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Application of Beta-Glucuronidase Transient Expression for Selection of Maize Genotypes Competent for Genetic Transformation

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Abstract—Genetic transformation of inbred maize lines and F_1 hybrids registered in Ukraine has been carried out. The study employed a biolistic method for genetic transformation of immature maize embryos that formed callus tissue and the pAHC25 vector containing the genes of phosphinothricin-N-acetyltransferase (*bar*) and β-glucuronidase (*uidA*). As a result of the transformation of callus tissue of maize genotypes, lines resistant to phosphinothricin and regenerated plants were obtained. The activity of β-glucuronidase in herbicide-resistant calli was detected. The presence of the *bar* gene in callus DNA was demonstrated by the PCR method. The rate of stable transformation ranged from 2.2 to 30% depending on the genotype. The relationship between the results of transient expression of the β-glucuronidase gene and stable genetic transformation was observed. The proposed protocol for genetic transformation of maize using the study of transient expression of the β-glucuronidase gene makes it possible to significantly simplify the process of selecting genotypes competent for genetic transformation and create transgenic organisms with new traits.

Keywords: Zea mays L., immature embryos, biolistic genetic transformation, *bar*, *uidA*
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INTRODUCTION

Maize (*Zea mays* L.) is one of the most common cereal crops in the world and in domestic agricultural production. Due to the limited cultivated area, genetic transformation is a powerful tool for improving existing genotypes to increase the economic attractiveness of their cultivation [1].

Various factors affect the expression of transgenes and biosynthesis of foreign proteins, in particular, the site of DNA incorporation, the number of integrated copies, regulatory elements of gene transcription, intracellular targetin, etc. [2]. In general, the integration of foreign DNA is difficult to control, and the effect that the plant genome exerts at the level of transgene expression can be significant. Therefore, an important stage of the study is the selection of genotypes competent for transformation. For this purpose, vectors containing reporter genes are used to visualize transformation events wherein the most often employed genes are *uidA* and *gfp* [3, 4]. The β-glucuronidase (*uidA*) gene isolated from the *Escherichia coli* genome is present not only in bacteria but also in vertebrate and invertebrate animals, while most species of higher plants do not have it [5]. The substrate of β-glucuronidase is β -glucuronides, which turn into an insoluble blue product in the presence of the enzyme. This reaction is used for histochemical localization of β-glucuronidase activity in cells and tissues.

In our work, we examined the competence for genetic transformation of maize genotypes (inbred lines and F_1 hybrids) of Ukrainian and foreign breeding, most of which were created and have been used in the breeding process in Ukraine. The genetic transformation of immature maize embryos was carried out by the biolistic method using the pAHC25 vector [6] containing the selective phosphinothricin-K-acetyltransferase (*bar*) gene and the reporter β-glucuronidase (*uidA*) gene under the control of the maize ubiquitin gene promoter.

MATERIALS AND METHODS

For the genetic transformation, we employed inbred maize (*Zea mays* L.) lines of Ukrainian (DK959, RS15, DK633266, and DK267) and foreign breeding (PLS61) and F_1 hybrids (PLS61 × DK959, DK959 × PLS61, PLS61 × DK633266, DK633266 × PLS61, and PLS61 × KP7).

The cultivation of donor maize plants, isolation of panicles and ears, and controlled artificial pollination of plants were carried out on the plots of the Institute of Grain Crops of the National Academy of Sciences of Ukraine (Dnipro). Immature embryos 1.0–1.5 mm long were isolated from 2–7 donor plants and inoculated in vitro on a nutrient medium to induce callusogenesis (medium no. 1), which contained macro- and microsalts, vitamins N_6, 100 mg/L casein hydrolyzate, 690 mg/L L-proline, 20 g/L sucrose, 10 mg/L $AgNO_3$, 1.0 mg/L dichlorophenoxyethyl acid (2,4-D), 0.5 mg/L dicamba, 0.1 mg/L abscisic acid (ABA), 7 g/L agar, shield up, as previously described [7]. After 10–20 days, immature embryos with calli formed on shields were subjected to biolistic transformation.

For genetic transformation, we used the pAHC25 vector [6], which contained the *uidA* and *bar* genes under the control of the maize ubiquitin gene promoter.

The biolistic transformation was carried out by our self-made particle inflow gun (PIG) [8] using a compressed helium pressure of 118 psi, pressure reduction in the gun chamber to 1 psi, and a distance of 19 cm from the macrocarrier with metal particles to the plant material. Plasmid DNA was carried by gold particles 0.6 μm in diameter (Bio-Rad). The vector DNA was adsorbed on metal particles by gradually adding up to 50 μL of a suspension of particles in 50% glycerol (0.03 mg/mL), 10 μL plasmid DNA (1 μg/μL), and 20 μL PEG/$MgCl_2$ solution (50% PEG 2000, 5 M $MgCl_2$, taken in a 4 : 1 ratio). After incubation for 30 min at room temperature, the particles were collected by centrifugation (2000 rpm for 1 min) and resuspended in 60 μL absolute ethanol. Then, 6 μL of the resulting suspension of metal particles with DNA was applied to the metal lattice and used for one shot. Preparation of plant material for transformation and further cultivation was performed according to [9]. The number of explants that were treated with one shot ranged from 15 to 35 depending on the size of calli. Five to six days after biolistic treatment, the plant material was transferred to a selective medium for induction of callusogenesis (medium no. 2), which corresponded to medium no. 1 in composition but did not contain casein hydrolysate, had reduced proline content (300 mg/L), 30 g/L mannitol, and 10 mg/L phosphinotricin, and was grown in the dark at the temperature of 27°C. Within 15 days from the start of selection, calli were transferred to the medium for the induction of regeneration (medium no. 3), which contained the salt component and MS vitamins [10], 300 mg/L L-proline, 20 g/L sucrose, 10 mg/L $AgNO_3$, 0.1 mg/L 2,4-D, 0.1 mg/L 6-benzylaminopurine (BAP), 7 g/L agar, and 5 mg/L phosphinotricin. From the next passage, $AgNO_3$ and 2,4-D were removed from medium no. 3 (medium no. 4).

To determine the specific callus increase after biolistic treatment, we used calli after 3 months of subculturing in the dark on control medium no. 5, which was similar in composition to medium no. 2 but without mannitol and phosphinotricin. We added 55 mg/L phosphinotricin to the selective variant of medium no. 5. For each experiment variant, the initial raw mass of each individual callus from a cycle of 25–126 pieces depending on the experimental variant and the raw mass of the same callus after 3 months of cultivation were determined. Callus cultivation was carried out at the temperature of 26°C on media nos. 1, 2, and 5 in the dark. The specific increase in calli (mg/callus) was calculated as the ratio of the average difference between the raw mass after 3 months of cultivation and the initial raw mass of each callus to the total number of calli analyzed.

The regeneration frequency (%) was calculated as the percentage of the number of immature embryos whose calli after cultivation on the regeneration-inducing medium formed plants-regenerants to the total number of immature embryos transferred to the selective regeneration-inducing medium. The analysis of this indicator was carried out on the 60th day after biolistic treatment (30 days in the callusogenesis-inducing medium + 30 days in the regeneration-inducing medium).

A histochemical analysis of the activity of β-glucuronidase in the plant material was carried out according to the procedure [5] 4 days after the biolistics to detect transient expression of the *uidA* gene and after 2–3 months to confirm the fact of stable transformation. The frequency of stable transformation (%) was calculated as the percentage of the number of callus lines that showed β-glucuronidase activity to the total number of calli transferred to the selective regeneration-inducing medium.

Phosphinotricin-resistant maize calli were used to isolate total DNA using the CTAB method [11]. The presence of the *bar* gene in plant DNA was determined by polymerase chain reaction (PCR) using primers barlf1 (direct) 5'-ACATC GAGAC AAGCA CGGTC-3' and bar1r2 (reverse) 5'-GCCAG AAACC CACGT CATGC-3' with an annealing temperature of 59°C for 30 s, followed by a synthesis step for 30 s at 72°C. The length of the expected fragment was 411 bp [12]. As a positive control, the common DNA of an already transformed rapeseed plant was used. The reaction products were fractionated on 1% agarose gel with ethidium bromide (0.5 μg/mL) in Tris-borate buffer at 8 V/cm for 90 min.

Statistical processing of the results was carried out according to the standard method [13]. Wherever possible, the mean value and confidence interval were calculated at the significance level ($p < 0.05$). The specific increments in the raw mass of calli grown on the medium containing 5 mg/L phosphinotricin, after biolistic treatment and without such treatment, were compared by the Student's test. The percentage of callus greening was calculated for each shot and the mean and confidence intervals ($p < 0.05$) were determined.

RESULTS

Transient expression of the uidA gene in maize calli after biolistic transformation. In the course of work with the biolistic transformation of maize calli, two series of experiments were carried out with 20 and 16 shots, and approximately 1000 immature embryos were processed (Table 1). After the biolistics, the embryos with calli were transferred to medium no. 2.

Four days after the biolistics, transient expression of the *uidA* gene in calli of various maize genotypes was studied. To carry out histochemical analysis, five embryos with calli were taken from one shot. Research was not carried out for all but only for part of the shots chosen randomly. Expression of the β-glucuronidase gene led to the appearance of blue staining, which differed in the intensity and occupied area of the callus (Fig. 1a). In the control calli, which were not subjected to transformation, no blue staining was observed. The presence of blue staining in the studied callus 4 days after transformation and its absence in the control untreated material indicates transient expression of the *uidA* gene.

In the studied inbred lines of maize, 20–90% of calli revealed transient expression of the *uidA* gene (Fig. 1b). Visual assessment of the intensity and area of callus staining showed the dependence of the level of transient expression of the *uidA* transgene on the callus genotype. Among the lines, PLS61 showed a high level of *uidA* gene expression, while calli of DK633266 and DK267 showed weak gene expression. Calli of all the studied hybrids showed transient expression of the *uidA* gene at the level of 100% (Fig. 1b), which testifies to the greater competence of the hybrids for biolistic transformation as compared to the lines. The most intense staining was observed in the hybrid PLS61 × DK959, while the weakest was in DK959 × PLS61.

Table 1. Biolistic transformation of maize of various genotypes

Genotype	Quantity	
	explants	shots
PLS61	178	6
DK959	58	2
RS15	127	5
DK633266	88	4
DK267	35	2
PLS61 × DK959	75	3
DK959 × PLS61	45	2
PLS61 × DK633266	124	4
DK633266 × PLS61	152	6
PLS61 × KP7	46	2
Total	928	36

Selection of transgenic maize lines and plant regeneration. The effect of the selective agent and transformation on callus proliferation was examined by studying the dynamics of the growth in the raw mass of callus tissue using the DK633266 line as an example (Table 2).

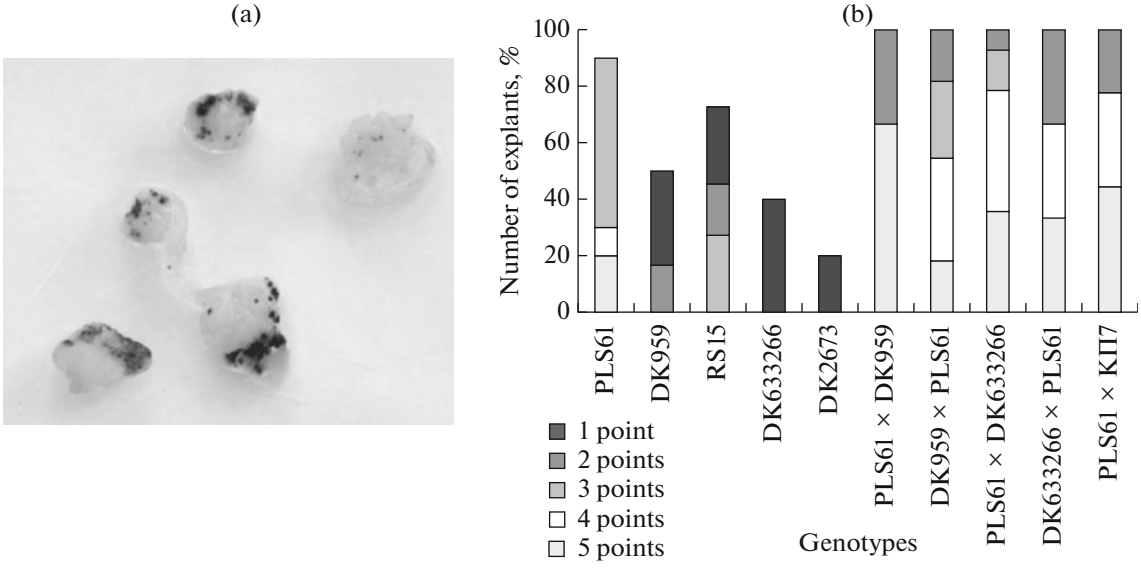

Fig. 1. Transient expression of the *uidA* gene in maize calli on the fourth day after biolistic transformation. (a) The appearance of callus of the inbred line PLS61 after histochemical analysis of the β-glucuronidase enzyme activity; (b) assessment of transient expression of the *uidA* gene for various maize genotypes. Points from 1 to 5 indicate the intensity and area of staining in the direction of their increase.

Table 2. Specific gain in raw mass of callus tissue due to selective action of phosphinotricin after 3 months of cultivation from biolistic treatment

Variant	Line DK633266	
	number of calli	specific gain in raw mass, mg/callus
1	29	282.0 ± 194.4
2	29	52.7 ± 29.4
3	38	555.8 ± 221.1
4	107	112.4 ± 18.6

1, calli without biolistic treatment, medium no. 5; 2, calli without biolistic treatment, medium no. 5 + 5 mg/L phosphinotricin; 3, calli after biolistic treatment, medium no. 5; 4, calli after biolistic treatment, medium no. 5 + 5 mg/L phosphinotricin. The confidence interval is indicated at the significance level ($p < 0.05$).

As can be seen from the data presented, variant no. 2 (without biolistic treatment, but under the action of phosphinotricin) showed a significant decrease in the gain in callus raw mass in relation to variant no. 1 (without biolistic treatment and without the influence of phosphinotricin), which indicates the inhibitory effect of the herbicide on cell proliferation in the dark, in contrast to the popular belief that phosphinotricin has no inhibitory effect in the dark [3]. As is known, phosphinotricin inhibits glutamine synthase, which plays a key role in the assimilation of ammonia, which is actively formed during photosynthesis. Therefore, the herbicide has the most negative effect on plant tissue under lighting conditions. Variant no. 3 (after biolistic treatment, without the effect of phosphinotricin) revealed a tendency to even more intensive callus growth than control variant no. 1. This stimulation of callus tissue growth can be explained by the very biolistic transformation procedure, since the blasting with metal balls creates a wound surface of the tissue and potentially can activate growth processes in response to injury. The callus raw mass gain in variant no. 4 (after biolistic treatment and cultivation with 5 mg/L phosphinotricin) was expectedly lower than in variant nos. 1 and 3. However, compared with variant no. 2, variant no. 4 showed a statistically significant ($p < 0.05$) increase in the raw mass gain. Such differences can be explained by the fact that part of the callus cells in variant no. 4 contain the *bar* gene and had genetically determined resistance to phosphinotricin.

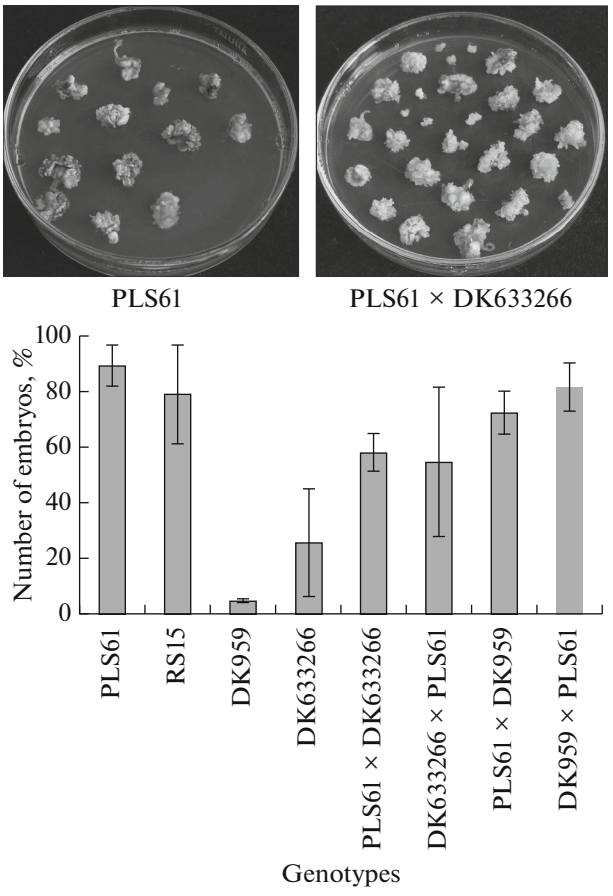

Fig. 2. Greening of maize calli after biolistic transformation on a selective medium with phosphinotricin. The confidence interval is indicated at the significance level ($p < 0.05$).

After transfer of calli, which underwent biolistic transformation, to the selective regeneration-inducing medium, their greening was observed (Fig. 2). The percentage of calli that turned green during selection differed for genotypes. In the control, after cultivation of non-treated calli, in the medium containing 5 mg/L phosphinotricin, no green calli were observed for 6 weeks.

After 2–3 months of cultivation on the selective medium, callus lines resistant to the herbicide were examined for the activity of the β-glucuronidase enzyme. Due to the risk of loss of the transgenic event, not all callus lines resistant to phosphinotricin were subjected to histochemical analysis but only those that had plant material in sufficient quantities to further obtain transgenic plants. After the histochemical analysis, we observed the appearance of blue staining for some calli (Fig. 3a, Table 3), which indicates the *uidA* gene expression and, accordingly, the transgenic nature of calli since no blue staining was observed in the control. Other researchers also did not reveal any activity similar to the action of the enzyme β-glucuronidase in the tissues of maize that was not transformed in any way [14–17].

Among the maize genotypes that underwent biolistic transformation, the presence of the *uidA* gene expression was shown for five genotypes: PLS61, DK959 × PLS61, PLS61 × DK959, DK633266 × PLS61, and PLS61 × DK633266 (Table 3). The most intense blue staining was observed for the genotypes PLS61 and PLS61 × DK959. The results of histochemical analysis of calli after prolonged selection are consistent with the results obtained after studying the transient expression of the *uidA* gene. Genotypes that

had β-glucuronidase activity 2–3 months after the transformation were selected as more competent in biolistic transformation compared to others based on the results of transient expression of the *uidA* gene. Taking into account the data of histochemical analysis, we calculated the frequency of stable transformation (Table 3).

The transformation frequency ranged from 2.2 to 30% depending on the genotype and was the highest for the genotype DK959 × PLS61. In general, the transformation frequency was quite high and corresponded to the best indicators obtained by other authors [17–19]. This testifies in favor of the fact that the proposed transformation protocol is sufficiently effective for the studied genotypes.

From calli that showed the *uidA* gene expression or phosphinotricin resistance, total DNA was isolated and analyzed by PCR for the nucleotide sequence of the *bar* gene. For all the callus lines, except for two, the presence of a fragment of the expected size was shown (Fig. 4). The absence of the expected amplicon for two callus lines of maize may be due to the low quality of DNA isolated from the callus or due to its chimerity.

Calli resistant to phosphinotricin were transferred to the selective medium to induce regeneration. After 30–45 days of cultivation, shoot regeneration was observed for four genotypes (Table 3, Fig. 5). The regeneration frequency varied from 2.2 to 6.8% and was the greatest in the PLS61 line and the DK633266 × PLS61 hybrid. When culturing the control nontransformed calli on the selective medium with phosphinotricin, regeneration was completely absent, while the regeneration frequency varied between 74.2–99.5% on the medium without the herbicide.

A histochemical analysis of leaves in regenerants revealed the activity of the enzyme β-glucuronidase (Fig. 3b).

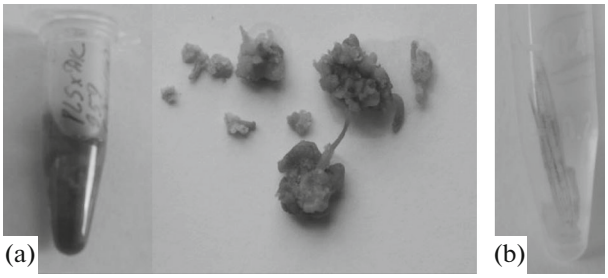

Fig. 3. Histochemical analysis for the presence of the β-glucuronidase enzyme activity for the callus line of the (a) hybrid PLS61 × DK959 and regenerants of the (b) PLS61 line after 2.5 months of cultivation on the selective medium after biolistic transformation.

DISCUSSION

Analysis of the transient expression of the *uidA* gene 4 days after biolistic transformation showed that it depends on the callus genotype. Hybrids showed a higher level of transgene expression compared to inbred lines. Among the lines, the highest, at the level of 90%, *uidA* gene expression was detected only in PLS61, which is known for its high regenerative ability in vitro [20]. Among the other maize lines involved in the study, which represent germplasms prevalent in Ukraine, only 20–70% of calli were found to express this gene. At the same time, all the studied hybrids demonstrated transient expression of the *uidA* gene at the level of 100%. These results are consistent with the data obtained from callus greening, plant regeneration on the selective medium, and histochemical analysis of β-glucuronidase activity in calli after prolonged selection. Thus, the genotypes that exhibited the highest level of transient expression of the β-glucuronidase gene turned out to be the most competent for biolistic transformation.

The issue of using F_1 lines or hybrids for genetic transformation is relevant in the context of expanding the genetic base for the transformation of maize and other cross-pollinated crops. It is generally believed that genetic modification should be carried out using inbred lines, since the result can be a line that is sister to the original one and that will contain a transgene that provides the original line with additional properties. The use of commercial lines for genetic transformation technologically faces the problem of low efficiency of callusogenesis induction, maintenance of callus tissue, and plant regeneration. At the same time, the use of F_1 hybrids for the genetic transformation of immature embryos, which combine a line in their gen-

Table 3. Regenerative and β-glucuronidase activity of maize calli after biolistic transformation

Genotype	Regeneration, number of lines	Regeneration frequency, %	GUS analysis,* number of lines	Transformation frequency, %
PLS61	5	6.8	9	12.2
DK959 × PLS61	1	5.0	6	30.0
PLS61 × DK959	0	0	6	12.2
PLS61 × DK633266	2	2.2	2	2.2
DK633266 × PLS61	4	6.8	9	15.3

* GUS analysis, histochemical analysis of calli for the presence of β-glucuronidase activity.

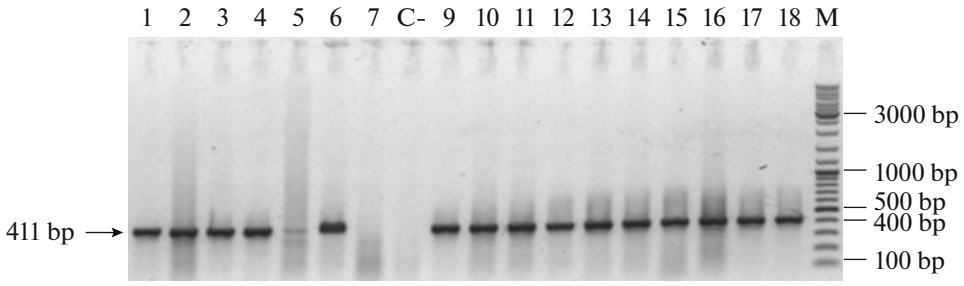

Fig. 4. Electrophoretic analysis of PCR products of callus DNA of maize lines and hybrids for the presence of the *bar* gene. Lanes 1–7, 9–18, biolistically treated calli: 1, PLS61; 2, DK959 × PLS61; 3, PLS61; 4–6, PLS61 × DK959; 7, DK959 × PLS61; 9, DK959 × PLS61; 10, PLS61; 11, 12, PLS61 × DK959; 13, PLS61 × DK633266; 14–18, DK633266 × PLS61; K, negative control, PLS61; M, Thermo Scientific™ GeneRuler™ DNA Ladder Mix molecular weight marker. The length of the expected fragment is 411 bp.

otype that constitutes the breeding value and a donor line of high callusogenesis and regenerative capacity, may be more efficient [21]. It is known that the Hi-II genotype, which was selected according to the backcross scheme with the hybrid A188 × B73, is widely used for the genetic transformation of maize in the world [22–25]. The frequency of the formation of embryogenic calli in the B73 maize line increased sharply due to introgression of chromosome segments of the A188 line in the classical backcross breeding [26]. In this work, only 0.2% of immature B73 embryos formed embryogenic callus. After crossing A188 × B73 and six backcrosses with B73 with selection in each generation for a higher frequency of callus formation and four self-pollinations, the average frequency of embryogenic callus formation reached 46%. RFLP analysis made it possible to identify segments of the A188 genome introgressed into the B73 genome. One of them, located in the long arm of chromosome 9, was closely associated with the efficiency of morphogenic callus induction and in vitro plant regeneration. The authors suggest that the main gene(s) responsible for callusogenesis and regeneration, whose introgression into other maize genotypes will expand the range of sensitive genotypes, is located in that region. These results and descriptions, including those in our studies, of the fact of a sharp increase in sensitivity in culture in vitro in first-generation hybrids as compared with inbred lines indicate the advantages of using namely hybrid genotypes for direct biolistic treatment that carry a fraction of the genetic material of both breeding-wise promising germplasms and donors of high sensitivity in vitro. When using calli of hybrids for genetic transformation, to turn both the transgene and other genes into a homozygous state, 5–7 generations of self-pollination of regenerated plants or the use of the method of matriclinous haploidy with obtaining doubled haploid lines within 2–3 years will be needed [27–29].

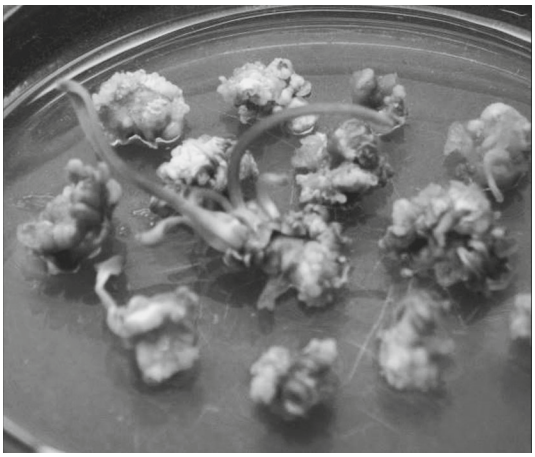

Fig. 5. Regeneration of shoots from maize callus of the PLS61 line on the selective medium.

In our study, PCR confirmed the *bar*-positive status of calli of the hybrids of the PLS61 line with commercial lines of Ukrainian breeding DK959 of germplasm Lakon and DK633266 of germplasm Lancaster. Thus, the use of hybrid immature embryos of specially selected genotypes and their formed calli as objects for introducing target genes will significantly expand the range of maize genotypes that can be modified directly, in particular, commercial germplasms common in Ukraine. It is also possible to apply the strategy of backcrossing the obtained transgenic plants in generations T_0–T_6 with the line of the commercial germplasm that is included in the formulas of the hybrid used for the transformation as well as selections for morphological and breeding traits of the commercial line. Among a wide variety of hybrid genotypes as compared with inbred lines, the method of studying transient expression of the β-glucuronidase gene will significantly narrow down their range and select those most competent for genetic transformation, and, thus, save effort, time, and costs for obtaining transgenic maize plants.

The proposed protocol for the genetic transformation of maize genotypes of foreign and domestic breeding prevalent in Ukraine, based on the use of the

ballistic method, immature embryos with formed callus tissue as explants, and phosphonotricin as a selective agent, turned out to be effective for producing transgenic maize callus lines and regenerated plants. Our study showed that phosphonotricin as a selective agent in media for inducing callusogenesis and regeneration has an inhibitory effect on the proliferation of callus tissue in maize both in the dark and under lighting. However, its negative effect is more significant precisely at the regenerative but not at the callusogenesis stage of in vitro development, which is understandable based on the mechanism of action of the herbicide, which results in the disturbance of the nitrogen metabolism in the plant cell and the accumulation of ammonia, which is produced in the process of photosynthesis in toxic amounts. Therefore, it was on the regeneration-inducing medium that the effect of selection for the use of phosphinotricin after genetic transformation was clearly manifested, when it was possible to achieve the plant regeneration frequency at the level of 2.2–6.8%, whereas it equals zero without genetic transformation on selective media with phosphinotricin.

CONCLUSIONS

As a result of biolistic genetic transformation of callus tissue from the maize genotypes registered in Ukraine—five inbred lines (PLS61, DK959, RS15, DK633266, and DK267) and five F_1 hybrids (PLS61 × DK959, DK959 × PLS61, PLS61 × DK633266, DK633266 × PLS61, and PLS61 × KP7)—transgenic calli and regenerated plants were obtained by the pAHC25 vector. According to the results of the study of transient expression of the β-glucuronidase gene, six maize genotypes were identified as the most competent for genetic transformation. The highest transient expression of the *uidA* gene among the inbred lines was demonstrated by PLS61 and among the hybrids by PLS61 × DK959. Callus lines of five maize genotypes resistant to phosphinotricin were selected. The activity of β-glucuronidase in callus cells resistant to herbicide was detected, and the presence of the *bar* gene in callus DNA was confirmed by PCR. On selective media, plants of four genotypes were regenerated: PLS61, DK959 × PLS61, PLS61 × DK633266, and DK633266 × PLS61. The PLS61 line and the DK633266 × PLS61 hybrid were characterized by the highest frequency of regeneration after biolistic treatment and cultivation with phosphinotricin. It was shown that phosphinotricin, as a selective agent in media for inducing callusogenesis and regeneration, has an inhibitory effect, and its action significantly blocks the activity of callus tissue of maize genotypes particularly at the regenerative stage of development in vitro. The frequency of stable transformation ranged from 2.2 to 30% depending on the genotype. The highest frequency of stable transformation was observed for the DK959 × PLS61 hybrid and the PLS61 line. It was shown that the results of transient expression of the β-glucuronidase gene in calli of various genotypes correspond to the results of obtaining stable transformational events. The study of transient expression of the *uidA* gene is useful for the selection of maize genotypes competent for biolistic transformation.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare no conflict of interest. This article does not contain any research using humans and animals as objects of study.

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