EXPERIMENTAL ARTICLES

UDC 577.21+604.6:582.983.2+631.348 doi: 10.15407/biotech8.02.052

COMPARATIVE MOLECULAR GENETIC ANALYSIS BETWEEN UKRAINIAN AND EU REGISTRATED GLYPHOSATE-TOLERANT RAPESEED TRANSGENIC PLANTS

A. M. Taranenko
L. O. Sakhno
B. V. Morgun
M. V. Kuchuk

Institute of Cell Biology and Genetic Engineering
of the National Academy of Sciences of Ukraine, Kyiv

E-mail: molgen@icbge.org.ua

Received 28.10.2014

The purpose of research was to analyze 10 developed at the Institute of Cell Biology and Genetic Engineering lines of rapeseed to confirm the presence and functionality of the transferred transgene CP4 *epsps*, as well as the differences among those lines from registered transformation events GT73 and GT200 (Monsanto).

During the study extraction of total rapeseed DNA, PCR analysis, electrophoretic separation and visualization of amplicons in agarose gel were conducted, as well as testing of green plants for resistance to glyphosate in greenhouse. The structural difference among 7 transgenic lines from registered transformation events GT73 and GT200 was revealed. Plants showing the presence of synthetic CP4 *epsps* sequence were resistant to the herbicide in a closed soil. The uniqueness of the obtained transformation events was confirmed, as well as the prospect of using them in breeding.

Key words: rapeseed, glyphosate, genetic transformation, CP4 *epsps* gene.

Taking into account an importance of rapeseed as agricultural crop, creation of new transgenic lines with improved properties is a timely task. Rapeseed (*Brassica napus* L.) is one of the most important oilseed crops. Rape oil is on the third place after palm oil and soybean oil on a scale of world production, and by import and export scales it takes the fourth place. Rape oil processing products have their use at food, medical, chemical, paint, fuel, printing, textile, metallurgy, machinery industries. The percentage of vegetable-based fuel derived from rape oil is 5.8% of the total scale, and till 2015 this index can increase to 8%. Rape oil meal after oil removing is a valuable component of cattle feed, because it contents about 32% of protein, 9% of fat, 30% of nonnitrogenous extractive substances. Digestive coefficient of those compounds is achieving to 70%. Green plant biomass also fits to be cattle feed starting from the middle spring.

Rapeseed is an important nectariferous plant too. During the flowering period honey production is about 80 kg from 1 hectare. Rapeseed is an optimal predecessor for cereal crops, because it improves the phytosanitary conditions of soil. Rapeseed biomass during its decomposition is producing glycosinolates — sulfuric compounds reducing weakness of following crops to fusariosis, black spot, septospyrosis and bacterial stem diseases [1].

Up to 2012 the leaders of world rapeseed production were China, Canada, European Union, India and United States [2].

The great importance of rapeseed as an agricultural crop stimulates for developing the new transgenic lines — as with changed biochemical characteristics, also with added mechanisms of resistance to multiple biotic and abiotic factors. In other way, the development of new methods for detection of transgenic sequences in plant material is no doubt an important direction.

Herbicides used to struggle the weeds in crop fields treatment, significantly harm to agricultural plants. Consequently the priority task is the development of rapeseed lines, resistant to herbicides, as an example — to glyphosate. Two lines with same resistance

were developed and commercialized by Monsanto Company. Now they are known as GT73 (RT73) and GT200. These lines were obtained by *Agrobacterium*-mediated transformation of *B. napus* cultivar Westar with vectors pMON17327 and pMON17209 correspondingly. The vectors contained the sequences encoding such enzymes as 5-enolpyruvylshikimate-3-phosphate synthase (*epsps* gene taken from *Agrobacterium tumefaciens* strain CP4) and glyphosate oxidase (*gox* gene taken from *Ochrobacterium anthropi*) [3].

In transformation event MON-ØØØ73-7, also known as GT73 and RT73 [4], the synthetic CP4 *epsps* gene is associated with regulatory sequences: 35S promoter taken from cauliflower mosaic virus (P-CmoVb), 3′-end of *rbcS* E9 gene taken from pea (E9 3′) as terminator, and sequence of chloroplast signal AEPSPS/CTP2, which directs a newly synthesized polypeptide to higher plant's chloroplasts. The expression of *gox* gene is controlled by the similar sequences.

The T-DNA of transformation event GT200 had the resembling structural plan, differing in chloroplast signal peptide sequence only — for this purpose the AEPSPS/CTP1 was used [3, 5].

Methods for rapeseed transformation were formerly developed and adapted at the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine [6]. Basing to this technology rapeseed transformation with a construct, containing synthetic CP4 *epsps* gene, was carried out. Eventually some potentially tolerant to glyphosate transgenic lines were regenerated [7, 8].

The purpose of our work was to analyze comparatively transgenic rapeseed lines $T_2$15/133/2/9, $T_2$15/133/2/3, $T_1$15/133/2/10, $T_0$15/133/5, $T_0$17/133/4, 18a/2, 18b/25, and to confirm the difference among these lines from the registered transformation events GT73 and GT200 (Monsanto).

Materials and Methods

Plant material. The particular genetic constructs with CP4 *epsps* gene giving resistance to glyphosate in model *Nicotiana tabacum* plants were developed at the Institute of Cell Biology and Genetic Engineering of NAS of Ukraine.

Genetic construct pCB133, developed at the Institute of Cell Biology and Genetic Engineering of NAS of Ukraine, was used to transform the spring rapeseed plants. The construct consisted of the following sequences: T*nos* — nopaline synthase gene terminator, P*nos* — nopaline synthase gene promoter, P*35S* — cauliflower mosaic virus gene promoter, T*ocs* — octopine synthase gene terminator, TP — transit peptide, *epsps* — 5-enolpyruvylshikimate-3-phosphate synthase synthetic gene, *bar* — phosphinothricin acetyl transpherase gene as selective one [7, 8]. Transformational frequency for the basic procedure reached 8–9% [6].

For the current study transgenic lines showing 3:1 mendelian segregation after self-pollination were taken on the basis of resistance to the selective agent, indicating that the gene insertion is a single copy [6–10].

For the analysis, leaf explants taken from 3-week old *B. napus* plants [7, 8], raised *in vitro* on MS medium [9] were used. A referent sample of rapeseed DNA with transformation event GT73 was used as a positive control in PCR tests [11].

Extraction of plant DNA was carried out according to the procedure [12, 13] using CTAB buffer (20 g/l CTAB (Serva), 1.4 M NaCl (Alfarus), 0.1 M Tris-HCl pH 8.0 (Merck), 20 мМ Na_2EDTA (Serva), 1,4 µl/ml β-mercaptoethanol (Sigma-Aldrich)), with following extraction of total proteins by chloroform:isoamyl alcohol (24:1) mixture, RNase A (Thermo Scientific) hydrolysis, dissolving of nucleic acids, and, finally, washing with 70% ethanol. The DNA sediment was diluted in 50 µl of TE buffer pH 8.0.

Evaluation of DNA purity. The fractionation of purified total DNA was confirmed by electrophoresis in 0.8% agarose gel with TBE buffer. The concentration of DNA in solution was determined spectrophotometrically by measuring the absorption of UV light at a wavelength of 260 nm (A_{260}) on BioPhotometer v. 1.35 (Eppendorf). The purity of DNA was evaluated by the ratio A_{260}/A_{230} and A_{260}/A_{280}.

The DNA concentration for all samples was normalized to 30 µg/ml with TE buffer.

Polymerase chain reaction (PCR) was conducted using Microcycler Personal thermocycler (Eppendorf). Reaction mixture (20 µl) contained 13.9 µl of sterile deionized water, 2 µl of 2 M dNTP (Thermo Scientific), 2 µl of 10-fold DreamTaq Green amplification buffer, 0.5 µl of 10 mM forward and reverse primers (Metabion), 0.1 µl of 5 u/µl DreamTaq™ DNA polymerase (Thermo Scientific). The characteristics of all primers used in our research are presented in a table below.

The primary PCR reaction was targeted to detect *cruA* gene sequence, encoding the *B. napus* reserve protein cruciferin A (GenBank accession X14555) [14]. The following reactions were directed to detect CP4 *epsps* sequences (GenBank accession JF445290) [15], GT73 and GT200 events.

The amplification program for *cruciferin A* gene sequence was: primary denaturation — at 94 °C for 4 min; 35 cycles: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 16 s; final elongation — at 72 °C for 10 min, with following cooling to 22 °C.

The amplification program for GT73 sequence was: primary denaturation — at 94 °C for 4 min; 35 cycles: 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s; final elongation — at 72 °C for 10 min, with following cooling to 22 °C.

The amplification program for GT200 sequence was: primary denaturation — at 94 °C for 4 min; 35 cycles: 94 °C for 30 s, 57 °C for 30 s, 72 °C for 17 s; final elongation — at 72 °C for 10 min, with following cooling to 22 °C.

The amplification program for CP4 *epsps* sequence was: primary denaturation — at 94 °C for 4 min; 35 cycles: 94 °C for 30 s, 59 °C for 30 s, 72 °C for 32 s; final elongation — at 72 °C for 10 min, with following cooling to 22 °C.

Agarose gel electrophoresis. PCR products were loaded into 1.2% agarose gel (MO BIO Laboratories) in TBE buffer with 5 μg/ml of ethidium bromide, with following separation at 5 V/cm for 45 min. The images of gels were taken in UV light using мLKB Bromma 2011 Macrovue Transilluminator with the digital camera Canon EOS 600D. The visual documents of electrophoresis were processed with the raster graphical redactor GIMP, additionally using Microsoft PowerPoint.

Glyphosate-tolerance testing. Spraying of 3-week old adapted plants was provided in a closed soil with «Hurricane Forte 500 SL, v.p.k.» commercially available herbicide (Syngenta). The concentration of its active component, potassium salt of glyphosate was 500 g/l. Plant treatment was processed with 2.5 μg/l working solution of glyphosate according to the producer's recommendations. The effect of treatment was evaluated in one week.

Results and Discussion

Young rapeseed leaves, taken from 3-week old rapeseed transgenic plants for lines 18a/2 (sample 4), 18b/25 (sample 8) [7], $T_2$15/133/2/9 (sample 1), $T_2$15/133/2/3 (sample 2), $T_1$15/133/2/10 (sample 5), $T_0$15/133/5 (sample 6), $T_0$17/133/4 (sample 9) [8], $T_2$12b (sample 10) [10], Bn12 (cultivar Mariya, sample 3), Bn15 (cultivar Exgold, sample 7), were used for the extraction of DNA. The quality of purified total DNA was determined with spectrophotometer.

Measurements showed that total DNA concentration in the samples fluctuated from 38.0 μg/ml ($T_2$15/133/2/3) to 362.8 μg/ml ($T_0$15/133/5), and the quantity varied from 1.9 μg ($T_2$15/133/2/3) to 18.14 μg ($T_0$15/133/5).

The correlations A_{260}/A_{280} and A_{260}/A_{230} showed the purity of DNA solutions was quite acceptable. Samples 2, 3, 7, and 10 showed some partial contamination of preparations of nucleic acids with proteins.

The pilot polymerase chain reaction was conducted for the evaluation of DNA quality with the purpose to determine *cruA* referent gene, which is known to be responsible for cruciferin A synthesis.

All analyzed samples showed the presence of a fragment, equal to the expected amplicon with all its characteristics (258 b.p. — Fig. 1).

The next stage of molecular genetic research was to confirm the presence of transferred CP4 *epsps* transgene in the DNA

Specifications of primers used in PCR

Name	Gene/event	Sequence	Amplicon length, b.p.	Reference
cruMPF1	*cruA*	5′ — TGGCT AAAGG TACGT GAATC TG — 3′	258	[14]
cruMPR1	*cruA*	5′ — CTCTC CCCAT AAGAC CTTCT CC — 3′	258	[14]
GT1F	GT73	5′ — TGAAC TTTCC TTTAT GTAAT TTTCC AGAA — 3′	522	[4]
GT1R	GT73	5′ — GCTAA TACGA AGGCA AGAAA AGGA — 3′	522	[4]
4EPSPSF	GT200	5′ — CAACG CAAAT CTCCC TTATC GG — 3′	274	[5]
4EPSPSR	GT200	5′ — GACCT CCAAA CATGA AGGAC CT — 3′	274	[5]
epsF	CP4 *epsps*	5′ — CAATA CGGGC AAGGC CATGC — 3′	488	[15]
epsR	CP4 *epsps*	5′ — CGACG CCGAT CACCT ACCGC — 3′	488	[15]

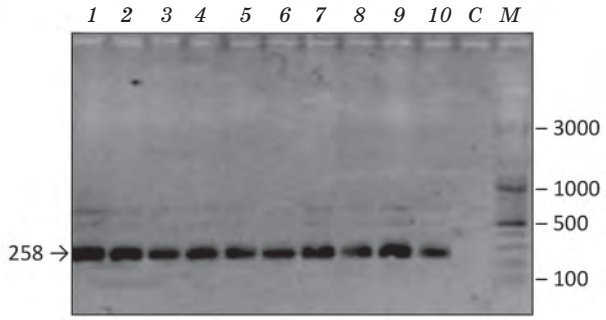

Fig. 1. **Electrophoregramm of the amplification products, directed to indicate cruA gene:** Lanes *1–10*, DNA samples extracted from lines *1–10*, respectively; *C* — negative control, TE buffer; *M* — molecular weight marker GeneRuler™ DNA Ladder Mix. An arrow shows the location of expected product and its length

of the studied lines. The DNA of transgenic *Zea mays* GA21 line was used as the positive control. In transgenic lines $T_2$15/133/2/9, $T_2$15/133/2/3, $T_1$15/133/2/10, $T_0$15/133/5, $T_0$17/133/4, 18a/2 the presence of the expected fragment with the length of 488 b.p. was noted (Fig. 2).

An arrow indicates the location of expected product and its length in b.p.

The next step was to confirm the difference between the inspected transgenic lines and registered transformation events GT73 together with GT200 developed by Monsanto Company.

Fragment with the length of 522 b.p. was not indicated in gel, which is pointing to the structural difference between the analyzed rapeseed lines and the transformation event GT73, developed by Monsanto Company (Fig. 3).

A DNA fragment with the length of 274 b.p. was not detected in agarose gel during the analysis of the samples for their possible similarity to the transformation event GT200 (the data is not shown). That testified about the non-similarity of analyzed lines to abovementioned transformation event.

Presence of the small-weight DNA fragments could be observed in both cases, pointing on the mutual activity of primers, which is usually typical for PCR.

Thus, the molecular genetic research had confirmed the uniqueness of the developed at the Institute of Cell Biology and Genetic Engineering of NAS of Ukraine glyphosate-tolerant rapeseed lines. The lines $T_2$15/133/2/9, $T_2$15/133/2/3, $T_1$15/133/2/10, $T_0$15/133/5, $T_0$17/133/4, 18a/2 showed the presence of CP4 *epsps* transgene in their genome. The difference among analyzed lines from registered foreign transformation events GT73 and GT200 had been proved.

The testing of transgenic plants for the resistance to glyphosate was conducted in greenhouse (Fig. 4). We revealed that transgenic plants remained green and vegetating, at the same time as control plants were turning yellowish and withering. That experimental data remained reliable during all time of observation.

Consequently this transgenic status of analyzed rapeseed plants was revealed. Structural difference between the analyzed lines $T_2$15/133/2/9, $T_2$15/133/2/3, $T_1$15/133/2/10, $T_0$15/133/5, $T_0$17/133/4, 18a/2 and the registered in European Union transformation events GT73, GT200 (Monsanto) was reliably proved. The expression of tolerance to «Hurricane Forte 500 SL, v. p. k.» herbicide, containing 2,5 μg/l potassium salt of glyphosate, was detected in greenhouse. For the purpose of future research we consider to evaluate the tolerance level of selected lines in opened soil, and to use the best forms for breeding work.

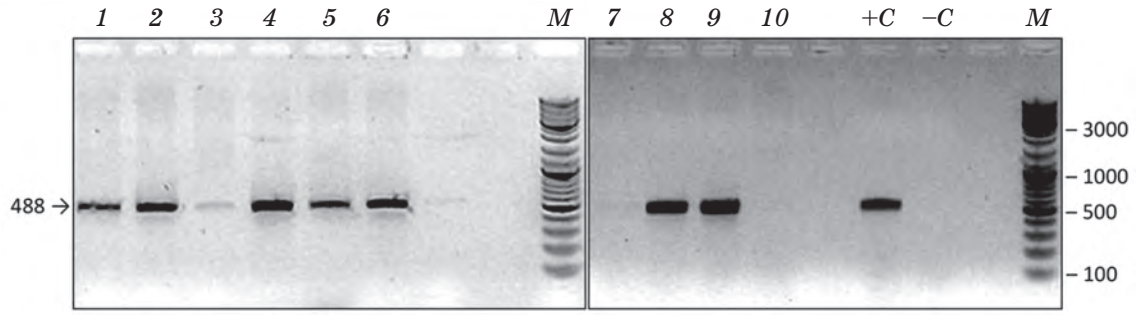

Fig. 2. **Electrophoregramm of the amplification products to indicate the presence of CP4 *epsps* gene.** Lanes *1–10*, DNA samples extracted from lines *1–10,* respectively; *+C* — positive control *Z. mays* GA21; *−C* — negative control, TE buffer; *M* — molecular weight marker GeneRuler™ DNA Ladder Mix

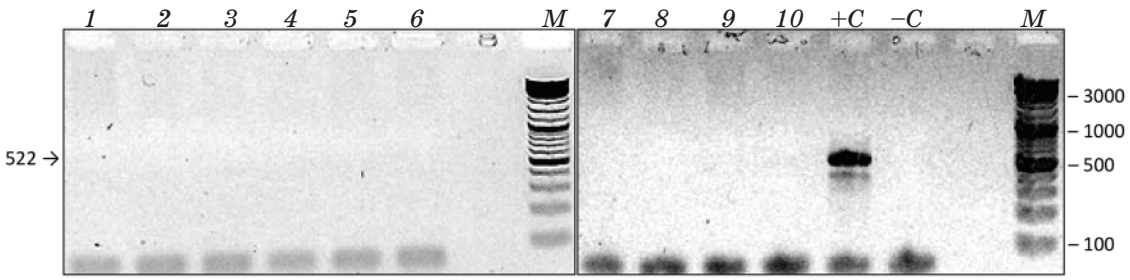

Fig. 3. **Electrophoregramm of the amplification products, directed to indicate GT73 transformation event:** Lanes *1–10*, DNA samples of lines *1–10*, respectively; *+C* — positive control; *–C* — negative control, TE buffer; *M* — molecular weight marker GeneRuler™ DNA Ladder Mix. Unmarked tracks were left empty on purpose as the buffer zone.
An arrow shows the location of the expected product and its length

Fig. 4. **The testing of rapeseed plants to glyphosate tolerance in greenhouse:**
the plants of non-transformed original Obriy cultivar (Bn18, on the left) and obtained on its base transgenic line 18a (on the right) after the spraying with «Hurricane Forte 500 SL, v.p.k.» herbicide

This research was financially supported with the project «Getting and studying of molecular biological and genetic peculiarities of glyphosate-tolerant agricultural crops» (state registration № 0110U006082) being part of the complex cross-disciplinary program of scientific research of the NAS of Ukraine «The fundamental bases of molecular and cellular biotechnologies».

REFERENCES

1. Pests and diseases of rape. Available at http://www.agrocounsel.ru/vyraschivanie-rapsa
2. Rape supply and demand balances, February 2012. Available at http://www.ukragroconsult.com/data/analytics/balansy-sprosa-i-predlozheniya-rapsa-fevral-2012g
3. Opinion of the Scientific Panel on Genetically Modified Organisms on a request from the Commission related to the Notification (Reference C/NL/98/11) for the placing on the market of herbicide-tolerant oilseed rape GT73, for import and processing, under Part C of Directive 2001/18/EC from Monsanto. *EFSA J.*, 2004, V. 29, P. 1–19.
4. GMO Detection method Database (GMDD) — Event Information GT73. Available at http://www.gmdd.shgmo.org/event/view/109
5. GMO Detection method Database (GMDD) — Event Information GT200. Available at http://www.gmdd.shgmo.org/event/view/108
6. *Sakhno L. A., Gocheva E. A., Komarnitsky I. K., Kuchuk N. V.* Stable expression of promoterless ***bar*** gene in transgenic rape plants. *Tsitol. Genet.* 2008, 42 (1), 21–28. (In Russian).
7. *Sakhno L. O., Gerasymenko I. M., Komarnitskii I. K., Sheludko Y. V., Goldenkova-Pavlova I. V.* Creation of glyphosate-resistant *Brassica napus* L. plants expressing DesC desaturase of cyanobacterium *Synechococcus vulcanus*. *Biopolymers and Cell.* 2012, 28 (6), 449–455. (In Russian).
8. *Sakhno L. O., Komarnitsskii I. K., Maystrov P. D., Kuchuk M. V.* Creation of rapeseed plants resistant to glyphosate-based herbicides by introduction a synthetic gene *epsps*. *Factors in Experimental Evolution of Organisms*. Collected Works, edited by Kunah V. A. *Kyiv: Lohos*, 2011, V. 11, P. 388–393. (In Ukrainian).
9. *Murashige T., Skoog F.* A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 1962, V. 15, P. 473–497.
10. *Sakhno L. O., Morgun B. V., Kvasko E. J., Kuchuk N. V.* Creation of transformed rapeseed plants expressing animal gene *cyp11A1* of $P450_{SCC}$ cytochrome. *Biotekhnolohiia.* 2010, 3 (5), 74–82. (In Russian).

11. *Morgun B. V., Stepanenko O. V., Stepanenko A. I.* Method for detecting the rapeseed transformation event GT73 by multiplexed PCR. *Ukraine Patent 2013 09296*, July 24, 2013.
12. *Somma M.* Extraction and Purification of DNA. Session 4. In: Training Course on the Analysis of Food Samples for the Presence of Genetically Modified Organisms — User Manual. Edited by M. Querci, M. Jermini, G. Van den Eede. *European Commission, DG Joint Research Centre, Institute for Health and Consumer Protection. Luxembourg*, 2006, 229 p.
13. *Sambrook J., Fritsch E. F., Maniatis T.* Molecular cloning: a laboratory manual. *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY*. 1989, 1626 p.
14. NCBI database. *Brassica napus cruA* gene for cruciferin. Available at http://www.ncbi.nlm.nih.gov/nuccore/X14555.
15. NCBI database. Synthetic construct CP4 EPSPS glyphosate tolerance protein gene. Available at http://www.ncbi.nlm.nih.gov/nuccore/JF445290.

ПОРІВНЯЛЬНИЙ МОЛЕКУЛЯРНО-ГЕНЕТИЧНИЙ АНАЛІЗ МІЖ УКРАЇНСЬКИМИ ТА ЗАРЕЄСТРОВАНИМИ В ЄС ГЛІФОСАТ-ТОЛЕРАНТНИМИ ТРАНСГЕННИМИ РОСЛИНАМИ РІПАКУ

А. М. Тараненко
Л. О. Сахно
Б. В. Моргун
М. В. Кучук

Інститут клітинної біології та генетичної інженерії НАН України, Київ

E-mail: molgen@icbge.org.ua

Метою дослідження було здійснення аналізу 10 ліній ріпаку, створених в Інституті клітинної біології та генетичної інженерії НАН України, для підтвердження наявності та функціональності перенесеного трансгена CP4 *epsps*, а також відмінності цих ліній від зареєстрованих трансформаційних подій GT73 і GT200 (Monsanto).

Проведено виділення загальної ДНК ріпаку, аналіз методом полімеразної ланцюгової реакції, електрофоретичне розділення та візуалізацію ампліконів у агарозному гелі, а також тестування зелених рослин на стійкість до гліфосату в умовах теплиці. Виявлено структурну відмінність 7 трансгенних ліній від зареєстрованих трансформаційних подій GT73 та GT200. У рослин, які містили синтетичну послідовність CP4 *epsps* відзначено стійкість до гербіциду за умов закритого ґрунту. Підтверджено унікальність новоотриманих трансформаційних подій і перспективність їх використання в селекційній роботі.

Ключові слова: ріпак, гліфосат, генетична трансформація, ген CP4 *epsps*.

СРАВНИТЕЛЬНЫЙ МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКИЙ АНАЛИЗ МЕЖДУ УКРАИНСКИМИ И ЗАРЕГИСТРИРОВАННЫМИ В ЕС ГЛИФОСАТ-ТОЛЕРАНТНЫМИ ТРАНСГЕННЫМИ РАСТЕНИЯМИ РАПСА

А. М. Тараненко
Л. О. Сахно
Б. В. Моргун
М. В. Кучук

Институт клеточной биологии и генетической инженерии НАН Украины, Киев

E-mail: molgen@icbge.org.ua

Целью исследования было осуществление анализа 10 линий рапса, созданных в Институте клеточной биологии и генетической инженерии НАН Украины, с целью подтверждения присутствия и функциональности перенесенного трансгена CP4 *epsps*, а также отличия этих линий от зарегистрированных трансформационных событий GT73 и GT200 (Monsanto).

Проведены выделение общей ДНК рапса, анализ методом полимеразной цепной реакции, электрофоретическое разделение и визуализация ампликонов в агарозном геле, а также тестирование зеленых растений на устойчивость к глифосату в условиях теплицы. Выявлено структурное отличие 7 трансгенных линий от зарегистрированных трансформационных событий GT73 и GT200. У растений, содержавших синтетическую последовательность CP4 *epsps*, отмечена устойчивость к гербициду в условиях закрытого грунта. Подтверждена уникальность вновь полученных трансформационных событий и перспективность их использования в селекционной работе.

Ключевые слова: рапс, глифосат, генетическая трансформация, ген CP4 *epsps*.