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INDIRECT SHOOT ORGANOGENESIS OF SOYBEAN *Glycine max* (L.) Merr. FROM STEM SEGMENTS AND USE OF THE EXPLANTS FOR *Agrobacterium*-MEDIATED TRANSFORMATION

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Abstract

Soybean *Glycine max* (L.) Merr. is an important oil, food and fodder crop for human and animals fodder. Currently, soybean lines genetically modified for improved resistance to herbicides and pests and for reduced linolenic acid content are widely grown. More than 85 % of transgenic soybean plants are obtained using *Agrobacterium*-mediated transformation method. Developed *Agrobacterium*-mediated protocols are based on somatic embryogenesis and direct or indirect shoot organogenesis. Cotyledons, cotyledonary nodes, hypocotyl and epicotyl segments, immature or mature embryos serve as explants. Despite the large number of *Agrobacterium*-mediated protocols, stable transformation of soybeans is still not a routine procedure because it depends on the genotype. Surprisingly, the data on the use of soybean stem segments in genetic transformation is practically absent, although stem segments widely and efficiently serve as explants in the production of most transgenic monocotyledonous plants. Thus, the purpose of the study was to develop a protocol for shoot organogenesis from stem segments of soybean and their application as explants for the production of transgenic plants by *Agrobacterium*-mediated transformation. Stem segments of aseptic soybean seedlings of breeding lines 1476 and 1477 were used for callus induction and shoot organogenesis. The explants were cultured on four various MS-based growing media which differed in 6-benzylaminopurine (BA) concentrations (0.5 and 1.0 mg/l) in combination with i) 0.1 mg/l indole-3-acetic acid (IAA), or ii) 0.1 mg/l indole-3-acetic acid (IAA) and 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2.4-D). It was shown that the studied soybeans genotypes differ significantly in morphogenetic ability. Out experiments confirmed that the addition of 2.4-D resulted in inhibition of shoot organogenesis in the both genotypes. It was found that 1 mg/l BA in combination with 0.1 mg/l IAA are the best growth regulators providing the highest frequency of indirect shoot organogenesis. As a result, an effective protocol of indirect shoot organogenesis from soybean stem segments of line 1476 seedlings was developed which ensures more than 50 % frequency of organogenesis. This protocol was applied in genetic transformation of soybean line 1476 by *Agrobacterium tumefaciens* strain AGL0 carrying the plasmid pCambia1381Z-pro-SmAMP1-771. By gradual selection on the induction medium supplemented with hygromycin B (1-10 mg/l), 8 independent lines of putative primary transformants were selected. PCR analysis confirmed the presence of the selective (*hpt*) and marker (*uidA*) genes in 4 independent transgenic lines. The transformation efficiencies calculated based on the results of PCR analysis was 2.0 %. These results indicate the successful involvement of stem segments as explants for genetic transformation of soybean.

Keywords: soybean, *Glycine max* (L.) Merr., in vitro culture, shoot organogenesis, *Agrobacterium*-mediated transformation

Soybean *Glycine max* (L.) Merr. is one of the most important food, in-

dustrial and fodder crops in the world. Soybean seeds contain about 40% of amino acid balanced protein, 20% of fat, as well as a large number of physiologically active substances, the vitamins, macro- and microelements and isoflavones [1]. According to the Food and Agriculture Organization of the United Nations (FAO), the global gross harvest of soybeans in 2014 amounted to more than 306.5 million tons with the cultivation in the territory of 117.5 million hectares, out of which Russia accounted for about 0.8% (2.3 million tons) from an area of 1.9 million hectares. At the same time, over 90% of soybean acreage in Russia is concentrated in the Far Eastern, Central and Southern Federal Districts. According to the International Service for the Acquisition of Agri-biotech Applications (ISAAA), genetically modified soybean lines, which have elevated resistance to herbicides and pests, as well as a reduced content of linolenic acid, were grown on the 78% of arable land in 2016 (91.4 million hectares), occupied by this crop in the world [2].

For the first time, two independent groups of researchers reported on the production of transgenic soybean plants in 1988 [3, 4]. To date, there are a large number of publications on this topic; however, despite the abundance of experimental data, the production of stable transgenic soybean plants has not become a routine procedure, since this process depends on the availability of effective protocols for shoot regeneration in vitro and under genetic transformation conditions. Over 85% of transgenic soybean plants are obtained by the method of agrobacterial transformation [4-8]. To introduce foreign genetic material into the soybean genome, direct methods are also used, i.e. bioballistic transformation [3, 9] and protoplast electroporation [10]. In recent years, the method of germ-line genetic transformation of soybean, in which elements of the plant's generative organs (germinating pollen, egg-cells, embryos and seeds) are used for introducing exogenous DNA, have become widespread [11, 12]. Each method of genetic transformation has its advantages and disadvantages. For bioballistic transformation, expensive consumables and equipment are required. Compared to direct methods of introducing exogenous DNA, the *Agrobacterium*-mediated method has a number of advantages: relative simplicity and cheapness, high competence of *Dicotyledoneae* plant cells to agrobacterial infection, the possibility of transferring large fragments of foreign genetic material given the low abundance nature of their integration in the genome.

Induction of morphogenesis in soybean tissue culture is a complex process, the regulation of which is executed at the cellular, tissue and organism levels [13]. The nature of morphogenesis (somatic embryogenesis or organogenesis), as well as its potential, are determined by the genotypic characteristics of the culture, the type and physiological age of the explant, the nutrient medium composition, physical factors, and many others factors [14, 15]. Thus, the research of C. Zhang et al. [16] demonstrated the functional role of the soybean transcription factor *GmESR1* in the regulation of genes responsible for the realization of the regeneration potential. It was suggested that differences in the ability to form somatic embryoids in vitro in soybean genotypes are most likely determined by the unequal content of endogenous auxins in cells and/or the degree of sensitivity to these hormones [17, 18].

Protocols for agrobacterial transformation of soybean with the use of somatic embryogenesis have been developed to date [19], although most often plants-regenerators are obtained by direct [7, 8, 20] or indirect [4-6, 9, 17-19] organogenesis using different types of explants, e.g. cotyledons [4, 17], hypocotyl [6, 15] and epicotyl segments [21], cotyledonary and leaf nodes [7, 8, 15, 20], immature [9, 14, 18, 19] and mature [5, 14] embryos. The selection of the basic composition of the nutrient medium, as well as the type and concentration of

growth regulators, is fundamentally important for the induction of the processes of morphogenesis in soybean tissue culture. Most often, explants are cultivated on media based on macro- and microelements according to Murashige and Skoog medium (MS) [5-8, 17, 18] or Gamborg medium [4, 15], supplemented by various growth regulators, particularly 6-benzylaminopurine (6-BAP) [4-6, 15, 20], thidiazuron [8, 14], 2,4-dichlorophenoxyacetic acid (2,4-D) [9, 17, 18] and indole-3-acetic acid (IAA) [8]. The positive effect of polyamines (spermidine) [5], as well as inhibitors of ethylene biosynthesis ($AgNO_3$) [15], on the increase in the frequency of shoot organogenesis in soybean was proved.

Available scientific publications show that segments of soybean stem are practically not used for genetic transformation, although such explants are effectively used in the genetic modification of most *Dicotyledoneae* plants. This type of explant can significantly reduce the amount of work for obtaining donor seedlings, which is especially important in case of limited seed material.

In this study, for the first time, experimental data on the model of two promising soybean breeding lines have been obtained, confirming the capacity of stem segments for shoot organogenesis in the culture in vitro, as well as their use as explants for genetic transformation by means of *Agrobacterium tumefaciens*.

The goal was to develop a protocol for the somatic organogenesis of shoots from stem segments in soybean tissue culture and their use as explants for the production of transgenic plants by the method of agrobacterial transformation.

Techniques. The initial plant material was soybean seeds *Glycine max* (L.) Merr. of two promising breeding lines (1476 and 1477), obtained at the All-Russian Scientific Research Institute of Leguminous and Cereal Crops (Orel Province, Russia). The seeds were surface sterilized for 10 min in 70% ethanol and then in 40% aqueous solution of sodium hypochlorite for 20 min, after which they were washed 3-4 times in sterile distilled water and germinated in culture vessels with a basic nutrient medium containing mineral components and vitamins according to MS medium [22] with sucrose (3%) and agar (0.8%). On days 12-14 of cultivation, 1.0-1.5-cm-long stem segments were isolated from aseptic donor seedlings. Then the stem segments were placed on the basic nutrient MS medium with the addition of various growth regulators to induce morphogenesis: MS_1 — 1 mg/l 6-BAP, 0.1 mg/l IAA; MS_2 — 1 mg/l 6-BAP, 0.5 mg/l 2,4-D, 0.1 mg/l IAA; MS_3 — 0.5 mg/l 6-BAP, 0.1 mg/l IAA; MS_4 — 0.5 mg/l 6-BAP, 0.5 mg/l 2,4-D, 0.1 mg/l IAA. Donor seedlings and explants were cultured in a climate chamber WLR-351H (Sanyo, Japan) at 18-21 °C, 4 klx illuminance and 16/8 h photoperiod (day/night). Passage to a fresh nutrient medium was performed every 14 days. On days 28 and 42 of culture, the frequency of shoot organogenesis was estimated to determine the variant of the nutrient medium providing the maximum yield of regenerants. The frequency of organogenesis, expressed as a percentage, was defined as the ratio of the number of stem segments, where at least one regenerated shoot originated, to the total number of explants. Each variant of the culture medium included at least 300 explants; the repetition was 3-fold.

For agrobacterial transformation, the previously obtained genetic construct pCambia1381Z-pro-SmAMP1-771 [23] was used; the plasmid pCambia1381Z-pro-SmAMP1-771 was transferred to the *A. tumefaciens* cells of the supervirulent strain AGL0 by the electroporation method [24].

Genetic transformation of plants was made by the method of co-cultivation of stem segments with a dilute suspension of *Agrobacterium*. Bacteria of AGL0 strain, bearing a genetic construct, were cultured on an orbital shaking incubator (180 rpm) for 12 hours at 28 °C in the dark in 20 ml of non-agar Luria-Bertani medium (LB) [25] supplemented with appropriate selective antibiot-

ics rifampicin (Sigma, USA) and kanamycin (JSC Biochemist, Russia) in concentrations of 25 and 50 mg/ml, respectively. The obtained agrobacterial culture was diluted with a non-agar medium MS to OD_{600} = 0.4-0.6. The optical density of the suspension was determined on a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). The explants were incubated in a bacterial suspension for 40 min, then dried with sterile wipes from filter paper and transferred to Petri dishes with agarized MS medium. Co-culture of explants with *Agrobacterium* was executed in the dark at 18 °C for 48 hours. Treated stem segments were washed 5-6 times with a non-agar MS medium supplemented with antibiotic timentin (ticarcillin + clavulanic acid) (SmithKline Beecham Pharmaceuticals, UK) in a concentration of 300 mg/l to eliminate *Agrobacterium*. The explants were cultured on MS_1 nutrient medium supplemented with 300 mg/l of timentin and 1 mg/l of hygromycin B (PhytoTechnology Laboratories, USA) to select shoots, which are resistant to the selective antibiotic. In subsequent passages, the concentration of the selective antibiotic was gradually increased to 10 mg/l. The transgenic status of soybean shoots resistant to hygromycin B was confirmed by polymerase chain reaction (PCR).

Total DNA was isolated using a DNA-Extran-3 kit (ZAO Sintol, Russia) according to the manufacturer's instructions. The DNA concentration was determined on a NanoDrop 1000 spectrophotometer. The total genomic DNA preparations obtained from wild-type plants and the plasmid DNA of pCambia1381Z-pro-SmAMP1-771 vector were used as a negative and positive control, respectively. PCR to identify the *hpt*, *uidA* and *virE2* genes was conducted using specific primers on a MJ Mini™ Personal Thermal Cycler amplifier (Bio-Rad, USA) in the following modes: total denaturation for 3 min at 94 °C; 35 cycles of denaturation, annealing of the primer and elongation for 30 s (*hpt* and *virE2* genes) and 1.5 min (*uidA* gene) at temperatures of 94, 62 and 72 °C respectively; total elongation for 5 min at 72 °C. The primers for the *hpt* and *uidA* gene sequences were selected using VectorNTI (Thermo Fisher Scientific, USA). The 25 μl PCR reaction mixture contained 2.5 μl of 10× PCR buffer, 0.5 μl of a 10 mM dNTPs mixture, 1 μl of forward and reverse primers at a concentration of 10 pM each, 1 μl of Taq DNA polymerase (5 IU/μl), 17 μl of bidistilled water and 2 μl (~ 60 ng) of DNA. The amplification products were separated in an electrophoresis chamber (Hoeffer, USA) in 1% agarose gel with 1× TAE buffer with ethidium bromide (Helicon LLC, Russia). The amplified fragments were visualized on the transilluminator UVT-1 (CJSC Biokom, Russia); their sizes were estimated with the molecular weight marker Gene Ruler 1kb DNA Ladder (Fermentas, USA).

Statistical processing of the results was made using the parametric tests of Student, Fisher and Duncan (α = 0.05). Before making the two-way analysis of the variance the mean values of the shoot organogenesis frequency were recalculated using the angle-inverse function $\sqrt{X}$. The calculations were made using the statistical program AGROS (version 2.11).

Results. One of the most important conditions for the production of transgenic plants is the capacity of cultured organs and tissues for organogenesis of full-fledged fertile shoots. It should be taken into account that while performing agrobacterial transformation, the morphogenetic potential of cultured tissues, regardless of the type of the explant used, is significantly reduced. The reasons for this are direct infection with a pathogenic microorganism, the insertion of T-DNA into functionally important parts of the genome, the inhibitory effect of selective antibiotics, prolonged cultivation in vitro, as well as a number of other stress factors that generate the formation of an excessive amount of active oxygen forms [27, 28]. In this connection, the initial task of the present study was

to develop a protocol for shoot organogenesis from the stem segments of the used soybean breeding lines. Callus induction and organogenesis was executed on 4 variants of nutrient media composed according to MS and supplemented with various concentrations of 6-BAP in combination with IAA (MS_1, MS_3) or IAA and 2,4-D (MS_2, MS_4). As a result, on days 8-10 of culture of stem segments of both soybean genotypes on all nutrient media, there was an increase in the size of explants. By the end of the first passage, callus tissue was formed. Qualitative characteristics of the callus tissue formed, as well as the place of its formation on the explant, significantly depends on the composition of the nutrient medium. Thus, with the simultaneous presence of two growth regulators of the auxin type in the composition of the nutrient medium, regardless of the concentration of 6-BAP (MS_2, MS_4), a light yellow pithy callus was formed on the entire surface of the explant. Then, the cells of the upper layers of light yellow callus produced a non-morphogenic unstructured callus of white color which necrotized during culture. At the same time, when culturing stem segments on nutrient media MS_1 and MS_3, the formation of callus tissue occurres mainly at the edges of the explant. The callus has a yellow-green or light green color and a denser structure (Fig. 1, A). In the callus tissue of this type, meristematic foci were formed, from which shoot organogenesis originated (see Fig. 1, B).

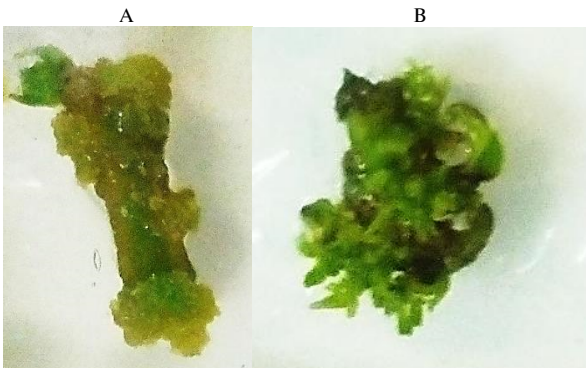

Fig. 2. Callus formation (A) **and mass shoot organogenesis** (B) **in culture of stem segments of soybean *Glycine max* (L.) Merr. line 1476 on Murashige and Skoog medium (MS_1) supplemented with 6-benzylaminopurine (1 mg/l) in combination with indole-3-acetic acid (0.1 mg/l).**

A two-way analysis of variance established the presence of significant differences at the 5% level of significance in terms of the frequency of shoot organogenesis between the studied genotypes and variants of nutrient media. In addition, the differences were also significant for the interaction of factors of the genotype × variant of the medium. The highest frequency of shoot organogenesis on days 28 and 42 was noted in the cultivation of soybean stem segments of the line 1476 on MS_1 nutrient media, containing 1 mg/l 6-BAP and 0.1 mg/l IAA. The frequency of organogenesis was 44.1% and 51.2%, respectively (Table 1). Reducing the concentration of 6-BAP in the nutrient medium (MS_3) led to a significant decrease in the frequency of shoot organogenesis in this genotype. Moreover, the addition of auxin 2,4-D to the nutrient medium inhibited the shoot organogenesis process. Similar results were noted with respect to the frequency of shoot organogenesis for the line 1477, except that this genotype was characterized by an extremely low

1. Frequency of organogenesis soybean *Glycine max* (L.) Merr. stem segments shoot as influenced by the genotype and composition of the nutrient medium

Nutrient medium	Organogenesis of shoots, %	
	day 28	day 42
	Line 1476	
MS_1	44.1^{e}	51.2^{g}
MS_2	20.8^{c}	35.1^{e}
MS_3	32.0^{d}	43.8^{f}
MS_4	14.5^{abc}	25.0^{cd}
	Line 1477	
MS_1	18.3^{bc}	27.0^{d}
MS_2	11.6^{a}	18.5^{b}
MS_3	13.5^{ab}	21.3^{bc}
MS_4	9.1^{a}	12.3^{a}

Note. See Techniques section for media. Differences between variants marked with at least one identical letter are statistically insignificant by the Duncan criterion ($\alpha = 0.05$).

capacity for morphogenesis in vitro. Thus, the frequency of shoot organogenesis on day 42 of explant culture on MS_1 medium did not exceed 27.0%. Thus, giving the low regeneration capacity of the line 1477, subsequent experiments on agrobacterial transformation with the use of this genotype seem inexpedient.

Within the framework of the present study, two independent experiments on the agrobacterial transformation of the soybean selection line 1476 were conducted. The genetic construct pCambia1381Z-pro-SmAMP1-771 [23] used for this purpose carries the selective gene *hpt* in T-DNA, which determines the resistance to hygromycin B, as well as a reporter gene *uidA* containing a modified intron of the castor catalase gene under the control of the 5′-deletion –771 bp of promoter *pro-SmAMP1* from *Stellaria media* (L.) (Fig. 2).

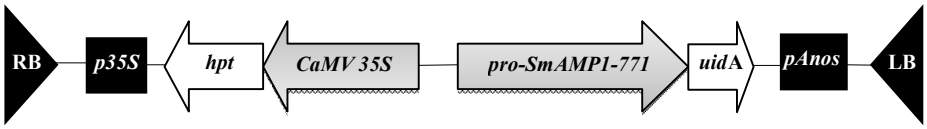

Fig. 2. Schematic representation of T-DNA of the genetic construct pCambia1381Z-pro-SmAMP1-771 used in experiments on the agrobacterial transformation of soybean *Glycine max* (L.) Merr.: RB, LB — respectively, the right and left flanking sequences of T-DNA, *CaMV 35S* and *p35S* — promoter and terminator of 35S RNA of cauliflower mosaic virus, *hpt* — hygromycin phosphotransferase gene of *Escherichia coli*, *pro-SmAMP1-771* — 5′-deletion variant (–771 bp) of *pro-SmAMP1* promoter from *Stellaria media*, *uid*A — reporter gene β-glucuronidase containing a modified intron of the castor catalase gene, *pAnos* — terminator of the nopaline synthase gene [23].

In total, 200 explants were inoculated while co-culture with a suspension of the *A. tumefaciens* AGL0 strain containing the plasmid pCambia1381Z-pro-SmAMP1-771. We used a strategy of gradual adaptation of explants to a selective agent, excluding shock and mass death (gradual increase in concentration in the selective environment of hygromycin B) which was successfully used in the agrobacterial transformation of tomato [29]. Despite the gradual increase in the concentration of hygromycin B in the selective medium MS_1, most of the stem segments (75.5%, or 151 explants) necrotized during the culture process. Moreover, bacterial contamination was observed for a part of the explants (36, or 18.0%). Necrotized and contaminated segments of the stem were excluded from the experiment. As a result, at the beginning of the third passage, the formation of light green callus-like tissue was observed for only 13 explants (6.5%). With an increase in the concentration of the selective agent in the nutrient medium, there was an increase in the size of the callus tissue. As a result, the frequency of callus formation on a selective nutrient medium supplemented with 10 mg/l of hygromycin B was 6.5%. However, only 8 of these 13 callus tissues (4.0% of the total number of explants) formed on a selective nutrient medium for the induction of morphogenesis were observed to form dense globular meristematic foci of green color, from which organogenesis of shoots subsequently originated. As a result, the frequency of organogenesis of shoots, resistant to hygromycin B, was 4.0%.

2. Nucleotide sequences of primers used in PCR to confirm the presence of *hpt*, *uidA* and *virE2* genes in hygromycin B resistant soybean *Glycine max* (L.) Merr. regenerants of breeding line 1476, and the expected size of amplicons

Gene	The nucleotide sequence of the primer (5′→3′)	The size of the amplicon, bp
hpt	F – TCTGATAGAGTTGGTCAAGACC	415
	R – CAAGGAATCGGTCAATACACTAC	
uidA	F – ATCGCGAAAACTGTGGAATTGATC	1628
	R – TTACCGCCAACGCGCAATATG	
VirE2 (26)	F – CGAATACATTCTCGTGCGTCAAACG	600
	R – TTTCGAGTCATGCATAATGCCTGAC	

Note. F, R — forward and reverse primers, respectively.

PCR analysis confirms the transgenic status of independent regenerants

resistant to hygromycin B. When amplified using specific primers (Table 2), for a sequence of the *hpt* selective gene, fragments corresponding to a positive control were obtained in 4 of the 8 regenerative plants (Fig. 3, A). Integration of the reporter gene *uidA* was established in all analyzed samples containing *hpt* gene (see Fig. 3, B). Also, all preparations of total genomic DNA in the studied samples were tested for the absence of the bacterial gene *VirE2* to exclude false positive results due to bacterial contamination (see Fig. 3, B). Thus, the efficiency of agrobacterial transformation of the soybean stem segments of the line 1476 when using the genetic construct pCambia1381Z-pro-SmAMP1-771 is 2.0%.

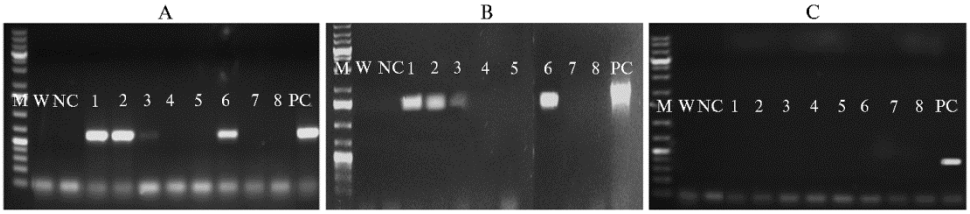

Fig. 3. Electrophoregrams of PCR products of *hpt* (A), *uidA* (B) and *VirE2* (C) genes in hygromycin B resistant regenerants of soybean *Glycine max* (L.) Merr. breeding line 1476: M — molecular weight marker (Gene Ruler 1kb DNA Ladder, Fermentas, USA), W — water, NC — negative control (total genomic DNA isolated from the 1476 soybean line), 1-8 — DNA of soybean regenerants resistant to hygromycin B, PC — positive control (plasmid DNA pCambia1381Z-pro-SmAMP1-771).

From PCR analysis, 50% of the regenerants resistant to hygromycin B are found to be so-called escapes. i.e. the plants adapted to exist on a selective medium with an antibiotic but not containing foreign DNA in the genome. In recent years, researchers have shown increased scientific and practical interest in this phenomenon. In particular, the escapes of fiber flax, which survived under the influence of stress factors after agrobacterial transformation, expanded the spectrum of genetic variability and served as a starting material for creating genotypes with an improved combination of selection characteristics [30].

Thus, as a result of the conducted studies, it is shown that the studied soybean breeding lines (1476 and 1477) differ significantly in their capacity for morphogenesis in vitro. It is experimentally confirmed that the addition of auxin 2,4-dichlorophenoxyacetic acid results in inhibition of shoot organogenesis. The growth regulators added to Murashige and Skoog nutrient medium and providing the maximum yield of regenerants are 6-benzylaminopurine (1 mg/l) in combination with indole-3-acetic acid (0.1 mg/l). For the soybean line 1476, an effective protocol is suggested for indirect somatic shoot organogenesis from stem segments with a frequency of over 50%. The protocol was used in subsequent experiments on genetic transformation performed by *Agrobacterium tumefaciens* AGL0 strain containing the plasmid pCambia1381Z-pro-SmAMP1-771. A stage-by-stage selection on a nutrient medium with increasing concentrations of hygromycin B (1-10 mg/l) resulted in eight independent lines. PCR method confirms the presence of the selective (*hpt*) and marker (*uidA*) genes in four of these lines. The effectiveness of agrobacterial transformation in our experiments is 2.0%. The obtained results testify to the successful application and prospects of using stem segments as explants for genetic soybean transformation.

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