Biotechnology in Russia, 2015, № 5, pp. 59-64

UDC 573.6:581.151:633.853.494

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Genetically engineered details of the commercial "Raudis" rapeseed variety with "natural tolerance" to glyphosate

It has been shown by PCR with various primers that the commercial "Raudis" Spring rapeseed breed presented as non-GMO naturally tolerant to glyphosate, and obtained by conventional breeding is indeed a genetically modified organism that contains genetic construct typical of GT73 line (Monsanto). This construct is most likely responsible for experimentally confirmed glyphosate tolerance of the breed.

Key words: 5-enolpyruvylshikimate-3-phosphate synthase, glyphosate tolerance, rapeseed

Genetically engineered tolerance to herbicides in crops is the trait that is widely accepted in agriculture along with other achievements in plant genetic engineering. According to ISAAA biotech crops tolerant to herbicides were cultivated on more than 100 million hectares worldwide in 2014 [1]. For the crops like soybean and cotton the share of biotech varieties is higher than that of the crops resulting from conventional breeding [1]. 25% share of biotech rapeseed was estimated in 2014 [1] and it continues to grow – ISAAA reported 21% in 2009 [2]. Economical benefit is most probably the factor providing for such a dynamics.

Our attention has been recently attracted by the Ukrainian Spring rapeseed variety "Raudis", which is widely advertised by Ukrainian agricultural companies and offered for sales, including for export [3-7]. It is positioned as the Ukrainian breed, which is «naturally tolerant to glyphosate» and that «glyphosate tolerance has been achieved through traditional breeding without use of genetic engineering». Occurrence of such natural tolerance is highly questionable.

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Abbreviations list: bp – nucleotide base pairs; PCR – polymerase chain reaction; dNTP – deoxyribonucleoside triphosphate; EPSPS - 5-enolpyruvylshikimate-3-phosphate synthase; GOX - Glyphosate oxidase; ISAAA – International Service for the Acquisition of Agri-biotech Applications; P-FMV – figwort mosaic virus 35S-promoter.

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The aim of this work was analysis of the commercial "Raudis" rapeseed variety in order to identify the origin of its glyphosate tolerance - either natural, or genetically engineered.

EXPERIMENT SETUP

Plant material. In this work we used seeds of the Spring rapeseed *var*. "Raudis" tolerant to glyphosate (originators – "RapsOil" LLC (Ukraine) and the National University of Life and Environmental Sciences of Ukraine). Rape seeds were germinated and grown in a greenhouse with the use of peat-sand soil mixture (neutralized peat moss), with natural light and +22-24 ^{o}C.

Detection of EPSPS protein in plant material. Testing plant material for the presence of CP4 EPSPS protein was carried out with the use of express method of immunochromatography on nitrocellulose layer (Lateral Flow Strip). We used RoundUp Ready® (CP4 EPSPS) ImmunoStrip® kits (Agdia Inc., USA) in accordance with the producer's user manual.

Isolation of plant DNA. DNA was isolated from rape leaves with the use of "DNeasy Plant Mini Kit" (Qiagen, Germany) according to the producer's protocol attached to the kit.

PCR-analysis. PCR primers were both designed using VectorNTI program and sequences published open source. Primers used in this work, their nucleotide sequences and the size of expected products is given in Table 1. Amplification of target fragments was carried out in a buffer containing 0.2 mM of dNTP, 0.2 μM of each of the primers, ca 30 ng of genomic DNA and 1,0 unit of Taq DNA polymerase («Fermentas», Lithuania) according to the producer's manual; reaction mix volume - 25μl. PCR was carried out under following conditions: denaturation - 95°C, 5 min; annealing - 60°C, 30 sec; elongation - 72°C, 40 sec, number of amplification cycles - 30 (amplifier - «Eppendorf», Germany). PCR products were analyzed in 1.5% agarose gel (Amersco, USA) in the horizontal electrophoresis chamber MINNIE GEL UNIT, 7x10CM (Amersham, Biosciences, USA). GeneRuler™ Express DNA Ladder #SM0333 and GeneRuler™ Low Range DNA Ladder #SM1191 («Fermentas», Lithuania) were used as molecular mass markers.

Molecular cloning was performed as given by Sambrook et.al. [10].

Table 1

Primers used in this work including for the detection of various structural elements of expression cassettes present in glyphosate tolerant rapeseed lines GT73 (RT73) and GT200 (RT200) (Monsanto)

Primer (target sequence)	Nucleotide sequence in 5'-3' direction	Length of amplified fragment, bp	Reference
RT73 Primer 1 (specific to line GT73)	ccatattgaccatcatactcattgct	108	[8]
RT73 Primer 2 (specific to line GT73)	gcttatacgaaggcaagaaaagga		
MDB510 (rape *cruA* gene)	ggccagggtttccgtgat	101	[8]
MDB511 (rape *cruA* gene)	ccgtcgttgtagaaccattgg		

CP4-EPSPS F (*cp4-epsps* gene) CP4-EPSPS R (*cp4-epsps* gene)	caacgcaaatctcccttatcgg gacctccaaacatgaaggacct	274	[9]
FMVfor (FMV promoter) FMVrev (FMV promoter)	ccaaagcctcaacaaggtcagggt cgcattagtgagtgggctgtcagg	364	This work
Clon For (*ctp2cp4-epsps* gene) Clon Rev (*ctp2cp4-epsps* gene)	atggcgcaagttagcagaatc tcaagcagccttagtgtcg	1596	This work

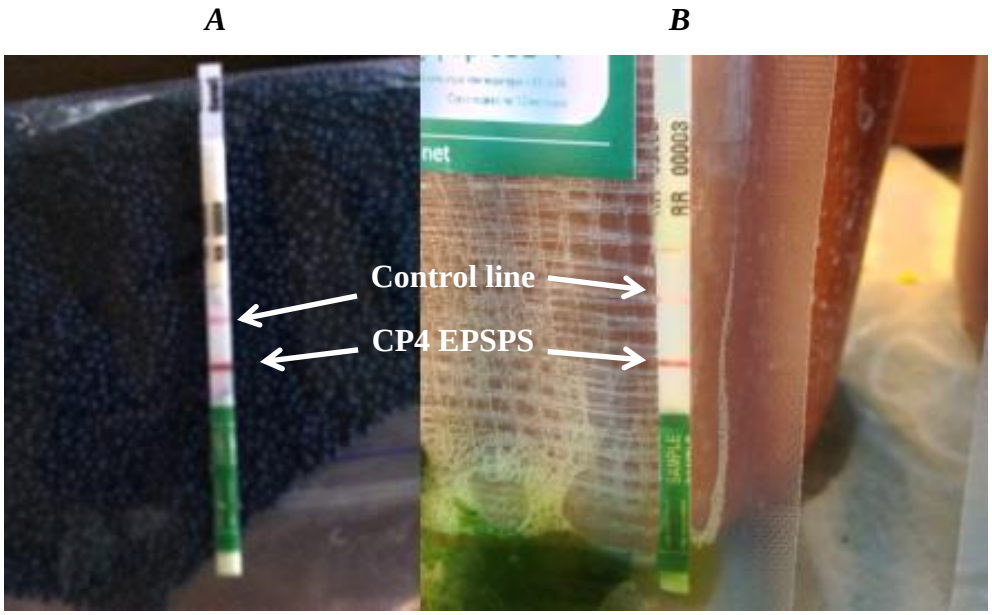

Fig. 1. Testing "Raudis" tissues for CP4 EPSPS protein; A – seed; B - leaves

RESULTS AND DISCUSSION

«Raudis» seeds were planted in a greenhouse performing perfect germination. On a vegetation stage corresponding to appearance of 5-6 leafs, the plants were excessively sprayed with Roundup® (Monsanto), 5g/l (by glyphosate) solution, showing no degradation within ten days, while control plants were totally suppressed (rapeseed variety "Ratnik", breed by the State Enterprise "All-Russian NIPTI of rapeseed" (Russia") was taken as the control plant). We then subjected glyphosate tolerant rapeseed to molecular biological analysis.

Testing the acquired seed of the "Raudis" variety with the Lateral Flow Strip technique clearly the presence of CP4 EPSPS protein, which clearly indicates the presence of genetically engineered insert in the analyzed rapeseed. The same result was obtained in testing rapeseed leaves grown of the analyzed seed (Fig. 1).

Since the presence of CP4 EPSPS protein in rapeseed tissues cannot occur due to any natural cause, we suggested that the "Raudis" variety was either an existing commercial line of genetically modified oilseed rape tolerant to glyphosate, or its derivative. According to ISAAA [11] there are six rapeseed lines with tolerance to glyphosate due to expression of cp4- epsps gene that were approved for commercial use (GT200, GT73 (RT73), MON88302, MON88302xMS8xRF3, MON88302xRF3, MS8xRF3xGT73 (RT73)). The first two were approved for commercial use in 1995 and 1996; the other four were approved much later (2010-2015). We therefore decided to focus on GT200, GT73 (RT73) lines. Genetic constructs of the transgenes for these two lines are schematically presented in Fig. 2.

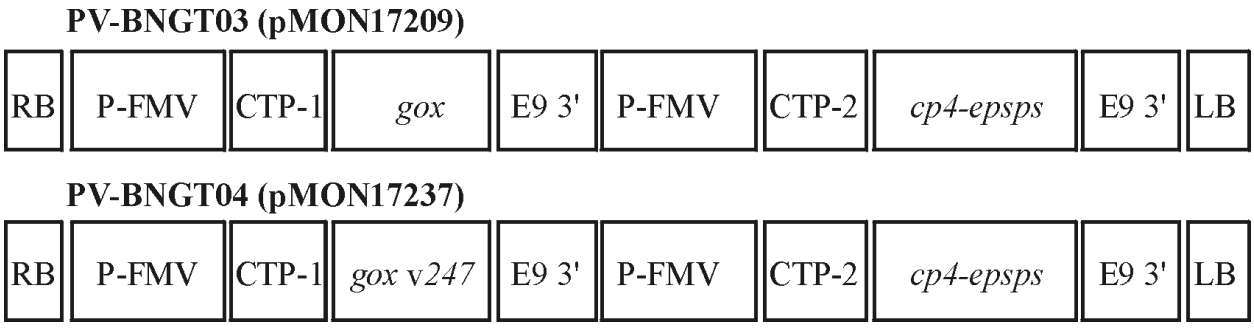

Fig. 2. T-DNA-vectors used by Monsanto to create glyphosate tolerant rapeseed lines [13,14]. FMV – 35S promoter of the modified figwort mosaic virus; CTP-1 – N-terminal chloroplast signal peptide of 1A Ribulose-1,5-bisphosphate carboxylase small subunit gene of *Arabidopsis thaliana*; *gox* and *goxv247* – synthetic genes coding for glyphosate oxidoreductase, *Ochrobactrum anthropi*; E9 3' – 3'untranslated part of *rbc*S E9 small subunit gene of pea; CTP-2 - N-terminal chloroplast signal peptide of EPSPS gene from *Arabidopsis thaliana*; *cp4-epsps* –gene synthetized on the base of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene from CP4 *Agrobacterium tumefaciens* strain; RB, LB – right and left vectors' borders, respectively.

PCR analysis was carried out on total plant DNA isolated from three "Raudis" plants. Primers RT73 Primer1 and RT73 Primer2 were used in the reaction. These primers were approved and recommended by The European Reference Laboratory for GM Food and Feed (EU-RL GMFF) of the Joint Research Centre (JRC EU) for specific detection of the line RT73 (GT73) [8]. The upper RT73 Primer1 is complementary to the sequence of the transgene insert, the lower RT73 Primer2 – to that of rapeseed genome DNA in the region of insert integration.

Through PCR of the "Raudis" DNA fragment of expected length was amplified, while it was not detected in the control reaction (Fig. 3A). At the same time carrying out PCR with MDB510 and MDB511 primers [8] specific to rapeseed gene *cruA* produced the expected fragment in both test and control samples (Fig. 3B). It points to presence of DNA sequence in "Raudis" genome analogous to DNA of line GT73 (Monsanto). We additionally carried out PCR using CP4-EPSPS F and CP4-EPSPS R primers described elsewhere [9], specific to *cp4-epsps* gene. Results given in Fig. 3C indicate the presence of synthetic *cp4-epsps* gene in the "Raudis" genome.

Basing on nucleotide sequence of FMV-promoter responsible for expression of the synthetic cp4- epsps gene [12] we synthesized a pair of primers – FMVfor (forward) and FMVrev (reverse) specific to the sequence of figwort mosaic virus 35S promoter (FMV). As a result PCR indicated presence of FMV promoter nucleotide sequence in "Raudis" genome (Fig. 3D). We additionally carried out PCR with the combination of primers FMVfor/CP4- EPSPS R (the upper primer is specific to FMV promoter, the lower primer is specific to *cp4-epsps* gene).

The analysis indicated that *cp4-epsps* was controlled by FMV promoter (Fig. 3E) similar to PV-BNGT04 used by Monsanto Company in creation of the line GT73 [13].

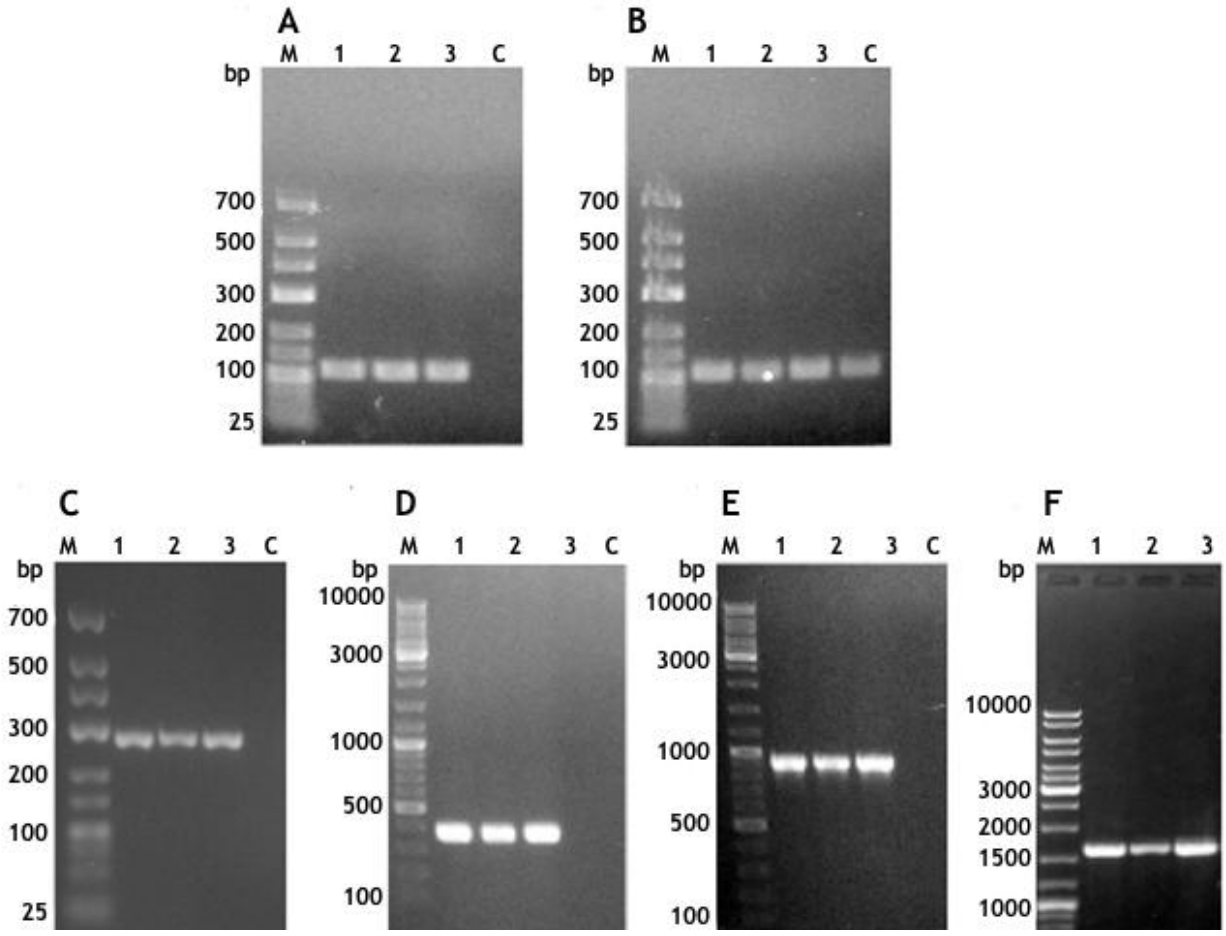

Fig. 3. Electrophoresis of products of PCR analysis of DNA isolated from rapeseed varieties "Raudis" and "Ratnik"; A – amplification with the primers specific to transgenic line GT73 (Monsanto); B – amplification with the primers specific to *cruA* rapeseed gene; C – amplification with the primers specific to the modified figwort mosaic virus 35S promoter, FMV; D – amplification with the primers specific to the gene encoding protein CTP2-CP4EPSPS; E – amplification with the combination of primers (forward primer specific to FMV promoter and reverse primer specific to the sequence encoding CTP2-CP4EPSPS); F – amplification of the nucleotide sequence encoding full-sized (1596 bp) CTP2-CP4EPSPS of genome DNA isolated from three "Raudis" plants; track C – amplified fragment of DNA of "Ratnik" rapeseed (control); M – molecular mass markers.

In order to identify nucleotide sequence of *cp4-epsps* gene from "Raudis" rapeseed we performed its cloning followed by sequencing. We used clon for/clon rev primers, which flank full translation area of *cp4-epsps* gene and amplified the sequence with the length corresponding to the translation area of *cp4-epsps* gene (1595 bp, Fig.3 F). This amplification product was cloned in pAT-TA vector (Evrogen, Russia). Cloning resulted in several clones used from which plasmid DNA was isolated and used for further sequencing. The results showed that DNA of the "Raudis" glyphosate tolerant rapeseed plants contained nucleotide sequence encoding *cp4-epsps* gene (clone pAT-grg6) identical to that published in GenBank under # GV597339 (Fig.4).

Fig. 4. Comparison of nucleotide sequence of CTP2/CP4EPSPS gene from line GT73 (GenBank #GV597339, 1 to 500 bp) and the sequence cloned from genome of "Raudis" rapeseed (clone pAT-grg6). *cp4epsps* sequence is given in bold font, chloroplast signal peptide is given in normal weight font.

Thus the study clearly indicates that the "Raudis" rapeseed variety is glyphosate tolerant genetically modified organism containing nucleotide sequences characteristic of canola line GT73 (Monsanto) [14].

It should be considered that the acreage of rapeseed planting in Ukraine is approaching 1 million hectares and the yearly export of rapeseed is estimated to be 1.2-2.1 million metric tons [15].

The obtained results convince us of the need for the speedy establishment of the state system of screening/monitoring of genetically engineered agricultural crops and their seed material.

Received on 10.09.2015

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