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GLYPHOSATE SELECTION OF MAIZE TRANSFORMANTS CONTAINING *CP4EPSPS* GENE

Aim. To study the selection conditions of maize transformants containing the *CP4epsps* gene using glyphosate as a selective agent. ***Methods***. Tissue culture *in vitro*, *Agrobacterium*-mediated transformation, selection of transgenic plants, isolation of total plant DNA, analysis of plant DNA by polymerase chain reaction (PCR). ***Results***. The morphogenic maize callus of immature embryos of the hybrid (PLS61)R_2×PLS61 was produced, which had a high regeneration rate (up to 95%), that persisted over long cultivation. *Agrobacterium*-mediated transformation of the morphogenic callus and selection of the transgenic material using glyphosate yielded maize transformants containing the *CP4epsps* gene at a frequency of 1%. ***Conclusions***. Maize genotype (PLS61)R_2×PLS61 is promising for studies on the maize genetic transformation, in particular for the production of transgenic maize resistant to glyphosate herbicide. The use of morphogenic maize callus (PLS61)R_2×PLS61 and glyphosate as a selective agent at a concentration of 0.1 mM and 0.25 mM in media for callusogenesis and 0.01 mM in the medium for regeneration was effective for the selection of transgenic plants with the gene *CP4epsps*.

Keywords: *Zea mays* L., morphogenic callus, *Agrobacterium*-mediated transformation, PCR, genetic engineering.

Introduction. Maize (*Zea mays* L.) is one of the major world crops. It is sensitive to weeds in the early stages of growth, which can lead to significant yield losses of 30 to 90% with uncontrolled cultivation [1]. The chemical method of controlling weeds using herbicides is an effective complement to conventional methods and integral part of modern agriculture. Most of the currently available herbicides provide weed control only in a narrow range [2]. Glyphosate N-(phosphonomethyl)glycine) – is a systemic broad spectrum herbicide, the most common in the world to kill weeds, especially annual broadleaf weeds and grasses. Glyphosate inhibits the enzyme of the metabolic pathway of shikimic acid 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) blocking the biosynthesis of benzoid aromatic compounds, including essential amino acids (phenylalanine, tryptophan, threonine), causing the death of plants [3]. Obtaining transgenic plants resistant to herbicides, is one of the main areas the creation of biotech crops for agriculture [4]. One way of obtaining plants resistant to glyphosate, is the introduction of the mutant gene *epsps*, a product of which has a low affinity for glyphosate [5-7]. The *CP4epsps* gene is one such gene that was isolated from the *Agrobacterium tumefaciens* of the CP4 strain [8]. In a transgenic plant, it provides resistance to the herbicide, completely compensating for the function of the native plant enzyme EPSPS, and can be used as a selective marker gene [5, 6, 9].

There is a natural variation in the sensitivity to glyphosate of maize different genotypes [10]. In this regard, to obtain glyphosate resistant plants, it is important to explore conditions for their selection on media with glyphosate. The aim of our study was to investigate the selection conditions for maize transformants of genotypes grown in Ukraine that would contain the *CP4epsps* gene using glyphosate as a selective agent.

Materials and methods

Plant material served maize morphogenic callus derived from immature embryos of hybrid F_1 (PLS61)R_2×PLS61 between somaclonal variant (PLS61)R_2 of PLS61 line, conducted through *in vitro* culture, and line PLS61. Callus was obtained by culturing immature embryos *in vitro* on modified N_6 medium [11] at 27°C in dark and maintained for 3 years, replanting to fresh nutrient medium every 3 weeks. To test the effect of different selective agents on the regeneration potential of the (PLS61)R_2×PLS61 callus, the following media were used: basic MSGR [9] containing 0.25 mg/l 6-benzylaminopurine (BAP) (No. 1); medium No. 1

with the addition of 500 mg/l cefotaxime to inhibit the growth of agrobacteria (No. 2); medium No. 2 with the addition of 5 mg/l phosphinothricin (No. 3); medium No. 2 with the addition of 100 mg/l of paromomycin (No. 4); medium No. 2 with the addition of 0.01 mM glyphosate (No. 5). The calluses cultured *in vitro* in dark at 27°C were transferred to regenerative selective media No. 1-5 and cultured under light conditions with a photoperiod of 14/10 h at 25°C with replanting into fresh medium every 3 weeks. After 6 weeks, the effect of antibiotics and herbicides added to the base medium No. 1 on the greening and regenerative capacity of the callus was evaluated. A vector pCB135 [12], which was in *A. tumefaciens* strain GV3101, derived from strain C58 [13], was used for maize genetic transformation. The vector contained *CP4epsps* gene under control of the 35S RNA promoter of the cauliflower mosaic virus and *npt*II gene under the control of nopalinsyntase promoter. The growth media for *A. tumefaciens* containing the vector pCB135 was supplemented with 100 mg/l kanamycin sulfate and 100 mg/l carbenicillin. The optical density of the bacterial suspension for transformation of explants (OD600) was adjusted to 0.4-0.5. *A. tumefaciens* preparation and *Agrobacterium*-mediated transformation of maize morphogenic callus were performed by the procedure [9]. After transformation, the calluses were transferred to a culture medium for callusogenesis, which contained macro-, microsalts, vitamins of the N_6 medium [14], 30 g/l sucrose, 3.5 mg/l $AgNO_3$, 1.0 mg/l dichlorophenoxyacetic acid (2.4 D), 0.5 mg/l dicamba, 0.1 mg/l abscisic acid, 7 g/l agar, 500 mg/l cefotaxime and 0.1 mM glyphosate and were cultured in dark at 27°C. After two weeks, the calluses were planted in a fresh medium in which the glyphosate concentration was increased to 0.25 mM and grown under the same conditions for two weeks. Selected herbicide-resistant calluses were transplanted to selective regeneration medium No. 5 and grown under illumination at 24°C and a 16-hour photoperiod. After two weeks of cultivation, the calluses were transferred to a fresh selective regeneration medium and cultured for another two weeks under the same conditions. After two weeks of cultivation, the calluses were transferred to a fresh selective regeneration medium and cultured for another two weeks under the same conditions. Subsequently, the glyphosate-resistant green calluses were transferred to regeneration medium No. 2 and continued cultivation, replanting the calluses to fresh medium every three weeks. The regenerating plants were separated from the callus and grown in jars containing bacteriostatic MS [15] without hormones, added 500 mg/l cefotaxime. The rooted plants were transferred to pots with soil and grown under standard greenhouse conditions.

Total DNA was extracted from regenerant leaves by the method using CTAB and PVP-40 [16]. Detection of *CP4epsps* and *npt*II gene sequences present in plant DNA was performed by PCR [12]. To exclude the possibility of contamination of plant material with agrobacteria, the amplification for bacterial *vir*-D1 gene was carried out. Transformation frequency was calculated as a percentage of the number of calluses that formed transgenic plants to the total number of calluses processed.

Results and discussion

Typically, calluses derived from immature embryos retain their regenerative capacity within 8-9 months of cultivation, and subsequently the callus regeneration activity attenuates [17]. We have isolated the genotype (PLS61)R_2×PLS61 which calluses from immature embryos have retained their regenerative capacity for more than two years. The calluses obtained from the immature embryos of the F_1 (PLS61)R_2×PLS61 hybrid have a regeneration frequency (75%) higher than the original PLS61 calluses (66.6%). Thus, the callus obtained from the hybrid line is promising for research on the genetic transformation of maize due to the high regenerative potential, which remains for a long cultivation.

To evaluate the effectiveness of different transgenic plant selection schemes after (PLS61)R_2×PLS61 callus transformation, we tested the effect of additional constituents of selective media, namely, cefotaxime, paromomycin, phosphinothricin, and glyphosate on the regenerative potential of callus (Table 1, Fig. 1).

Adding to the medium of cefotaxime at a concentration of 500 mg/ml had no negative effect on the regenerative capacity of the callus. On the contrary, the rate of regeneration increased from 75% in the control to 94.4%. Paromomycin at a concentration of 100 mg/l had a slight inhibitory effect on the morphogenetic potential of the callus (Table 1, No. 4).

Table 1. Effects on the regenerative capacity and greening of maize (PLS61)R_2×PLS61 calluses by different selective agents

Medium, No.	Planted calluses, pcs.	Calluses that formed shoots		Green calluses	
		pcs.	%	pcs.	%
1	20	15	75.0	17	85.0
2	18	17	94.4	17	94.4
3	20	0	0	4	20.0
4	19	13	68.4	14	73.7
5	28	3	10.7	7	25.0

Therefore, its use in transgenic plant breeding schemes can lead to the large number of "false" transformants that we had seen previously [12] and, consequently, the expense of effort, time, money to maintain, grow and analyze them. Paromomycin concentration of 100 mg/l was taken based on the results of other researchers [9], but for this genotype, the use of paramomycin at such a concentration may not be effective for transgenic plants selection. The use of 5 mg/l phosphinothricin in selective medium completely suppressed regenerative ability of callus (Fig. 1). When using glyphosate at a concentration of 0.01 mM as a selective agent we observed the regeneration of shoots from three calluses (Fig. 1). However, shoots were mostly discolored, and those that looked green were subsequently discolored during further cultivation for the next 6 weeks. Therefore, glyphosate at a concentration of 0.01 mM had a negative effect on the regenerative capacity of the maize callus. Cultivation of the maize callus for 5-6 weeks on regeneration medium, which contained 0.01 mM glyphosate followed by cultivation during the same period in the absence of herbicide, did not lead to full plant recovery, indicating that this concentration of glyphosate was sufficient for transgenic plant selection.

Four hundred and eight (PLS61)R_2×PLS61 hybrid calluses were processed by *Agrobacterium*-mediated transformation (Table 2, Fig. 1).

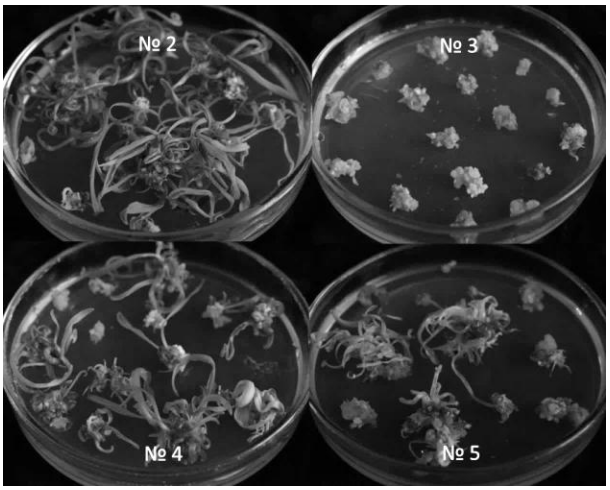

Fig. 1. Appearance of hybrid (PLS61)R_2×PLS61 calluses after cultivation on selective regeneration media No. 2-5 for 6 weeks.

Table 2. Results on *Agrobacterium*-mediated transformation of (PLS61)R_2×PLS61 maize callus using pCB135 vector

Processed calluses					Plants in jars, pcs.	Plants in soil, pcs.	PCR, samples		
Total pcs.	SRM*		Regeneration**				Total, pcs.	«+»****	
	pcs.	%	pcs.	%***				pcs.	%
408	225	55.2	47	20.9	129	25	81	5	6.2

Notes: * SRM – quantity of calluses transplanted to selective regeneration medium; ** – the number of calluses that formed plants; *** – in relation to the quantity of calluses transplanted to the selective regeneration medium; **** – the quantity of samples in which the amplicons of the expected length were observed.

After transformation, multiple plant regeneration was observed on the glyphosate containing medium (Fig. 2). The regeneration frequency was 11.5%.

One hundred and twenty nine regenerants obtained after transformation using the vector pCB135 were planted into jars with MS medium containing 500 mg/l cefotaxime. Plants that formed roots were transferred into pots with soil and grown in greenhouse (Fig. 3). In total, 25 plants were planted in soil, representing 19.4% of all plants planted in jars.

Eighty-one DNA samples extracted from plant regenerants of 29 lines derived from 23 callus lines were analyzed by PCR method. In the study on presence of the *CP4epsps* and *npt*II genes, amplicons of the expected length were found in five DNA samples of regenerants obtained from four callus lines (Fig. 4, 5). PCR for the presence of the bacterial gene *vir*-D1 did not reveal contamination of these samples with bacterial DNA (Fig. 5).

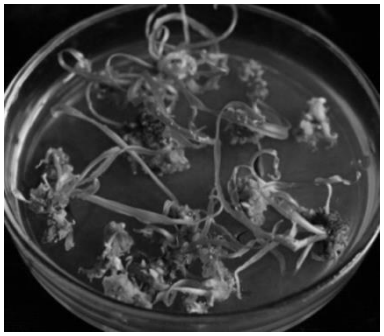

Fig. 2. (PLS61)R_2×PLS61 plant regeneration after *Agrobacterium*-mediated transformation by vector pCB135 and carrying out selection using glyphosate.

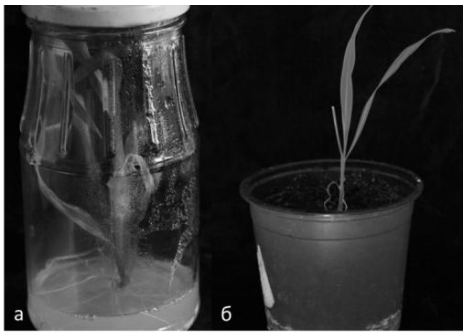

Fig. 3. Appearance of (PLS61)R_2×PLS61 maize plants *in vitro* (a) and *in vivo* (b) obtained after *Agrobacterium*-mediated transformation using vector pCB135.

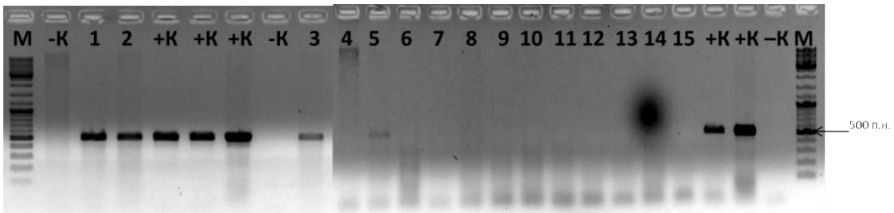

Fig. 4. Electrophoregram of products of plant DNA amplification of (PLS61)R_2×PLS61 maize regenerants, obtained as a result of *Agrobacterium*-mediated transformation using vector pCB135 after PCR for the presence of the *CPepsps* transgene. The length of expected fragment was 498 bp. Lanes 1-15 – maize DNA samples: lane 1 – line 6 (regenerant from the Petri dish), 2 – 8 (regenerant from the dish), 3 – 9 (regenerant from the dish), 4 – 6-1-1 (greenhouse plant), 5 – 6-1 (regenerant from the jar), 6 – 4-1 (regenerant from the jar), 7 – 9-1 (regenerant from the jar), 8 – 7-1 (regenerant from the jar), 9 – 13-1 (regenerant from the jar), 10 – 13-1-1 (regenerant from the jar), 11 – 6-4 (regenerant from the jar), 12 – 1-2 (regenerant from the jar), 13 – 13-1-2 (regenerant from the jar), 14 – 13-1-8 (regenerant from the jar), 15 – 7-1-3 (regenerant from the jar); +K – positive control, -K – negative control, M – molecular weight marker Leader Mix.

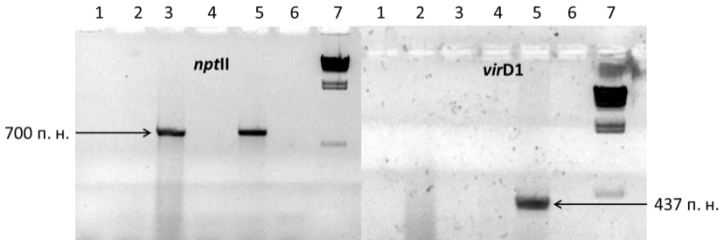

Fig. 5. Electrophoregrams of plant DNA amplification products of (PLS61)R_2×PLS61 maize regenerants obtained by *Agrobacterium*-mediated transformation using vector pCB135 after PCR for the presence of the *npt*II transgene and the bacterial *vir*-D1 gene. The expected fragment length for the *npt*II gene was 700 bp and for the *vir*-D1 gene – 437 bp. Lanes 1-7 – DNA samples: lane 1 – maize regenerant, line 14-1; 2 – 6-5; 3 – 5; 4 – 8-4; 5 – positive control; 6 – negative control; 7 – DNA marker λ/HindIII.

Interestingly, the signal intensity of amplicons corresponding to *CP4epsps* transgene, decreased as the regenerants were growing without selection pressure until complete disappearance in greenhouse plants (Fig. 4, lanes 1, 4, 5; lanes 3 and 7). Perhaps this was due to transgene methylation and, thus, a weaker affinity of DNA to the appropriate primers [18]. The maize genome has its native *epsps* gene, which can compete with the mutant transgene in the absence of selective pressure. In addition, a vector that did not contain nucleotide sequences of monocotyledonous plants to increase transgene expression in cereals was used in the study. These factors could lead to "knock out" of the transgene after removal of selective pressure as the unnecessary foreign genetic material.

Thus, we obtained transgenic maize regenerants containing the *CP4epsps* gene, after *Agrobacterium*-mediated transformation of the (PLS61)R_2×PLS61 morphogenic callus by pCB135 vector. The frequency of transformation was 1%. In our view, the low transformation frequency of maize in this study compared to others [7] was caused by using a vector that did not contain regulatory sequences to enhance transgene expression in monocotyledonous plants.

Conclusions

We have identified the maize genotype (PLS61)R_2×PLS61, which is promising for studies on the genetic transformation of maize, due to the high regenerative potential of callus derived from immature embryos that remains for a long cultivation. Callus tissue obtained from immature embryos of the (PLS61)R_2×PLS61 genotype, has a resistance to temporary agrobacterial contamination and the ability to retain its regenerative potential after glyphosate selection, which makes it particularly attractive for biotechnology studies to produce glyphosate-resistant maize plants. The use of maize morphogenic callus (PLS61)R_2×PLS61 and glyphosate at a concentration of 0.1 mM and 0.25 mM in media for callusogenesis and 0.01 mM in regeneration medium as a selective agent proved effective for the selection of transgenic plants with the *CP4epsps* gene after *Agrobacterium*-mediated callus transformation.

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СЕЛЕКЦІЯ НА СЕРЕДОВИЩАХ ІЗ ГЛІФОСАТОМ ТРАНСФОРМАНТІВ КУКУРУДЗИ З ГЕНОМ *CP4EPSPS*

Мета. Дослідити умови селекції трансформантів кукурудзи, які б містили ген *CP4epsps*, з використанням гліфосату в якості селективного агенту. ***Методи***. Культура тканин *in vitro*, *Agrobacterium*-опосередкована трансформація, селекція трансгенних рослин, виділення загальної ДНК рослин, аналіз рослинної ДНК методом полімеразної ланцюгової реакції (ПЛР). ***Результати***. Продуковано морфогенний калюс кукурудзи з незрілих зародків гібрида (PLS61)R_2×PLS61, який має високу частоту регенерації (до 95%), що зберігається впродовж тривалого культивування. В результаті *Agrobacterium*-опосередкованої трансформації морфогенного калюсу та селекції трансгенного матеріалу з використанням гліфосату були отримані трансформанти кукурудзи, які містили ген *CP4epsps* з частотою 1 %. ***Висновки***. Генотип кукурудзи (PLS61)R_2×PLS61 є перспективним для досліджень з генетичної трансформації, зокрема отримання трансгенної кукурудзи, стійкої до гербіциду гліфосату. Використання морфогенного калюсу кукурудзи (PLS61)R_2×PLS61 та гліфосату в якості селективного агенту в концентрації 0,1 мМ та 0,25 мМ у середовищах для калюсогенезу і 0,01 мМ у середовищі для регенерації виявилось ефективним для відбору трансгенних рослин з геном *CP4epsps*.
Ключові слова: *Zea mays* L., морфогенний калюс, *Agrobacterium*-опосередкована трансформація, ПЛР, генетична інженерія.