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# Secondary metabolites synthesis in transformed cells of Glycyrrhiza glabra L. and Potentilla alba L. as producents of radioprotective compounds

P. G. Kovalenko, V. P. Antonjuk*, S. S. Maliuta[1]

*Institute of Molecular Biology and Genetics, NAS of Ukraine, 150 Zabolotny Str., Kyiv-143, Ukraine*
*[1]Biospirtprod, Kyiv, Ukraine*

**Abstract.** Following the Chernobyl explosion, screening for new drugs of radioprotective activity has been initiated in Ukraine. The European licorice (*Glycyrrhiza glabra L.*,) is a pharmacologically important species which is rich in flavonoids and saponins, especially in the roots. To increase the pharmacological potential of this plant, the authors have obtained transformed licorice protoplasts with higher production of target compounds. Isolated licorice *G.glabra L.* protoplast suspension was electroporated by the chimeric plasmid pDNt23-CaMV35S-nos-npt-II-cat (pDNt23-root-specific and CaMV 35S promoters, nos-nopaline synthase terminator, and selectable NPT-II gene). On the selective medium with addition of kanamycine sulphate, proorganogenic clusters have been obtained. These obtained cell clusters have been additionly inoculated by *Agrobacterim rhizogenes* (15834). In general, the level of secondary metabolites in these transformed cells was 2-fold higher than in nontransformed control cells. We found that flavonoids isolated from transformed *G.glabra* cells possess aldose reductase inhibitory activities; these compounds have been tested on the rat lens. The intact root segments of *Potentilla alba L.* have been also inoculated by this strain too, to obtain «hairy roots» as terpens producent. These compounds have shown inhibitory effect on the thyroxine levels in white rat thyroid glands. These results suggested that metabolites obtained from transformed pharmaceutical plant species can be used as radioprotective compounds after γ-irradiation.

**Keywords:** *Glycyrrhiza glabra; Potentilla alba;* foreign DNA; electroporation; hairy roots; radioprotectors.

**Introduction.** Plant tissue cultures are at present attracting worldwide attention, because plant cells are able to synthesize specific compounds, especially various secondary metabolites useful as medicines and food additives. Cultured plant cells can grow far more rapidly than their original plants; this means that increased productivity is expectable. The productivity may be also enhanced by optimizing cultivation conditions, cell selection and genetic transformation [1—3].

Plant roots contain various useful compounds, such as pharmaceuticals and pigments, and the effective utilization of such compounds is of great interest [4, 5].

The European licorice (*Glycyrrhiza glabra L., Leguminosae*) and other members of this pharmacologically important genus are rich in isoflavonoid constituents and contain glycyrrhizin, an olcane type triterpene glucuronide, being used as a natural sweetener as well as a source for anti-inflammatory drugs. There are data suggesting that glycyrrhizin in combination with methyluracil possesses a radioprotective effect [6]. Flavonoid complexes are known to be the major components with high biological activity against inflammatory diseases and pyrexia [7—9]; they are used as oral hypoglycemic drugs [10, 11]. At first licorice roots (*Glycyrrhiza glabra L.*) have been used very intensively as a flavouring in food industries. The diversity of secondary

***Corresponding author.**
Tel.:+38044-2660729; fax: +38044-2660759
**E-mail address:**
omg@imbg.org.ua.

metabolites found in roots shows the immense biochemical potential contained in this organ. The full understanding of the biosynthetic pathways and biological functions of the licorice root secondary metabolites production is still in the search. For example, β-amyrin, a possible biosynthetic precursor of glycyrrhizin synthesis, was detected in callus mass of transformed licorice cells [12, 13]. However, the root cultures are not suitable for large-scale production of secondary metabolites because of their relatively slow growth in different in vitro conditions. Licorices (*Glycyrrhiza species*) are very popular medical plants in the world. In particular, the dry roots of these plants have been used as a source of crude drugs medicine «KAMPO».

After Chernobyl atomic explosion in Ukraine, the levels of chronic diseases (peripheral neuropathy, retinopathy, cataract, diabetics and other ones) have been increased. Recently, the increased activity of the polyol pathway resulting in the accumulation of sorbitol from glucose by an enzyme aldose reductase (RLAR) has been shown to be implicated in the pathogenesis of these complications.

For this purpose, flavonoid compounds isolated from *Glycyrrhiza sp.* cells have potent aldose reductase inhibitory activity [10].

The genetic engineering of licorice will be very important in future as an alternative means for scale production of high-value plant secondary metabolites from transformed cell lines.

It is important that flavonoids contained by licorice roots possess cytotoxic and anti-tumour activity [14]. For example, flavone-8-acetic acid is a synthetic derivative of the basic flavonoid skeleton with a unique form of preclinical antitumour activity, but its action mechanism is still not completely known [15].

The second plant species, white cinquefoil (*Potentilla alba L., Rosaceae*), contains up to 30 % of tannines, glycosides and possesses a cytostatic effect. *P. alba* roots are used in Ukrainian folk medicine to reduce the thyroxin level in blood plasma as an important drug against thyroid gland diseases; they stimulate thyrotropin secretion and are recommended as radioprotective remedy after Chernobyl atomic explosion [16].

Recently, transgenic cell clusters and «hairy root» cultures have become of interest because of their continuous and active proliferation in phytohormone-free medium and their capacity to produce valuable materials synthesized and accumulated in vitro, their levels being comparable to their concentrations in original plants [17].

The «hairy roots» affecting a wide range of dicotyledonous species are caused by a soil bacterium, *Agrobacterium rhizogenes*. The induction mechanism of hairy roots and expression of the diseased phenotype were studied by many investigators [4, 8].

One of the possible approaches to manipulate the secondary metabolism of the intact suspension cells is a direct useful gene transfer to isolated protoplasts by electroporation [18]. The transient expression of the reporter chloramphenicol acetyltransferase (CAT) gene under the control of CaMV35S promoters has been demonstrated to be a powerful tool to study the introduction of foreign DNA into plant protoplasts [19—22].

The genetic manipulations with licorice intact cells depend significantly on the current success of protoplasts isolation procedure. A range of marker genes, most commonly under the control of regulators elements, such as the organ-specific pDNt23 and 35S promoters, have great interest for improving of these licorice cell lines. However, nowadays, there is limited information on the relationship between electroporation conditions and CAT expression in licorice protoplasts. Our results presented below are obtained both with a rectangular pulse generating-system and more commonly used capacitor discharge system delivering pulses of exponentially decaying voltages.

The aim of this work was to characterize factors influencing the expression of introduced genes via electroporation to *G. glabra* protoplasts and to study the initiation of the *P. alba* «hairy roots» culture to increase the secondary metabolite productions. We describe the effects of these obtained bioactive compounds on aldose reductase activity and the effect of the total triterpen preparations on the thyroxin levels in experimental rats.

**Materials and methods.** ***Plant material and cell culture.*** The stolon and root of the *G. glabra* were taken for the callus culture initiation and permitted subsequently to obtain cell suspen-

sion growth and protoplasts isolation. The field plants of *G.glabra* for in vitro manipulation have been obtained from Crimea Botanic Garden (Ukraine).

The callus initiated was detected in modified MS (Murashige and Skoog) medium [23] supplemented with phytohormones benzyladenine (BA, 0.2—1.0 mg/l, Sigma Chemical Co.) and naphthalene acetic acid (NAA, 0.5—5.0 mg/l, Sigma Chemical Co); the medium was agarized by the 0.9 % agar (Difco, USA).

About 67 % of the explants in this work initiated their callus growth within 3 weeks. The growth was slightly better at the medium with the pH 5.7—6.0. A well-growing callus cell line was used for further experiments in modified liquid MS medium supplemented with BA (0.2—2.0 mg/l) and NAA (0.2—2.0 mg/l), and the licorice suspension cells growth has been obtained in this medium. These suspension cell clusters consisted of clumps containing 10—30, their shapes being usually spherical.

These licorice cell clusters were agitated on a reciprocal shaker (110 strokes/min) in the dark at 25 ˚C and subcultured with intervals of 24 days. Cell culture suspensions grown during a month were treated with an enzyme mixture. The evaluation of range of enzyme formulation shows that almost all cells could be converted to viable protoplasts in 9 h with a combination of 0.5 % cellulase R-10 «*Onozuka*» (Yakult Honsha Co, Japan), 0.5 % macerozyme R-10 (Sigma Chemical Co, USA), and 0.3 % dricelase (Sigma Chemical Co,USA) in osmotic (0.6 M mannitol and MS salts). After purification on a sucrose-mannitol gradient, the protoplasts were resuspended in the culture medium. The protoplasts density was determined by counting of a known volume of culture on a haemocytometer slide, and their viability was measured by the exclusion of phenosafranine (Aldrich, USA) according to the protocol [24]. Isolated protoplasts yields were 0.7—1.0 x $10^6$ cells/g of fresh weight and with viabilities of 70—80 %. Electroporated and non-electroporated protoplasts were cultured in the modified liquid MS medium.

The root cultures of *P. alba* were initiated from natural soil growing young root segments received from the Lviv Forest Academy (Ukraine). For the initiation of the young root growth in in vitro culture, we have sterilized original roots according to standard methods and transferred on modified MS medium with addition of 2,4D (dichlorophenoxyacetic acid, 1.0 mg/l), adenine (3 mg/l), kinetin (1mg/l), sucrose (3 %); the medium was agarized by the Phytogel (Sigma Chemical Co, USA; 0.2 %). These cultures were incubated in the thermostatic room with constant temperature (26 ˚C) and under fluorescent light (2000 lux) with the photoperiod 8/16 (light/darkness). The fresh young roots have been initiated after 5 weeks of culture. Such isolated root explants have been used for the *A. rhizogines* 15834 inoculation procedure.

**Electroporation.** To perfect the electroporation procedure for licorice suspension protoplasts, we have studied the following conditions: capacitor discharge system (capacitance from 50 μF to 200 μF), and time constant 80 mS. Protoplasts were electroporated at a density from 4x105 to 106 cells /ml in a pulse medium containing 20 mM KCI, 6 mM MgCI2- 2.06 M sorbitol, pH 6.0. Pulses from electroporator were delivered in to 340 μl of protoplasts suspension in a cylindrical electrodes chamber (4 ˚C, the resistance being 1,7 kΩ with stainless steel electrodes).

To optimize the electroporation process, the protoplasts were subjected to three electrical pulses (RC pulse duration was 80 mS) of field strength 200—350 V/cm, with 30 s intervals between the pulses. After electroporation the protoplasts samples were kept on ice for 8 min before dilution and culturing. The electrode chambers were sterilized with absolute ethanol.

**Plasmid construction.** The pDNt 35SCaMV-CAT-npt-II-nos-3'-cat gene construction [25] was used for the direct gene transfer into the licorice protoplasts by electroporation. Both linearized and supercoiled forms of this plasmid were used in our experiments.

The plasmids were propagated in *Escherichia coli* cells and purified according to the standard protocol. The plasmid DNA was linearized by means of digestion with appropriate restriction enzymes. After phenol-chloroform extraction and ethanol precipitation the DNA was resuspended in water under sterile conditions. Non-linearized plasmid DNA was treated in the same way as the linearized DNA but without any digestion. The DNA concentrations were meas-

ured by spectrophotometry at 260 nm. The carrier DNA (calf thymus DNA, Sigma Chemical Co, USA) in concentrations of 0, 20, 50 or 150 μg/ml was added. The plasmid was sterilized by ethanol precipitation and dissolved in the electroporation buffer.

**Protoplasts viability and CAT assay.** After 48 h of culturing the number of surviving protoplasts was counted in ten random fields using a LOMO (RU) microscope. Dead protoplasts were clearly distinguishable and appeared condensed and destroyed. The cell viability was also confirmed by staining with fluorescein diacetate. The CAT activity was determined essentially as described in the protocol [26]. The extracts were run at high speed for 5 min and the supernatant was decanted for CAT assay. Briefly, protoplasts (3x105) were collected by centrifugation and the pellets were resuspended in 500 μl of a buffer (0.225 M Tricine, pH 7.8, 5 mM phenylmethyl sulphonyl fluoride and 5mM EDTA). The extracts were clarified by centrifugation, and the supernatants were then transferred to a fresh tube and heated (10 min, 65 °C). Acetyl CoA (final concentration 1 mM) and $^{14}$C-chloramphenicol (0.2 μCi) were added and the reaction was allowed to proceed for 1 h at room temperature before being terminated by the addition of 10 volumes of ethyl acetate. As a control 0.5 unit of commercial CAT (Sigma Chemical Co, USA) was used. The washed protoplasts were dried and redissolved in 30 μl of ethyl acetate and submitted to ascending chromatography in a chloroform/methanol mixture (95:5, v/v) on silica gel plates. Separated spots of $^{14}$C-chloramphenicol and its acetylated forms were visualized by autoradiography. Results are expressed as the conversion per cent of chloramphenicol to its acetylated forms. Calibration experiments with commercial CAT enzyme showed the relationship between the number of enzyme units used and the conversion per cent, 1 CAT unit having given 93 % of conversion. All results obtained were within this range. Activity in «negative controls» was determined either after incubation of protoplasts in plasmid and carrier DNA without electroporation, or after electroporation in the presence of the carrier without plasmid DNA, as described [27].

**Selection of the electroporated licorice protoplasts.** After electroporation, licorice cells were diluted with the fresh modified liquid MS medium containing BA (0.2—0.5 mg/l), NAA (0.5—1.0 mg/l), and 2,4D (005 mg/l) and stored for 2 days at 22 °C in the dark. After this time, the electroporated protoplasts were transferred to a fresh modified MS medium supplemented with kanamycin sulphate (100 mg/l) and cultivated at 25 °C in the dark during 7 days. The fresh medium with the half reduced kanamycin sulphate concentration were used for every new passage. Well-growing protoplast suspensions were selected to produce lines of kanamycin-resistant cell aggregates being a source of total flavonoids production. Non-electroporated (control) licorice protoplasts were cultured on the modified MS medium without kanamycin sulphate.

**Obtaining of G.glabra and P.alba hairy root cultures.** To obtain transformed root culture of *G.glabra* and *P.alba*, we used *Agrobacterium rhizogenes strain* 15834 received from the IBG (Berlin, Germany).

This strain containing agropine-type plasmid pRiA4b was cultured in 40 ml of YEB medium (pH7.2) containing yeast extract (1.0 g/l), beef extract (5.0 g /l), peptone (5.0 g/l), sucrose (5.0 g /l), $MgSO_4 \cdot 7H_2O$ (0.49 g/l); the cultures were incubated in the dark at 28 °C on a rotary shaker (160 rpm). When the $OD_{600}$ value had reached approximately 1.0, the bacteria suspension was collected and run at 4,000 rpm for 5 min and resuspended in growth regulator-free liquid MS medium for inoculation.

For the induction of the hairy root cultures from organogenic licorice cell clusters and from *P.alba* root segments, the following procedures have been used.

We have taken an organogenic licorice cell clusters obtained from licorice cell suspension previously electroporated by the plasmid pDNt23-35SCaMV-npt-II-nos3'-cat. We used also *P.alba* root segments explants. We have taken old *P.alba* cultures (3rd and 4th month of growth) explants which were pre-cultured on solid growth regulator-free MS medium during 24 h. Subsequently, these licorice cell clusters and *P.alba* root segments were infected by dipping them into MS medium-suspended *Agrobacterium* solution for 20 min. Following infection, these inoculants were washed once with sterile water and blotted with filter paper to remove the excess of *Agrobacteria.*

After two days of co-cultivation at 28 ˚C in the dark, these inoculated explants were transferred onto MS medium containing carbenicillin (500 mg/l, Sigma Chemical Co) and kept in an air conditioner-controlled chamber at 25 ˚C, under 14h/ day light photoperiod (3000 lux) to induce hairy roots. Control explants (non-inoculated licorice cell clusters and *P.alba* small root parts) were given the same treatment but dipped into the sterile YEB medium. Each treatment took place in 100 ml Ehrlenmayer flasks and the infection experiments were repeated twice.

On the $28^{th}$—$31^{st}$ days post infection, hairy roots were excised from infected *G.glabra* clusters and from *P.alba* root explants. These inoculated explants were cultured on growth regulator-free MS medium agarized by the Phytogel (0.2 %) and supplemented with carbenicillin (500 mg/L) to eliminate agrobacteria; after several days of culture, the elongated root tips were cut off and transferred to growth regulator-free MS agar medium without carbenicillin. This procedure was repeated 3—4 times until no colony of bacteria appeared. Sterile hairy root cultures were maintained at 25 ˚C in the dark on growth regulator-free MS medium without antibiotic.

To maintain the subsequent hairy root culture growth, we have transferred every week these hairy roots clones obtained into fresh modified (hormone-free) MS liquid medium supplemented with cefotaxime (0.25 g/l) and ampicillin (1 g/l) (Sigma Chemical Co, USA). These hairy roots could grow rapidly on liquid and growth regulator-free MS medium and had characteristics of transformed roots such as quick growth and high lateral branching (Fig. 1). Paper electrophoresis revealed that bacteria-free hairy roots of *P.alba* could synthesize agropine and mannopine (Fig. 2).

The total levels of metabolites, as such flavonoids and terpens, are summarized in tables 3 and 4. Bioextracts obtained from these transgenic roots have been used for screening tests on experimental animals.

**Analytical methods.** For the preparing of bioextracts from intact *G.glabra* and *P.alba* root tissues, we treated them by following enzymatic solutions: Pectinase (0.5 %, Sigma Chemical Co), Cellulase (1 %, Sigma Chemical Co). After washing the root tissues have been treated by boiled water (400 g) and the compounds were put on a Sephadex LH-20 column and eluted successively with $H_2O$-methanol (1:1) and acetone-$H_2O$ (1:1). Each eluted fluid, except for that washed with $H_2O$-methanol, was evaporated and centrifuged. These fractions were collected from the Sephadex LH-20, MCI gel CHP20P. Effects of these compounds on rat lens aldose reductase (RLAR) were assayed according to the special protocol [10].

Concentrations of total flavonoids were determined by using the HPLC («Pharmacia») and a detector (270 nm) equipped with a stainless steel column. The mobile phase contained 145 ml of tetrahydrofuran, 125 ml of dioxan, 50 ml of MeOH, 20 ml AcOH, 2 ml of 5 % $H_3PO_4$, and deionized water (up 1 liter), the flow rate

*Fig. 1. Hairy roots of Potentilla alba cultured on liquid growth regulator-free MS medium for 28 days.*

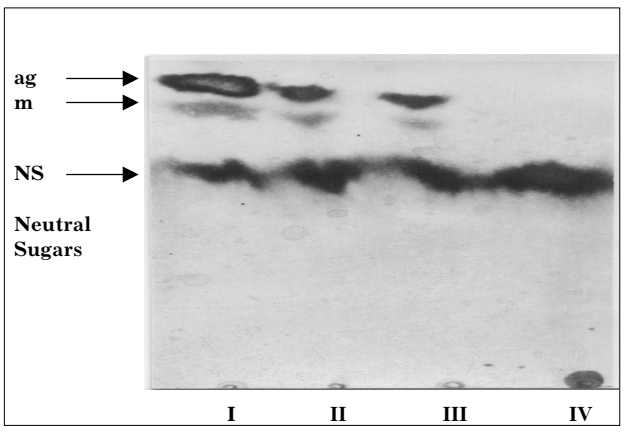


*Fig. 2. Detection of opines (agropine (ag) and mannopine (m) by paper electrophoresis in extract of P. alba hairy «root cultures». Lane I: Agropine and mannopine standards; (lanes II and III) — detected opines in «hairy root» strains; IV: non-transformed (control) roots.*

being 1 ml/min. Flavonoids were separated after the retention of 9 min. All experiments were triplicated and averaged.

For the total terpens analysis in transformed P.alba, air-dried and lyophylized hairy roots have been used. These root materials were extracted with hot $CCI_4$ (20 ml/g tissue), and the constituents were initially determined by silica-gel TLC (Kiesel gel 60 F254; solvent, toluene/EtOAc =4/1) together with standard samples for preparative TLC, and aliquots were applied to an HPLC column (Simpack CLC-ODS, 0,15 m x 6 mm, (Shimadzu, Japan); the fractions were read at 205 nm.

**Experimental animals.** Male Wistar rats (150-200 g) were used. They were housed in plastic cages in an air-conditioned room at 24 ˚C and received standard diet. These rats have been irradiated (9,5 *Gy*) in the γ-ray apparatus (model RUM-17, Ru). After irradiation procedures, the first post-treated experimental animals group received water and food from Chernobyl zone, and were mixed with metabolites as total flavonoids mixtures. Second experimental group of rats received the same diet but mixed with a triterpens preparation. The control animal group had the same water and food without flavonoids and terpens.

Metabolites obtained from *G.glabra* and *P.alba* transformed tissues were dissolved in distilled water. The rats received daily doses of flavonoids (80 mg/kg/day) and terpens (50 mg/kg).

A month later the effect of secondary metabolites on the RLAR activity in rat lens was found in the first experimental animal group. The changes of the thyroxin levels were seen in the second animal group fed by isolated terpens contained by their diet.

**Results and Discussion.** For the direct gene transfer to isolated licorice protoplasts, we have used electroporation. In other experiments, we have shown electroporation to permits the successful protoplast transformation and we have applied two electroporation systems based either on low voltage/long pulses or on high voltage/short pulses [27, 28].

We have used square wave pulse generators and capacitor discharge systems, which deliver exponentially decaying voltage pulses. Typical values of exponential decay for isolated protoplasts with lower voltage pulse method are 200—450 V/cm and 10—90 ms (exponential decay). This method has resulted in stable transformed cell lines and high viability of cells after electroporation [16, 17]. The optimal voltage and time constant depend on the protoplasts diameter and physical parameters of electroporation, such as plasmid concentration and forms, cells density and physiological properties of protoplasts isolated.

The electroporation therefore requires a baance between factors increasing the membrane permeability and factors resulting in membrane breakdown and loss of protoplasts viability. The mechanisms of the electrically induced permeability membrane charges, new lipid bilayer configuration and the penetration of plasmid DNA adsorbed by the cell surface are not yet well known.

The discharge system used for the CAT activity monitoring in carrot protoplasts with the study of their survival after electroporation depends on the salt composition of buffer used in electroporation [29].

To determine the optimal electroporation conditions, we took mean licorice cell size (29—36 $^{1}$μM), and these protoplasts were isolated from exponentially dividing suspension cells (3—4 days of culture). This population of suspension cells would obviously contain the largest proportion of mitotic cells, and freshly isolated protoplasts appeared to be the most appropriate for electroporation. The older (5—7 days) licorice suspension cells do not divide synchronously; so they are not good for electroporation. It has been shown previously that suspension protoplasts culture was permeabilised at a range of voltages. It was proposed to be due to the cell size heterogeneity in the population. The voltage required for membrane breakdown depends on the cell size, such that at lower field strengths larger protoplasts would be permeabilized, the higher field strengths leading to the cell disruption. Viabilities of electroporated and non-electroporated licorice protoplast suspensions were 38.5 ± 1.3 % at 250 V/cm and 27.2 ±3.3 % at 350 V/cm: 27.2 ±3.3 %, the control values being 64 ± 1.2 %. For better viability of electroporated suspension protoplasts, we have added to the cultures a modified MS medium with some effective natural plant growth regulators: «*Emystym*» obtained from endophyte myc-

orrhiza fungi of *Panax ginseng* (Timiryazev Academy, Ru) (0.002 mg/l). The maximum effect of field strength on the expression of pDNt23-35SCaMV-npt-II-cat-nos was observed within 50 h after electroporation.

The optimum field strength was 250 V/cm with 30 s intervals between 3 pulses (RC pulse duration = 80 ms), the best capacity being 95 μF. The plasmid DNA concentration of 100 μg/ml was sufficient for routine measurement of CAT activity. The effects of field strength in CAT expression in licorice protoplasts with linearized and supercoiled DNA forms are shown in the Table 1.

The concentration of the pDNt-35Scat-npt-II-cat-nos plasmid used during electroporation was an important factor influencing the transient CAT activity in licorice suspension protoplasts. The addition of 0, 20, 50 or 150 μg/ml of carrier DNA to licorice protoplasts (in the presence of 0, 50 or 100 μg/ml plasmid DNA) did not result in any increased CAT expression (Table 2).

From the results presented in this paper, it is apparent that the transient expression of CAT in licorice suspension protopalasts to be dependent on two categories of parameters. The using of a rectangular pulse generating system has been a very efficient method to determine the lowest CAT activity. Poorly considered physiological characteristics of the intact licorice protoplasts prevent the higher CAT expression

Thus, the licorice protoplast suspension contained large starch granules and the suspension cells were with small nuclei and large cytoplasm. The extractable activity in licorice protoplast suspension was the greatest, under the optimal electroporation conditions tested. Such a combination of electrical parameters appears to induce more pores in the plasma membrane, allowing more DNA to be taken up to nucleus, or perhaps a longer pulse duration facilitates the electrophoretic movement of plasmid DNA molecules into the nucleus.

The levels of transient expression conversion (%) of chloramphenicol to its acetylated products) were very similar to results obtained with protoplast suspensions of many other plant species. The pulse generator with the rectangular pulses and their duration were under precise control.

These results show that the highest CAT activity in protoplast suspension ($10^4$ cells/ml) was obtained at 250 V/cm and with the linearized plasmid concentration 100 μg/m1. Similary, the using of linearized plasmid DNA compared with supercoiled DNA resulted in higher levels of transient expression. In several species, linear DNA has been found to be better than circular for plasmid uptake and integration leading to the increased gene expression.

The increased permeability of the cell wall to DNA molecules depends on the treatment of licorice suspended cells with a pectolytic enzyme, macerozyme (0.5 %, w/v). This evidence suggests that this effect may be due, in part, to the breakdown of cell clumps into smaller aggregates, in which a larger proportion of the cells may be permeabilized, and presumably also to a removal of pectin from the cell wall. The cold pretreatment of licorice protoplast suspension and regenerated cell lines plays an important role to achieve the highest degree of electroporation efficiency and to increase the protoplasts viability after electroporation.

*Table 1*

*Transient CAT activity in licorice suspended protoplasts electroporated with pDNt23-35S-nos at various forms*

| DNA forms | Plasmids μg/ml | Repeat | Protoplasts[a] cells/ml (x$10^4$) | cpCM[b] per (x$10^4$) | CpCM[c] total |
|---|---|---|---|---|---|
| lin. | 50 | 3 | 37 | 0.830 | 29.0 |
| lin. | 100 | 3 | 44 | 1.950 | 72.0 |
| sup. | 50 | 3 | 35 | 0.027 | 0.6 |
| sup. | 100 | 3 | 39 | 0.850 | 31.0 |

**Linearized — lin; supercoiled — sup; a — of surviving protoplasts, viabilities of non electroporated controls were 62.8 % ±17.6 %; b — this is the cpm chloramphemcol (CM) at 60 mmol/l; c — the cpm acetylated/cpm total CM x 100. CAT enzyme control gave 92.6 % ± 6.4 % CM, per $10^4$ surviving licorice cells. Non electroporated control was:= 0.003 %, per $10^4$ surviving licoricecells.*

*Table 2*

*Effect of carrier DNA on CAT activity in licorice suspension protoplasts*

| Carrier DNA (g/ml) | *Plasmid DNA ($\mu$g/ml) | [a]% cpCM total |
|---|---|---|
| 0 | 0 | 0.3 |
| 20 | 0 | 0.3 |
| 50 | 0 | 0.26 |
| 0 | 100 | 67 |
| 20 | 100 | 62 |
| 50 | 100 | 73 |
| 20 | 50 | 26 |
| 50 | 50 | 23 |
| 100 | 50 | 28 |
| 150 | 100 | 64 |

** – plasmid pDNt23-35S- npt-II-nos-cat;*

*[a] – the – cpm acetylated/cpm total CM x 100 (per $10^4$ protoplasts). Voltage was 250 V/cm (95 $\mu$F).*

In conclusion, the results presented here support the view that electroporation of an important pharmaceutical species, *G.glabra*, can be defined as the transfer of foreign genes isolated from plants into a new genetic background. Further investigation is needed to determine the factors causing enhanced cellular storage of secondary metabolites products, and especially, their influence of flavonoids and glycyrrhizin increase in in vitro culture.

Experiments are now aimed at obtain stable transformed cell lines with increased total metabolites production. These results show that both cell culture and electroporation conditions are important for transformation rates of licorice species.

The higher level of total flavonoids in *G.glabra* hairy root culture comparing to dedifferentiated plant cells depends on properties of genetic cell lines; in some of them these levels are often lower than in donor plants. That is why it is necessary to develop high yield cell lines and to design the optimal culture conditions to improve the productivity. A correlation of morphological differentiation and biosynthesis/accumulation of secondary metabolites is often found in plant cells and usually interpreted as a result of organ-specific expression (root-specific one in our experiments) of biosynthetic genes.

In Ri-transformed *G.glabra* cell lines there are increased levels of some flavonoids — liquiritin, naringenin, luciraside, (unpublished date); they may also induce higher level of the RLAR inhibition.

In summary, the results presented in this paper demonstrate the first example of direct

*Table 3*

*Total flavonoids production from electroporaited, Ri-transformed and non transformed root cells of Glycyrrhiza glabra and its RLAR effect on rat lens*

| Type of culture | Flavonoids production (g/l) | Aldose reductase* inhibition (%) | |
|---|---|---|---|
| | | 0,1 $\mu$kg/ml | 1,0 $\mu$kg/ml |
| Control (untransformed cells) | 0.2 | 0.6 | 21.4 |
| Electroporated licorice proto-plasts by plasmid pDNT23 35ScaMV-npt-II-nos-cat | 1.9 | 2.1 | 3.7 |
| Licorice cell clusters inoculated by *A. rhizogenes* 15834 | 3.42 | 11.8 | 58.6 |

** — RLAR activities were measured from rat lenses which have been homogenized and centifugated at 1000000 x g, and then the supernatant was used for enzyme fraction. RLAR activities were used assayed using 1 mM dl-glyceralaldegide as a substate by determinigf the increase of fluorescence NADP in the presence of 6N NaOH by a Hewlett-Packard fluorescent spectrophotometer.*

*Table 4*

*Total terpens production from electroporated cells and hairy roots of Potentilla alba and their effect on thyroxine level in rats*

| Type of culture production | Terpens (mg/g dry wt.) | Concentration of total terpens (%) | Thyroxine nmol/l, M±m, n=7 |
|---|---|---|---|
| Control (untransformed roots) | 0.45 | 0.0 | 37±16.1 |
| Hairy roots induced by *A.rhizogenes* 15834 (agarized medium) | 0.67 | 0.3 | 14.0±3.3 |
| Hairy roots induced by *A.rhizogenes* 15834 (liquid medium) | 1.2 | 0.3 | 9.8±5.2 |

**Thyroxin levels have been tested on thyroid glands on young white rats, $p < 0,05$.*

gene transfer to licorice isolated protoplasts as well as of the electroporation conditions necessary for successful transient gene expression. This technology is promising for biotransformation of important metabolites (flavonoids and glycyrrhizin) by bacteria or plant cells.

The present investigation demonstrates that flavonoids obtained from genetically transformed licorice cells and hairy root cultures of *G.glabra* possess a potent RLAR inhibiting activity. They are promising agents for the prevention of inflammatory reaction after γ-irradiation of experimental rats. It has been also shown, the isolated triterpens from transgenic *Potentilla alba L.* «hairy roots» culture to possess thyroidoprotective effects after γ-radiation.

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**Отримання вторинних метаболітів із трансформованих клітин Glycyrrhiza glabra і Potentilla alba — продуцентів сполук радіопротекторної природи**

П. Г. Коваленко, В. П. Антонюк, С. С. Малюта[1]

Інститут молекулярної біології і генетики НАН України,
вул. Академіка Заболотного, 150, Київ, 03143, Україна
[1] Біоспиртпрод, Київ, Україна

**Резюме.** Використовуючи сучасні біотехнологічні методи, було проведено скринінг вторинних метаболітів радіопротекторної природи, що дуже актуально після Чорнобильської катастрофи. З цією метою досліджували накопичення вторинних метаболітів у генетично трансформованих суспензійних клітинах кореня солодцю Glycyrrhiza glabra L. Дібрано оптимальні умови електропорації ізольованих протопластів солодцю за допомогою плазміди, яка містить коренеспецифічний промотор (pDNt23). Отримані клітинні агрегати було додатково інфіковано агробактеріальним штамом (Agrobacterium rhizogenes 15834). Таким чином вдалося отримати культуру «hairy roots» солодцю зі збільшеним виходом вторинних метаболітів флавоноїдної природи. У культурі клітин перстачу білого Potentilla alba L. також вдалося отримати штами трансгенних клітин зі збільшеним виходом терпенів. Ізольовані метаболіти було протестовано на щурах як потенційні біологічно активні радіозахисні сполуки.

**Ключові слова:** Glycyrrhiza glabra, Potentilla alba, екзогенна ДНК, електропорація, Ri-плазміди, культура кореневих волосків.

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