transformation.

From the very beginning, auxin-induced adventitious roots of tobacco had rather high levels of expression of genes *NtCDKB1-1* and *NtEXPA5* and they actively grew on hormone-free medium right after the excision from the explants. In two weeks, these roots probably switched on the adaptation mechanisms, since the content of mRNA of genes *NtCDKB1-1* and *NtEXPA5* considerably increased at that time. However, in 1-month-old adventitious roots, the level of expression of the examined genes started to decrease, which subsequently led to a reduction in the growth rate of these root crops. Thus, auxin-induced roots

differed from the normal roots by more rapid growth only in the beginning of culturing, but they also were not able to grow on hormone-free media for a long time even after a reduction in the duration of culturing up to two weeks. In the most rapidly growing auxin-induced tobacco roots, we detected mRNA of gene *rolC*, which may be accounted for by activation of expression of a homologous tobacco gene *trolC* [29]. In fact, the primers we chose to determine the level of expression of *A. rhizogenes* gene *rolC* turned out to be complementary to a fragment of tobacco gene *trolC* (Gene ID: 107814767). The gene *trolC* is probably auxin-inducible since we never detected its expression in normal roots.

Hairy roots of tobacco grew more rapidly than all the other types of examined root crops both on solid and liquid nutrient media. Moreover, the roots genetically transformed with pRi did not tend to reduce the growth rate in the course of morphological analysis lasting for a month. Molecular analysis of these roots showed a very high level of expression of gene *NtCDKB1-1*, whose mRNA content was seven times greater than that of expansin gene *NtEXPA5*. These results suggest that the growth of hairy roots much more depends on cell divisions than on their elongation. At the same time, growth of other types of root crops we examined almost equally depended on cyclin-dependent protein kinase and expansin. It is well known that expression of gene *NtCDKB1-1* is chiefly regulated by cytokinins [10, 30], which may point to a more active participation of this group of phytohormones in the regulation of hairy roots' growth in isolated cultures. Actually, the level of expression of gene *rolC* whose product is involved in activation of cytokinins in hairy roots was 128 times greater than the expression of gene *rolB*. At the same time, the product of gene *rolB* is mainly involved in the activation of auxins that also play an important role in the maintenance of growth of roots genetically transformed with pRi-plasmid. Since transformation with gene *rolB* alone is sufficient for induction of hairy roots [5], one should not underestimate the role of this gene and auxin signaling as a whole in the maintenance of active growth of root crops.

Growth characteristics of adventitious roots of tobacco obtained after biolistic transformation did not differ from growth parameters of auxin-induced roots. Moreover, by the expression profiles of genes *NtCDKB1-1* and *NtEXPA5*, these two types of roots were identical. At the same time, it is impossible to account for development of the adventitious roots after bombardment solely by hormonal changes, since auxin-induced adventitious roots ceased growing on hormone-free media as early as after one month of isolated culturing. We also performed numerous experiments on bombardment of tobacco leaf explants with gold particles free of DNA and managed to obtain merely one line of adventitious roots that did not differ from normal roots by their growth parameters and ceased growing in isolated culture in two weeks. Thus, one can think that the emergence of adventitious roots after bombardment is related to the used DNA containing all four *rol*-genes with all the regulatory sequences; however, PCR analysis showed lack of genes *rolB* and *rolC* in the genomes of the examined lines of adventitious roots. Induction of adventitious roots from the explants after bombardment could be promoted by a transient expression of *rol*-genes. This assumption does not solely rest on the fact that bombardment of cotyledon explants with DNA-free gold particles did not induce adventitious roots. The point is that we found mRNA of genes *rolB* and *rolC* in some lines of adventitious roots produced as a result of bombardment, which may also point to a transient expression of *rol*-genes in the adventitious roots.

Thus, we worked out a bacterium-free and plasmid-free method of hairy roots' production by means of biolistic transformation of leaf disks, which, however, needs improvement in view of a very low efficiency. In most cases, transformation performed in this way brought about development of adventitious roots with a presumably transient expression of *rol*-genes. Investigations conducted with tobacco showed an important role of transgene *rolC* and host gene *CDKB1-1* in the maintenance of rapid growth of hairy roots; however, one cannot rule out participation of other genes in positive regulation of hairy roots' growth.

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