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L.O. SAKHNO [1], O.Y. KVASKO [1],
Z.M. OLEVINSKA [2], M.Y. SPIVAK [2], M.V. KUCHUK [1]

[1] Institute of Cell Biology and Genetic Engineering NAS of Ukraine, Kyiv
E-mail:sakhno@icbge.org.ua

[2] Zabolotny Institute of Microbiology and Virology NAS of Ukraine, Kyiv

CREATION OF TRANSGENIC *BRASSICA NAPUS* L. PLANTS EXPRESSING HUMAN ALPHA 2b INTERFERON GENE

Spring rapeseed transgenic lines expressing human interferon alpha 2b were created by Agrobacterium-mediated transformation of aseptic plant leaf explants. The maximum antiviral activity of the leaf extracts reached 4500 IU/g fresh weight. It was determined that the antioxidant activity and the activity of an enzyme of plant antioxidant system – superoxide dismutase (SOD) – in the leaf tissues of transgenic plants increased compared to controls. There were no correlations between the interferon and antioxidant activities, as well as between SOD and interferon activities. Using the obtained transgenic rapeseed plants with high interferon and antioxidant activities as a feed additive for animals might have preventive effect on their body, increasing resistance to infections of various origins.

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Introduction. Over the last decade has intensified research on obtaining plants that synthesize biological molecules with pharmaceutical orientation – antibodies, cytokines, recombinant enzymes, hormones, vaccines for human and farm animals [1–3].

Rapeseed has been successfully grown for the production of animal feed (green mass, silage, haylage, grass meal) in different crops in pure form and in mixtures with other cultures [4]. Cattle have grazed at rapeseed sowing because it has the ability to intensive regrowth after cattle nibble or mowing. For biochemical characteristics rapeseed exceeds most forage crops. In 1 kg of green mass is usually synthesized up to 30 g protein, easily digested, a significant amount of ascorbic acid (505–1070 mg/kg for winter cvs and 515–786 mg/kg for spring cvs) and carotene (19,4–42,9 mg/kg for winter and 20,6–37,4 mg/kg for spring) [4, 5]. Along with standard measures, changes in their diet could help to prevent the animal incidence: use as feed additives to green mass rapeseed plants that produce pharmacological proteins and have increased biochemical properties.

There are many rapeseed and rapeseed biotechnological studies on fatty acid changes [6–8], herbicide, pest, disease and abiotic stress resistance [9–13], pharmaceuticals production [14, 15].

Our experiments aimed to create rapeseed plants with human interferon alpha 2b gene and to characterize some of their biochemical parameters such as antiviral, antioxidant and superoxide dismutase (SOD) activities.

Materials and methods. *Plant material.* Aseptic plant leaves (temperature 24 °C, 4000–5000 lux, 16/8 light photoperiod) were used as a raw material. There are spring rapeseed cv Magnat (National Agrarian University of UAAS selection) and cv Magnat (Science-practical center of NAS of Belarus on agriculture selection). Seeds were kindly provided by M.V. Slisarchuk (department of linseed and rapeseed selection and seed production of National Scientific center «Institute of UAAS on agriculture») and by A.M. Shishlova (department of molecular genetics and biotechnology of Institute of genetics and cytology of NAS of Belarus), respectively.

Genetic transformation, bacterium strain and vectors. Transformation of precultivated leaf explants from 3–4 week plants were carried out according to our method proposed earlier [16]. *Agrobacterium tumefaciens* strain GV3101 was used

for plant transformation. *Escherichia coli* strain XL1Blue was applied for cloning of binary plasmid vectors. Plasmid vectors pICH5290 and pICH17311 were generously donated by Icon Genetics GmbH (Germany). Restriction endonucleases (REs) and T4 DNA ligase were used with supplied buffers («Fermentas», Lithuania). Bacterial cell transformation, plasmid DNA isolation and electrophoretic analysis were carried out as described in [17]. A short nucleotide sequence containing *Bam*HI and *Xba*I recognition sites was added to the pICH17311 construct digested with *Pst*I RE. Recombinant *HuIFN-α2b* gene was excised with *Nco*I and *Xba*I REs and ligated into pICH5290 vector predigested with the same REs. The obtained vector were designated as pCB125 (Fig. 1, *a*) [18].

PCR analysis was carried out with primers INTFOR 5'-ctcctgcttgaaggacag-3' and INTREV 5'-ggagtcctccttcatcag-3' amplifying the 264 bp fragment of *HuIFN-α2b* gene. To detect agrobacterial contamination, primer pair (VirD1-1 5'-atgtcgcaaggcagtaagccca-3' and VirD1-2 5'-ggagtctttcagcatggagcaa-3') was used for amplification of 264 bp fragment of *virD1* gene. The reaction mixture contained 1 μg of total plant DNA; 0.25 μM of each primer; 0.5 u of Taq DNA polymerase and corresponding buffer («Helicon», Russia); 0.5 mM of deoxynucleotide triphosphates (dNTPs) (0.125 mM each). Amplification was conducted under following conditions: 94 °C 5 min → (94 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec) ×30 → 72 °C 5 min [19].

The total soluble protein (TSP) content was measured by the method of Bradford [20]. Extracts from plant leaves were prepared in double volume of 100 mM Tris/HCl buffer, pH 8.0, containing 5 mM Na_2EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol, and 2.5 % PVP.

Antiviral activity of leaf extracts. The interferon activity was measured by microtitration method [21] based on the studied extracts ability to protect established piglet testicular cell culture (from cell collection of Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine) against cytopathic effect of vesicular stomatitis virus (Indiana strain, from collection of Danilo Zabolotny Institute of Microbiology and Virology, NAS of Ukraine).

Antioxidant activity was detected by Semenov et al. [22] with slight modification. Leaves (300 mg) were ground with 1.5 ml of 0.25 M phosphate buffer (pH 7.4). Homogenate were transferred to Eppendorf tube and centrifuged 10 min at 4000 r/min. Reagent mixtures were consisted of 1.5 ml of 0.25 M phosphate buffer (pH 7.4), 0.5 ml of 0.8 mM 2,6-dichlorphenolindophenolat Na (DCPP Na), 0.5 ml 3.2 mM $FeSO_4$ and 0.5 ml supernatant (experiment) or 0.5 ml distil water (control). They were transferred into cuvette (1 cm) and solution optical density (D_τ, 510 nm) was detected each 30 sec during 5 min. Also solution optical density (D_∞, 510 nm) was detected when distil water were used instead of $FeSO_4$. D_∞ characterised total DCPP Na oxidation. As indexes of plant material antioxidant activity were considered the means of inhibition constant (K_i) of DCPP Na oxidation. They were calculated by formula:

$$K_i = \frac{K_{control} - K_{exp}}{C},$$

$K_{control}$ and K_{exp} were constant of speed oxidation of DCPP Na in control and experimental solution, respectively, ml/l min. They were rated as the slope on the graph of the natural logarithm ΔD_τ ($\Delta D_\tau = D_\infty - D_\tau$) from time. C – plant material concentration in cuvette, mg/ml.

Superoxide dismutase (SOD) activity. Photochemical oxidation of nitro blue tetrazolium method were used for determination of SOD activity [23]. Processed liquid nitrogen plant material (100 mg) in Eppendorf tube (1.5 ml) was rubbed with 1 ml of Tris-HCl buffer (pH 8.0) and was centrifuged at 13000 g (4 °C) during 15 min. Supernatant was used for analyses. Reaction was held in Eppendorf tube (1.5 ml). One tube for each probe was retained in the dark. The other was illuminated by white light lamp (fluorescent lamp T5/G5, model ELI-230A-T5-8W) during 5 min. in the thermostat at 23 °C. The solution optical density of illuminated probe was measured at 550 nm («BioPhotomether Eppendorf», Germany) versus optical density of dark probe. Null probe had in its composition leaf extract. The calculation was carried out by the formula:

$$\text{SOD (relative unit/ml suspension)} = (OD_1/OD_2 - 1)\,[DF],$$

OD_1 – optical density of null probe; OD_2 – optical density of experimental probe; DF – dilution factor = reaction mixture volume, ml/used plant

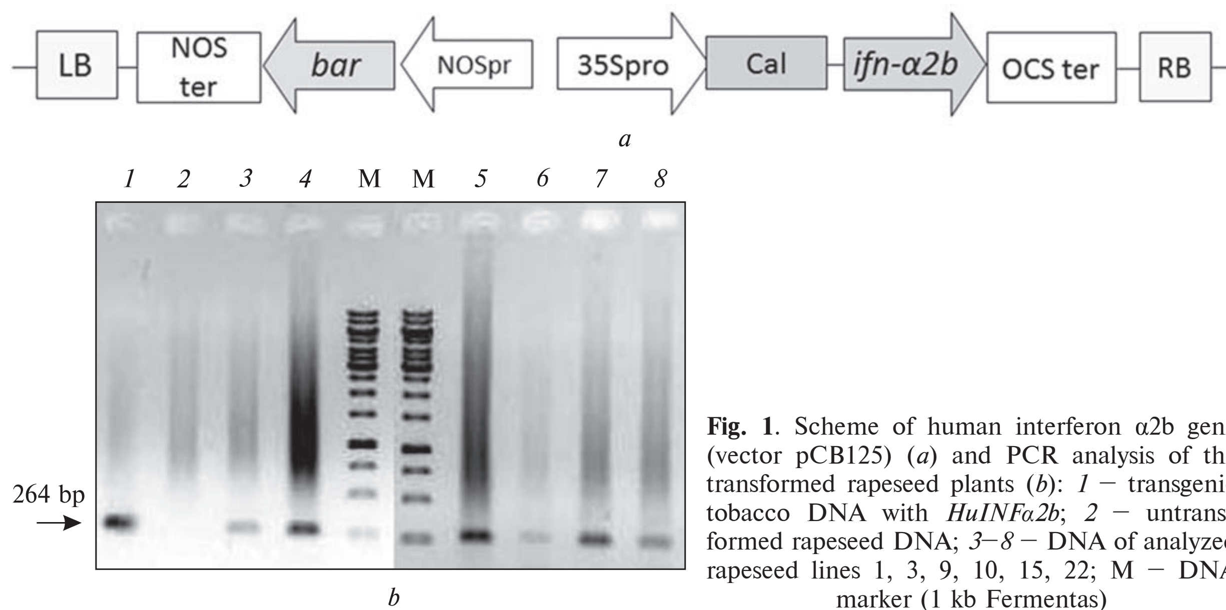

Fig. 1. Scheme of human interferon α2b gene (vector pCB125) (*a*) and PCR analysis of the transformed rapeseed plants (*b*): *1* – transgenic tobacco DNA with *HuINFα2b*; *2* – untransformed rapeseed DNA; *3–8* – DNA of analyzed rapeseed lines 1, 3, 9, 10, 15, 22; M – DNA marker (1 kb Fermentas)

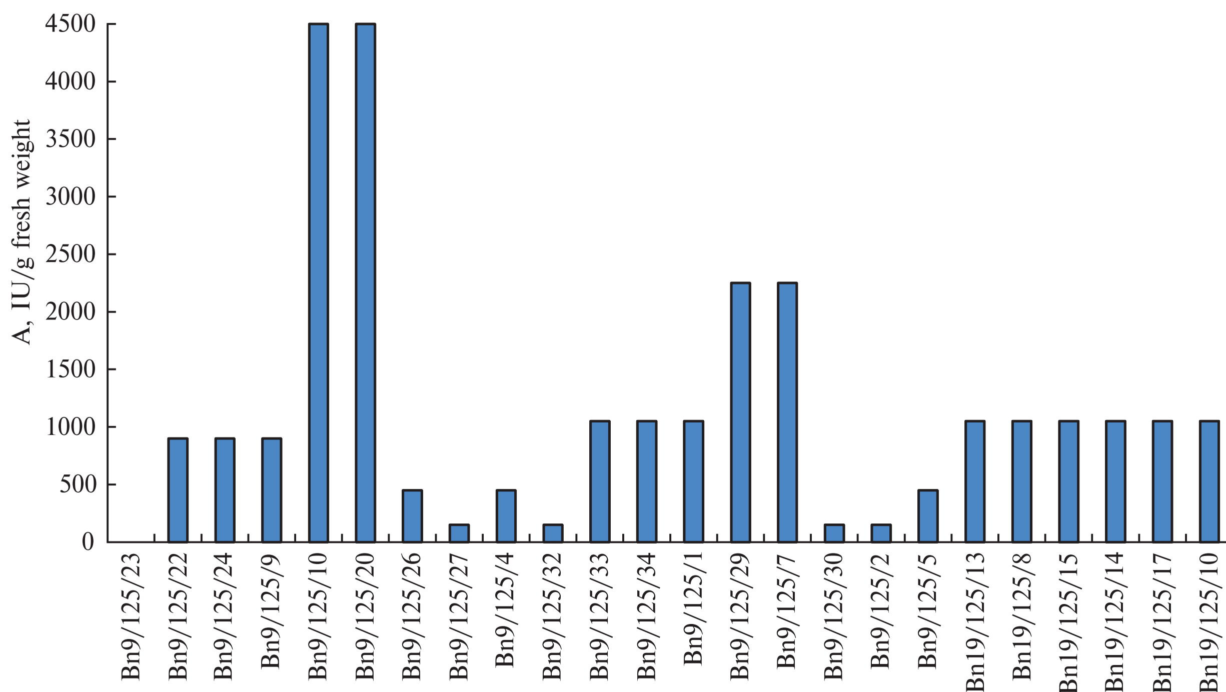

Fig. 2. Interferon activity in leaves of primary rapeseed transformants

extract volume, ml; SOD activity was expressed in relative unit/mg protein.

The statistical design was carried on for the obtained results in way of interferon antiviral activity, antioxidant and SOD activity measurement. Differences from control values were significant at $P < 0.05$ according to Duncan's multiple range test.

Results and discussion. The first regenerants were formed in selective condition (phosphinothricin, 5 mg/l) after 5–6 weeks from the beginning

of experiments. It took 7–14 days for complete formation of shoots. Root formation took place without additional induction after regenerant passage on MS culture medium [24] without hormone during 2–3 weeks.

As a result of experiments 52 phosphinothricin resistant rapeseed lines were created. There are 18 lines on basis of cv Magnat (National Agrarian University of UAAS selection) and 32 ones derived from cv Magnat (Science-practical center of NAS of Belarus on agriculture selection).

Plant lines were propagated *in vitro* by graftage and tested for *HuINFα2b* gene presence and *Agrobacterium* contamination absence. PCR analyses detected presence of target DNA in obtained plants (Fig.1, *b*). Bacterial contamination was absent.

Aseptic leaves of transformed plants were used for protein extraction to test the activity of human interferon α2b. It was determined difference of interferon activity between transgenic line extracts (Fig. 2).

Interferon activity in rapeseed primary transformants growing under *in vitro* conditions varied from complete absence (line Bn9/125/23) to 4500 IU/g fresh weight. The maximum protein activity for this group of plants was recorded for the two lines – Bn9/125/20 and Bn9/125/10. In seven rapeseed lines interferon activity was lower than 500 IU/g fresh weight. In most lines (14) it varied from 900 up 2250 IU/g fresh weight.

Rapeseed lines with high interferon activity were planted into soil under greenhouse conditions. They are easily adapted, and blossoms without anomalies formed viable seeds.

Four weeks after planting the parts of second from apex leaf were extracted for interferon activity evaluation. It was found that in the lines Bn9/125/20 and Bn9/125/10 interferon activity remained at the same level as under aseptic culture.

To study the changes in the adaptation ability of obtained rapeseed plants biochemical parameters such as leaf tissue antioxidant activity and the activity of one of the enzymes of plant antioxidant system – superoxide dismutase – were investigated. It was shown that some transgenic rapeseed lines had increased antioxidant activity (Fig. 3).

Antioxidants protect cell structures from damage by free radicals [25]. The main natural

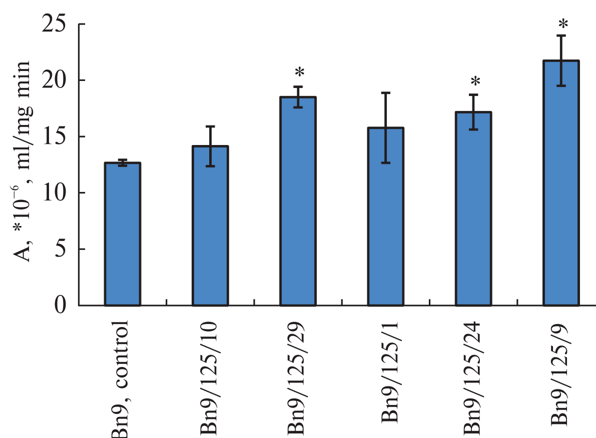

Fig. 3. Leaf tissue antioxidant activity in control (Bn9, cv Magnat) and carrying *HuINFα*2b gene biotechnological rapeseed lines

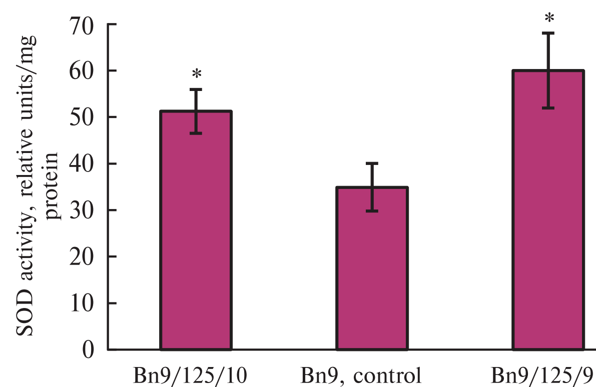

Fig. 4. SOD activity in leaf tissue of aseptic rapeseed plants

antioxidants are vitamins E and C, fatty acids, polyphenols. Increased antioxidant activity in plant tissues positively affects their ability to resist stress factors of various origins [26, 27].

Rapeseed leaves were shown to have antioxidant activity, which exceeds that in many traditionally cultivated plants [28]. In recalculation on ascorbic acid it was 710 μg/g raw weight. This index was lower for the letter lettuce and spinach (446 and 434 μg/g raw weight, respectively), in red cabbage leaves was almost twice higher (1217 μg/g raw weight).

Using the obtained rapeseed plants with high antioxidant activity as a feed additive for animals should have preventive effect, increasing resistance to infections of various origins.

Comparison of α2b interferon and antioxidant activity in leaf tissues of plants grown under

Antioxidant and interferon activities in leaf tissue of biotech rapeseed lines with *HuINFα2b* gene

Lines	Antioxidant activity ×10^{-6}, ml/mg min	Interferon activity, IU/g fresh weight
Bn9, control	12,67 ± 0,27	0
Bn9/125/10	14,13 ± 1,76	4500
Bn9/125/29	18,50 ± 0,82	2250
Bn9/125/1	15,77 ± 3,11	1050
Bn9/125/24	17,17 ± 1,55	900
Bn9/125/9	21,73 ± 2,23	900

aseptic conditions showed that there was no correlation between these two indicators (Table).

Formasan (violet color substance) formation occured as a result of photochemical oxidative reaction of nitro blue tetrazolium. In the null probe oxidation was complete and plant extracts could inhibit formasan formation due to SOD activity. SOD activity calculations showed that it increased in the analyzed biotech rapeseed lines compared with the control one (Fig. 4).

In transformed rapeseed plants, which expressed the wheat mitochondrial Mn SOD (*Mn SOD3.1* gene), an increased ability to resist stress of various origin was observed [29]. They germinated in laboratory and field conditions faster than control plants and were kept drought, the influence of high and low temperatures. Sensitivity to stress of our obtained transgenic rapeseed plants needs further investigation.

It should be noted that both SOD activity and antioxidant activity in leaf tissues of Bn9/125/9 line was higher than in controls. Increased antioxidant activity was not observed for Bn9/125/10 line, although SOD activity in its tissues was raised (Fig. 2 and 4). Perhaps the increased SOD activity in this case was leveled by a decrease of some other component of the antioxidant system (e.g. changing the content of ascorbic acid or tocopherol, the activity of enzymes catalase or peroxidase, etc.).

Conclusions. We obtained transgenic rapeseed plants, which are characterized by the presence in their nuclear genome of human interferon α2b gene. We showed that biotechnological lines had different activity of introduced gene, the maximum value reached 4,500 IU/g fresh weight. Using the obtained plants with high antioxidant activity as a feed additive for animals might have preventive effect, increasing resistance to infections of various origins.

Created rapeseed lines possessed also such biochemical characteristics as high antioxidant activity of leaf tissue under *in vitro* cultivation and increased activity of antioxidant system enzyme – superoxide dismutase. This gives reason to hope for increased resistance of transgenic lines to biotic and abiotic stresses.

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Л.А. Сахно, Е.Ю. Кваско,
З.М. Олевинская, Н.Я. Спивак, Н.В. Кучук

СОЗДАНИЕ ТРАНСФОРМИРОВАННЫХ РАСТЕНИЙ *BRASSICA NAPUS* L., ЭКСПРЕССИРУЮЩИХ РЕКОМБИНАНТНЫЙ АЛЬФА 2b ИНТЕРФЕРОН ЧЕЛОВЕКА

Методом агробактериальной трансформации листовых эксплантов асептических растений ярового рапса получены линии, экспрессирующие альфа 2b интерферон человека. Максимальная противовирусная активность экстрактов листьев достигала 4500 МЕ/сырого веса. Установлено, что антиоксидантная активность и активность одного из ферментов антиоксидантной системы растений – супероксиддисмутазы (СОД) – в тканях листьев трансгенных растений повышена по сравнению с контрольными. Не выявлено корреляции как между активностью интерферона и антиоксидантной активностью, так и между активностью интерферона и активностью СОД. Использование полученных трансгенных растений рапса с высокой активностью интерферона и повышенной антиоксидантной активностью как добавки к кормам животных могло бы оказывать профилактическое влияние на их организм, повышая устойчивость к различным инфекциям.

Л.О. Сахно, О.Ю. Кваско,
З.М. Олевінська, М.Я. Спивак, М.В. Кучук

СТВОРЕННЯ ТРАНСФОРМОВАНИХ РОСЛИН *BRASSICA NAPUS* L., ЯКІ ЕКСПРЕСУЮТЬ РЕКОМБІНАНТНИЙ АЛЬФА 2b ІНТЕРФЕРОН ЛЮДИНИ

Методом агробактеріальної трансформації листових експлантів асептичних рослин ярого ріпака отримано лінії, що експресують альфа 2b інтерферон людини. Максимальна противірусна активність екстрактів листків сягала 4500 МО/сирої

ваги. Встановлено, що антиоксидантна активність і активність одного з ферментів антиоксидантної системи рослин – супероксиддисмутази (СОД) – в тканинах листків трансгенних рослин підвищена в порівнянні з контрольними. Не виявлено кореляції ні між активністю інтерферона і антиоксидантною активністю, ні між активністю інтерферона і активністю СОД. Використання отриманих трансгенних рослин ріпака з високою активністю інтерферона та підвищеною антиоксидантною активністю як добавки до кормів тварин мало б профілактично впливати на їхній організм, підвищуючи стійкість до інфекцій різного походження.

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