EFFECT OF THE ELICITORS ON SECONDARY METABOLITES PRODUCTION BY LICORICE (*Glycyrrhiza uralensis Fisch*.) HAIRY ROOTS CULTURES

P.G.KOVALENKO, **S.S.MALIUTA**

Department of Molecular Genetics, Institute of Molecular Biology and Genetics NASU, Zabolotnogo #150, Kyiv 03143, UKRAINE.

E-mail:p.g.kovalenko@imbg.org.ua

Plant roots are used as a source of pharmaceuticals, dietary products, agrochemical, flavors, fragrances and many others specially chemicals. However, the roots of licorice have been shown to contain more than 100 other bioactive compounds and especially such as antileukemic inductors [1-4]. Licorice root contains a variety of compounds. The most prominent is the water-soluble triterpenoid glycoside glycyrrhizin (GL). The root content of this compound varies from 2%-4% depending upon growing conditions . GL has been shown in several studies to have antiviral activity against the human immunodeficiency virus (HIV) both in vitro and in vivo. Gl, has been known as an antiviral agent, its IC_{50} for HIV-1 in MT-4 cells being 0.15 mM. The mechanism of action of Gl may at least partially be attributed to an interference with virus-cell binding [2]. It blocked plaque formation and HIV-specific antigen expression in one in vitro study. In a subsequent study by this same group GL sulfate was found to be four times stronger than GL and was also an effective inhibitor of HIV reverse transcriptase . In addition, several phenolic compounds isolated from licorice, especially licopyranocoumarin, inhibited the cytopathic activity of human HIV in cell culture. Glycyrrhizic acid (GA) is the major bioactive triterpene glycoside of licorice root (Glycyrrhiza Radix) extracts possessing a wide range of pharmacological properties (anti-inflammatory, anti-ulcer, antiallergic, anti-dote, anti-oxidant, anti-tumor, anti-viral etc.). Official sources of GA are *Glycyrrhiza glabra* L. and *G. uralensis Fish. (Leguminosae*). GL is one of the leading natural compounds for clinical trials of chronic active viral hepatitis and HIV infections (preparation Stronger Neo-Minophagen C, SNMC), and its monoammonium salt (glycyram, tussilinar) is used as an anti-inflammatory and anti-allergic remedy. Gl is an inhibitor of lipoxygenase and cyclooxygenase, inhibits protein kinase C, and downregulates the epidermal growth factor receptor, and also has anticarcinogenic properties as to induce a variety of enzymes (phase II catalysts) involved in the detoxification and excretion of carcinogenic and toxic substances. Licorice polyphenols induce apoptosis in cancer cells. Furthermore, as licorice plants can grow only in limited regions in the world. The objective for this purpose it is very important to improve biotechnological potential in *G.uralensis* Fischer, *in vitro* culture**.** The accumulation of GL by licorice tissues *in vitro* has been detected in callus and in hairy roots culture. Hairy root cultures induced by transformation with the soil-borne pathogen *Agrobacterium rhizogenes* are an attractive experimental system as they are long-term aseptic root clones, genetically stable, with growth rates comparable to those of the fastest growing cell suspension cultures. Manipulation of the culture environment allows us to study the production of the important plant natural product compounds in the defined culture. And the main objective of this work was to examine the hairy roots culture and elicitor-like effect for the increase of the GL production on licorice cells. We report here on the isolation and identification of GL from Ri T-DNA

transformed roots of licorice plants species and elicitor's effect, which have been successfully, used for increase the secondary metabolites production.

Materials and methods

Seeds of Glycyrrhiza species were obtained from the Crimea Botanical Garden (Ukraine). In our studies on licorice hairy root cultures to produce useful compounds, we investigated the constituents of *G.uralensis* hairy root cultures which were established by induction of *A. rhizogenes* strains (LBA9402, 15834, 8196) and *A. tumefaciens* strain *R1000(pRiA4/pBI121)*. Explants were immersed in *Agrobacterium* diluted 1:1 (v:v) with liquid MS medium [1:5; 1:10 and 1:20 (v:v)]. Production of hairy roots was observed on the leaf and petiole explants from cultivated plants when inoculated with *A. rhizogenes* strains in combination with acetosyringone. Opine analysis of the cultures transformed by the strain 8196 and 15834 were performed . Expression of the *GUS* reporter gene was detected on the explants from *in vitro* seedlings and inoculated with strains R1000 of *A. tumefaciens* and LBA 9402. The measure the growth and secondary metabolite production, the hairy roots were harvested by filtration, washed twice with distilled water, and lyophilized. The ability of the total phenolics production was evaluated by analyzing the total phenolics contents of the transformed cells and the media. Lyophilized hairy roots were analyzed by TLC chromatography.

Results and discussion

In the present research we have confirmed a method to obtain transgenic licorice roots in which the foreign genes expressed quite efficiently (Fig.1). And the present studies demonstrated that *Glycyrrhiza* species is highly sensitive to *Agrobacterium*-mediated transformation, and especially when used a super virulent *Agrobacterium rhizogenes* strains (Table 1).

Table 1. Effect of different strains of *Agrobacterium rhizogenes* on the frequency of infection and the growth of licorice hairy root cultures

***Agrobacterium* strain**	**Infection frequency[a] *(%)***	**Number of hairy roots (or tumors)**	**Root length (cm)**
LBA 9402	35	3.3±1.4	2.5±0.3
15834	31	3.2±1.1[b]	2.2±0.3
8196	28	3.7±1.2[b]	2.7±0.2
R1000	46	4.3±1.5	3.3±0.2

[a]Percentage of seedlings from which antibiotic-resistant tissues emerged.[b]Caused the induction of tumours. Values represent the mean±SD of three independent measurements 28 d after inoculation. Approximately 50 seedlings were examined for each measurement. Wounded hypocotyls and true leaves were susceptible to infection by strains R1000 and LBA 9404. Other strain as 15834 infected more than 25–30% of explants, but, in contrast, strain 8196 infected 20–28% of the exposed tissue. The transformed status of the hairy root cultures were analyzed by paper electrophoresis for the transgenic lines after inoculation by strains as 15834 and 8196. Agropine and mannopine are well known opines detectable in hairy roots transformed with these Ri

plasmids (Fig.2). On the other hand, the complete and stable transformation by strains LBA 9402 and R1000 were evaluated by determining (1) the histochemical localization of GUS activity in various root tissues.Strong GUS staining was visible in the growing root tips and vascular tissues of young transgenic roots, and *NPTII*-positive (kanamycin sulphate concentration was 50 mg/l) hairy roots of *G.uralensis* produced after co-cultivation of wild-type seedlings with *A. rhizogenes* strain R1000. The observed distribution in GUS transcript and enzyme activity levels is a common phenomenon in transformed plant tissues due to a combination of several factors including transgene copy number, the location of chromosomal insertion, or a variety of post-translational effects. An efficient *A. rhizogenes*-mediated protocol has been developed for the establishment of transgenic *G.uralensis* hairy root cultures. From the four *A. rhizogenes* strains tested, LBA 9402 and R1000 were found to be the most virulent strains and caused the formation of hairy roots exhibiting the most rapid growth rates. Strain 15834 was less virulent and efficient for hairy root development in *G.uralensis*. Obtained results were shown, what genetically engineered root cultures are suitable model system to study various aspects of the metabolic and molecular regulation of several natural product pathways. Methods of Ri T-DNA transformation established in this study could be used to develop transgenic plant of pharmaceutical important licorice plant species (Table 2). It should be pointed that a major interest in the transformation study with these licorice species are due to the possibilities in genetic manipulation of the hairy roots secondary metabolites production as a Gl and flavonoids.

Table 2. Flavonoids and glycyrrhizin (Gl) production from hairy roots of *G.uralensis* elicited by biotic elicitors.

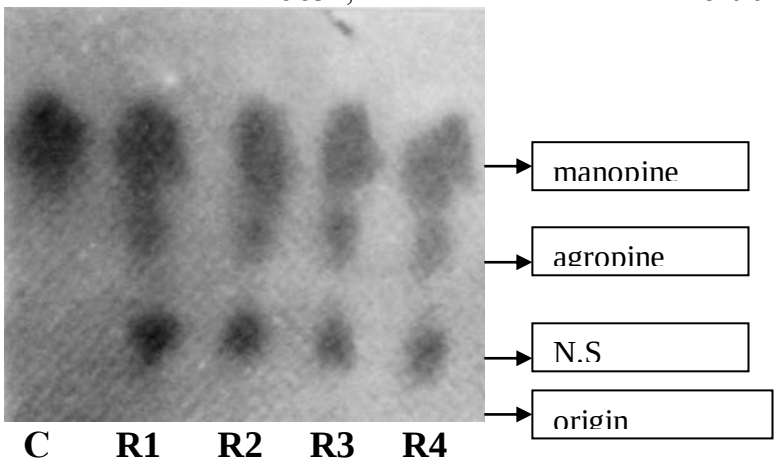
Strains number	**Used *A.rhizogenes* strains**	**Elicitors (mg/l); fungal extract as diluted**	**Total flavonids (%, dry weight)**	**Gl (%, dry weight)**
1-MS	LBA/9402	50-YE+10^{-4} f.extr.	13.4	Non-ident.
2-MS	LBA/9402	50 0-YE+10^{-6} f.extr.	13.6	Non.ident.
3-MS	8196	50-YE+10^{-6} f.extr.	13.9	2.1
4-MS	8196	50 0-YE+10^{-6} f.extr	14.4	3.6

The roots on free growth regulators medium showed fast highly branched growth characteristic of hairy roots, filing the cultures dishes after 21 days. Obtained results were shown, what soil-borne pathogens of the genus *Agrobacterium* are able to transfer part of their DNA, the T-DNA carried on a large plasmid, to the genome of a host licorice cells. *Agrobacterium rhizogenes* is the causal agent of ‘hairy root’

diseases in other plant species too. Over the last decade, transformed root cultures from plants have attracted considerable attention because of their genetic and biochemical stability, rapid growth rate and ability to synthesize secondary products at levels comparable to wild-type roots. Clearly, the selection of an effective *Agrobacterium* strain for the production of transformed root cultures is highly dependent on the plant species, and must be determined empirically. The differences in virulence, morphology and growth rate are at least partially related to the variety of plasmids contained within each bacterial strain. It should be pointed that a major interest in the transformation study with these licorice species are due to the possibilities in genetic manipulation of the hairy roots secondary metabolites production as a Gl and flavonoids. Quite important what in this our work we presented novel *Acremonium sp*. fungus as highly efficient biotic elicitor for secondary metabolite accumulation in transformed licorice roots. Significant increase in Gl production by elicitated hairy roots cultures of *G.uralensis* was observed in the presence of this fungal elicitor. It is considered that this new fungal elicitor may be applicable to other pharmaceutical plant cell culture systems for efficient production of the important secondary metabolites.. However, the relationship between the chemical structure of elicitor and its stimulating activity needs further investigation for rational design and synthesis of more potent elicitors for the specific flavonoids and Gl production. The elicitation effect signaled plant defense responses, which assumes that environmental stimuli are perceived at receptors on the plasma membrane and trigger the biosynthesis of specific proteins. However, so far little is known about putative elicitor receptors that bind fungal metabolites specifically. The mechanism of signal perception and transduction process evoked by an exogenous elicitor remains to be verified through experiments.

A **B**

Fig. 1 Transgenic roots of *G. uralensis* (28 th day of culture). Lane A: transformation with 15834; lanes B: transformed with 8196.



C **R1** **R2** **R3** **R4**

Fig.2. Paper electrophoretic analysis of extracts from hairy root cultures of G.*uralensis* C -control (non transformed roots) ; R1-R4 licorice hairy root clones.

analyzed.

References

1. *Kovalenko P.G., Antonjuk V.P., Maliuta S.S*. Secondary metabolites production from transformed cells of *Glycyrrhiza glabra* and *Potentilla alba* as a producents of radio protective compounds // Ukrainica Bioorganica Acta.-2002.-**1**, N1.-P.21-31.
2. Ito M., Sato A., Hirabayashi K., Tanaka F., et al. Mechanism of inhibitory effect of glycyrrhizin on replication of human immunodeficiency virus (HIV) // Antiviral Res.-1988.-**10**.-P.289-298.
3. Yoshikawa M., et al. Effect of glycyrrhizin on immune-mediated cytotoxicy// J.Gastroenterol.Hepatol. –1997.-**12**.-P. 243-248.
4. *Logemanna W., Lauria F*. Antileukaemic activity of glycyrrhetinic acid //Nature.1960.-**187**.-p.607-608.
5. *Hatano T, Yasuhara T, et al*. Anti-human immunodeficiency virus phenolics from licorice // Chem Pharm Bull. 1988.-**36**.-p.2286-2288.
6. *Ito M, Nakashima H*. Inhibitory effect of glycyrrhizin on the in vitro infectivity and cytopathic activity of the human immunodeficiency virus// Antiviral Res. 1987.-**7**.-127-137.