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RNA ISOLATION AND REACTION OF REVERSE TRANSCRIPTION IN THE DETERMINING OF THE TRANSGENES DISPLAYING EFFECTIVENESS IN SUGAR BEET PLANTS

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Were represented the results of studies on choosing selection techniques of total RNA from transgenic sugar beet plants using two approaches: the method of phenol extraction and RNA isolation on silicate sorbent. Method of reverse transcription reaction in order to determine the expression of transgenes in sugar beet plants was described.

RNA isolation, polymerase chain reaction, reverse transcription, sugar beet

Introduction. Nowadays, genetic engineering of plants is an integral part of modern molecular and cellular biology, the main objectives of which are: genetic transformation of plants, expression of the transferred genes and its regulation in the cells of transgenic plants. However, the problem of variability in gene expression is essential in transformed plants growing [6, 7]. In particular, the stability of genetic structures in genome of transgenic sugar beet plants and their expression is not well understood, so determination of the transgenes displaying effectiveness in sugar beet plants is relevant. One way of the estimation of a built genes expression is polymerase chain reaction with reverse transcription (RT-PCR), which allows to establish the presence of specific RNA in cells of transgenic plants.

The aim is the selection procedure of total RNA extraction from transgenic sugar beet plants and conduction of the reverse transcription reaction.

Materials and methods. Genetic structure, glyphosate tolerant sugar beets that were studied, consists from *35S* promoter, *NOS* terminator and *CP4 EPSPs* gene (*5-nolpyruvylshikimate-3-phosphate synthase gene*). Plant material for the extraction of total RNA obtained with the method of *in vitro* cultivation [4]. Sterile sugar beet plants were analyzed for the presence of the studied genetic construction components through the usage of the polymerase chain reaction (PCR).

Extraction of DNA from the aseptic sugar beet plant leaves was made with usage of cationic detergent CTAB (cetyltrimethylammonium bromide) [3]. In DNA

preparations spectrophotometric analysis for PCR conduction ratio values of the absorption maxima at wavelengths 260/280 nm was close to the value of 1.8 [5].

In sugar beet plants *35S* promoter of cauliflower mosaic virus was detected by PCR with further electrolytic reaction products separation using a set of reagents *GenPac GMO-35S PCR test* ("Izogen Lab", Russia). For the *NOS* terminator and *EPSPs CP4* gene identification in sugar beet plants multiplex PCR real-time test system was used ("Syntol", Russia).

During the total RNA extraction from sugar beet plant two fundamentally different ways were used: the method of RNA isolation with phenol and RNA extraction using sorbent [1, 2].

RNA extraction using phenol. In this paper, a set of reagents *Trizol RNA Prep 100* ("Izogen Lab", Russia) was used. The procedure of the total RNA extraction consists of the following steps: cell lysis in the presence of guanidinium thiocyanate and phenol, purification of RNA preparation with chloroform and sedimentation of nucleic acid with isopropyl alcohol. As the solvent ExtraGene *E* was used.

RNA extraction using sorbent. To isolate total RNA set of reagents for RNA isolation "Ribo - sorb" (LLC "InterLabservis", Russia) was used. During the RNA selection with sorbent the following steps were conducted: lysis of cell membranes, adsorption of RNA on silicate adsorbent with a certain pH and in the presence of saline buffer, RNA washing from proteins and phenolic compounds with alcohol solutions, sorbent removal and RNA solution in the buffer.

For the total RNA quality and quantity estimation spectrophotometer was used. RNA preparation is considered to be clean if the rate of adsorption on photometric wavelengths 260/280nm close to 2.8 [5].

For the obtaining of complementary DNA (cDNA) first chain reverse transcription reaction (RT) was performed with usage of a commercial reagent set GenPak® RT Core ("Izogen Lab", Russia) according to the manufacturer's guidelines, using obtained total RNA as a matrix. The reaction mixture contained 100 units of reverse transcriptase M-MLV, 20 units of RNAase inhibitor, hexanucleotide random primers, deoxynucleoside triphosphates and optimized buffer system for a standard reverse transcription reaction [5]. The reaction was carried out in the

following stages: dried contents of the finish mixture was dissolved by adding 5 ml RT solvent (part of the set), added 5 ml of RNA samples, positive and negative controls, the mixture was incubated for 40 min. at 50°C, the reaction was stopped with adding to the tubes 10 ml of stop solution (part of the set) and heated at a temperature 95°C for 10 minutes. As a positive control were used RNA that was isolated from non-transgenic sugar beet plants, and as a negative control 5 ml of the ExtraGene *E* solvent was used.

Results. During the obtaining of a sterile seedlings and vegetative plants of sugar beet sterilization efficiency was 96%, the number of viable explants – 88%. Sterile plants were used for the total DNA extraction.

For investigated samples DNA concentration was between 500-700 mg/ml, the rate of purity 1,8-1,93. Thus, the quantity and quality of DNA give evidence of obtaining ability of a highly purified DNA, which can be used for PCR with the aim of genetic sequence detection, through the usage of DNA isolation with cationic detergent CTAB.

Visualization of PCR reaction products was performed by electrophoresis in 2.5 % agarose gels (Fig. 1).

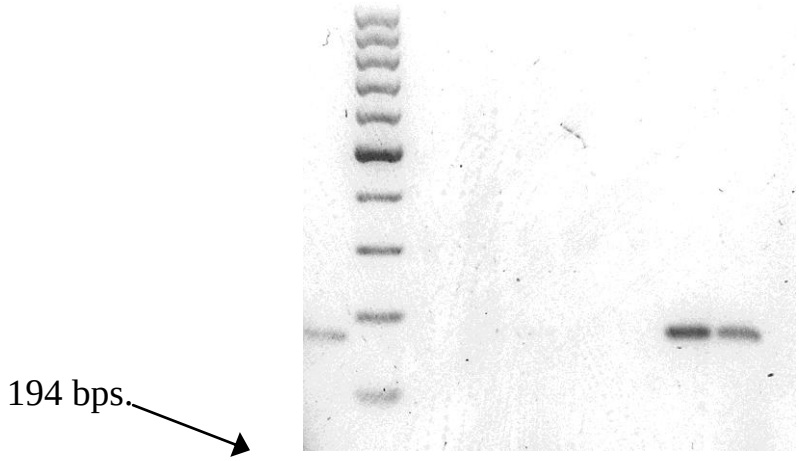

Figure 1. The results of the distribution of amplified reaction products in agarose gel: 1 - positive control, 2 - molecular weight marker (*GeneRulerTM 100bp*), **3 - negative control, 4-8 - investigated DNA samples.**

As a result, electrophoretic separation of the reaction products in agarose gel the presence of amplicon with size of 194 bp was established, which are correspond to the sequence of *35S* promoter in the positive control sample and the samples that correspond to tracks 4 and 5. The absence of the amplicon with mentioned size on

negative control this track indicates the reliability of the data and the absence of contamination.

The result of real time PCR analysis is a graph of studied DNA samples fluorescence and data of graphs comparing the amplification products accumulation (Fig. 2).

B03	FAM	Neg Ctrl	ОК-	1	N/A	00,00	N/A	N/A
B04	FAM	Neg Ctrl	ОК-	1	N/A	00,00	N/A	N/A
B07	FAM	Pos Ctrl	ПК+	1	27,87	27,93	0,077	N/A
B08	FAM	Pos Ctrl	ПК+	1	27,98	27,93	0,077	N/A
F03	FAM	Unkn	НТр1/2	5	N/A	00,00	N/A	N/A
F04	FAM	Unkn	Тр1/1	6	N/A	00,00	N/A	N/A
F05	FAM	Unkn	Тр1/2	7	35,77	35,77	N/A	N/A
F06	FAM	Unkn	Тр3/1	8	38,64	38,64	N/A	N/A
F07	FAM	Unkn	Тр3/3	9	N/A	00,00	N/A	N/A
F08	FAM	Unkn	В.1	10	N/A	00,00	N/A	N/A
B03	Cy5	Neg Ctrl	ОК-	1	N/A	00,00	N/A	N/A
B04	Cy5	Neg Ctrl	ОК-	1	N/A	00,00	N/A	N/A
B07	Cy5	Pos Ctrl	ПК+	1	27,46	27,51	0,064	N/A
B08	Cy5	Pos Ctrl	ПК+	1	27,55	27,51	0,064	N/A
F03	Cy5	Unkn	НТр1/2	5	35,56	35,56	N/A	N/A
F04	Cy5	Unkn	Тр1/1	6	36,67	36,67	N/A	N/A
F05	Cy5	Unkn	Тр1/2	7	33,66	33,66	N/A	N/A
F06	Cy5	Unkn	Тр3/1	8	35,91	35,91	N/A	N/A
F07	Cy5	Unkn	Тр3/3	9	41,72	41,72	N/A	N/A
F08	Cy5	Unkn	В.1	10	38,69	38,69	N/A	N/A

Figure 2. Items of the amplification products accumulation graphs comparison for the four samples of sugar beet plants

The data presented in Fig. 2 indicate the presence of DNA sequences of *NOS* terminator in two samples (35 and 38 threshold cycles for dye *FAM*) and *CP4* gene *EPSPs* in four samples (threshold cycles for dye *Cy5* - 35, 36, 33 and 35 respectively).

As a result of the studies genotypes of sugar beet plants with different genetic structures composition that are used for RNA isolation were selected: samples that contain all of the genetic structure (*35S* promoter, *NOS* terminator gene and the *CP4 EPSPs* gene) and with absence both promoter and regulatory sequences.

Based on the data, the highest number of total RNA was obtained using phenol extraction (500-900 mg/ml), with purity from 1.5 to 1.9. The concentration of RNA obtained with the usage of sorbent was 40-70 mg/ml, and the purity rate was 1.8-2.0. Thus, the purity of RNA in the extraction on the sorbent is significantly higher than the phenol extraction (1,5-1,9), but low concentration shows that this method is less suitable for RNA isolation from plant sugar beets for RT reaction.

Visualization of reverse transcription reaction was performed by electrophoresis in 1.5% agarose gels (Fig. 3).

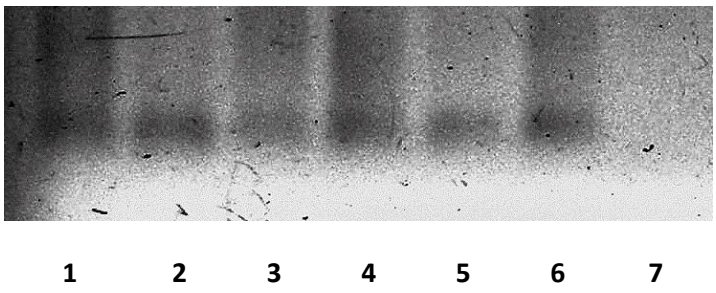

Figure 3. Results of reverse transcription reaction products visualization in agarose gel: 1 - positive control, 2-6 - cDNA samples, 7 - negative control.

On successful undergoing of the reverse transcription reaction indicates compliance of studied transgenic sugar beet track samples (№ 2-6) reference control sample (№ 1) and the absence of any reaction products on the track of negative control (№ 7) led to the conclusion about the reliability of the data and to avoid possibility of contamination.

Conclusion

As a result of the research sample of transgenic genotypes of sugar beet plants with different variants of introduced construction composition that include useful gene was formed. These data suggest that during the RNA extraction from transgenic sugar beet plants for reverse transcription reaction, phenol extraction method suits the best, because it provides additional purification of nucleic acids from proteins and phenolic compounds. Thus, the obtained cDNA allow to make assessment of gene expression efficiency by PCR .