BIOLOGIJA. 2018. Vol. 64. No. 4. P. 321–330

"Floral-dip" transformation of *Amaranthus caudatus* L. and hybrids *A. caudatus* × *A. paniculatus* L.

Olha Yaroshko[1],

Maksym Vasylenko[1],

Alena Gajdošová[2],

Bogdan Morgun[1],

Olesia Khrystan[1],

Liudmyla Velykozhon[1],

Mykola Kuchuk[1]

[1] *Institute of Cell Biology and Genetic Engineering, National Academy of Sciences of Ukraine, 148 Akademika Zabolotnogo St., Kyiv, Ukraine*

[2] *Institute of Plant Genetics and Biotechnology of the Slovak Academy of Sciences, P. O. Box 39 A, Akademická 2, 950 07 Nitra, Slovak Republic*

After "floral-dip" transformation of *Amaranth* plants with *Agrobacterium tumefaciens* strain GV3101 carrying pCBV19 gene vector that contained *bar* and *gus* genes, transgenic seeds were obtained. The functioning of the tran+sferred genes in *Amaranthus* tissues was confirmed with herbicide selection (PPT herbicide – *phospinotricin*) and *gus* gene activity. Positive results were obtained for cultivars "Karmin" and "Kremoviy rannii". The percentage of GUS positive samples was 1% (for "Karmin"), 2.2% (for "Kremoviy rannii") from the total initial quantity of plants that was prior to selection with the herbicide. The seeds of six amaranth cultivars were received after treatment with *A. tumefaciens* by the method "floral dip". The lowest lethal dose of herbicide PPT was established – 40 mg/l. After spraying with herbicide, resistant plants were obtained for cultivars: "Kremoviy rannii" (21%) and "Karmin" (20%). After conduction of PCR analysis, positive results were obtained for four cultivars. The percentage of *bar* positive plants was 0.3% ("Helios"); 0.26% ("Sterkch"); 0.06% ("Kremoviy rannii"); 0.3% ("Rushnichok") from total initial quantity of plants.

Keywords: *Agrobacterium*, transformation, "floral-dip", transgenic *Amaranthus*

INTRODUCTION

Amaranth (food of the Aztecs) is still not widely used as an agricultural plant despite its many advantages such as a high protein content (13–29%) (Zeleznov et al., 2009), and high yield, when one plant can produce up to 50,000 seeds (Munusamy et al., 2013). Although highly valuable, genetic transformation of amaranths has not been developed. One of possible application of transgenic amaranth plants might be "molecular farming", that is, production of pharmaceutical proteins in plants.

* Corresponding author. Email: 90tigeryaroshko90@gmail.com

The aim of this work was to evaluate the functioning of transiently transferred *gus* gene in amaranth tissues, to detect the lowest lethal dose of PPT herbicide on amaranth seedlings, and to obtain transgenic plants of amaranth after the "floral-dip" genetic transformation by using *A. tumefaciens* (pCBV19).

MATERIALS AND METHODS

The transient expression of the transferred genes in the leaves of *Amaranthus*

The objects of research were cultivars of species of *Amaranthus caudatus* L.: "Helios", "Karmin", "Kremoviy rannii", "Rushnichok", hybrids: *A. caudatus* × *A. paniculatus* L. – cv. "Sterkh", *A. caudatus* × "Sterkh" – cv. "Zhaivir", the seeds were obtained from M. M. Grishko Botanical Garden of the National Academy of Sciences of Ukraine.

The goal was to check and evaluate the functioning of pCBV19 gene vector of *A. tumefaciens* (Fig. 1) in transiently transformed *Amaranthus* leaves.

For this purpose we used the method of vacuum infiltration (Martins et al., 2015) and detection of *gus* activity (Jefferson, 1987). As an infection agent, we used *A. tumefaciens* gene construction pCBV19 which contained *bar* and *gus* genes.

The seeds were germinated in pots with soil and were grown in a greenhouse (22–26°C, 14-hour light period, illumination 3000–4500 lx).

After two months of growing in the greenhouse, plants were infiltrated with strain *A. tumefaciens*.

First, *A. tumefaciens* was sown in the liquid LB medium (for 24 h mixing on shaker). We added 1 ml of *A. tumefaciens* into 50 ml of medium with 0.2 mM of acetosiringone. Next, *Agrobacterium* was centrifuged for 12 min, 5000 rpm and was resuspended into the medium with sucrose (50 g/l + 0.2% super wetting agent Silwet) (Munusamy et al., 2013).

Infiltration of whole plants was carried out in the flask with the medium containing *A. tumefaciens* (for 5–10 min, 22–24°C) in the vacuum chamber under pressure of 0.1 mPa.

Detection of *gus* genes (β-*glucuronidase* activity) was carried out by histochemical assay on fourth day after infiltration in the presence of its specific substance, X-gluc (5-bromo-4-chloro-3-indolyl-β-*D*-glucuronide by Jefferson (Jefferson, 1987). The leaves of the infiltrated and control (without infiltration) plants were taken and incubated in *gus* histochemical buffer (50 Mm sodium phosphate, pH 7.0; 50 mM EDTA, pH 8.0; 0.5 mM $K_3Fe(CN)_6$; 0.5 mM $K_4Fe(CN)_6$; 0.1% Triton X-100; 1 mM X-gluc).

Gus reaction was stopped after 24 h of incubation at 37°C in the dark, then rinsed five times in 70% ethanol at 1 h intervals. After that, leaves were placed on microscope slides for observation. Specific activities were detected visually by the appearance of staining of plant tissues in blue colour.

The possibility of endogenous *gus* expression was tested by subjecting uninfiltrated leaves (negative control). Leaves of stably transformed *Nicotiana tabacum* were used as positive control.

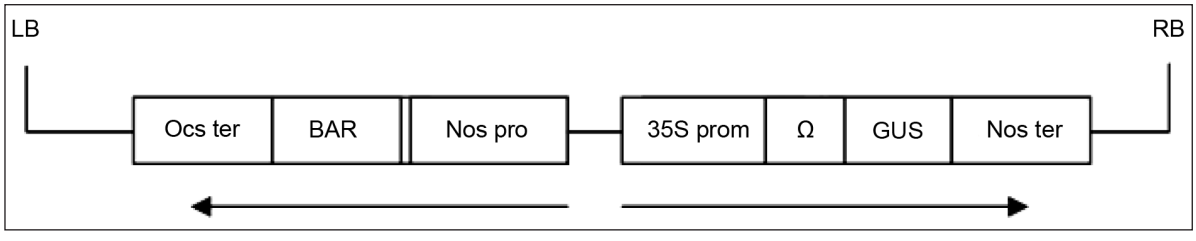

Fig. 1. Schematic representation of the T-DNA site of the pCBV19 vector LB – left border sequence, RB – right border sequence; Nos pro – nopaline synthase promoter, Nos ter – nopaline synthase terminator; 35S prom – 35S promoter; BAR – *bar* gene, GUS – *gus* gene; Ocs – octopine synthase; Ω – regulatory sequence enhancer

Determination of the lowest lethal dose of PPT herbicide (phosphinothricin) on amaranth seedlings

Seeds obtained from plants not subjected to agroinfiltration were sown in plastic pots with soil and grown in greenhouse conditions (22–26°C, 14-hour light period, illumination 3000–4500 lx). At the age of two weeks, the seedlings were sprayed with PPT herbicide of different concentrations of (10 mg/l, 20 mg/l, 40 mg/l). The amaranth plants were used as negative control grown without PPT spraying. Determination of the lowest lethal dose of PPT herbicide on uninfiltrated with *Agrobacterium* seedlings was conducted in order to fulfil further selection of assumedly transformed seedlings with the same herbicide in the presence of *bar* gene.

The "floral-dip" transformation of *Amaranthus* and selection of transformed plants

First, the seeds of the above-mentioned cultivars were sown in open ground. For two months the plants were grown under natural daylight in open ground and then transferred into greenhouse conditions.

A. tumefaciens with the recombinant vector from the glycerol stocks was sown in the liquid LB medium (incubated at 28°C for 24 h mixing on shaker). After 24 h of incubation, 1 ml of *A. tumefaciens* (bacterial cell density 10^9 cell ml^{-1}) was added into 50 ml of medium with 0.2 mM of acetosiringone. Next, *Agrobacterium* was centrifuged for 12 min, 5000 rpm. Then, it was resuspended into medium with sucrose (50 g/l + 0.2% super wetting agent Silwet) (Munusamy et al., 2013).

At the age of two months, amaranth inflorescences (when the size of inflorescences was in the range of 5–8 cm) were infiltrated according to the "floral-dip" method (Zhang et al., 1999). For this purpose the inflorescences of *Amaranthus* were dipped into a flask with medium containing sucrose (50 g/l) + 0.2% super wetting agent Silwet and *A. tumefaciens* (for 10 min, 24–26°C). An equal quantity of *Amaranthus* plants were infiltrated by the "floral-dip" method (50 plants for each variety; 20 plants were not treated with a suspension to obtain seeds of control plants). The amaranth plants were infiltrated in a shaded area away from direct sunlight. Then, a cellophane package was put on each inflorescence after the infiltration. The plants were left in similar conditions overnight. On the next day, the cellophane packages were changed to paper packages. Plants were allowed to grow in greenhouse conditions until 50% of the plants became yellow (the seed maturity phase).

The same varieties of *Amaranthus* grown under the same conditions as those of the infiltrated plants, but without agrobacterial infiltration, were used as negative control.

More than 10,000 mature seeds (T_1) were harvested and stored according to Curtis (Curtis, 2004) and Bent (Bent, 2006). The seeds were harvested when the age of plants was 82 days for variety "Zhaivir", 102 days for variety "Karmin" and "Helios", 113 days for variety "Rushnichok" and "Kremoviy rannii", and 123 days for variety "Sterkh".

The post-harvesting period for the seeds from "floral-dipped" plants continued during 120 days. After this period, the seeds were sown in plastic pots with soil and grown in greenhouse conditions (22–26°C, 14-hour light period, illumination 3000–4500 lx).

To screen for the presence of *bar* and *gus* genes, 2500 seeds and 200 seeds obtained from control plants (grown without treatment with *Agrobacterium*) were sown separately. The seedlings obtained from "floral-dipped" and control plant seeds were treated at the age of two weeks with a PPT herbicide at a concentration of 40 mg/l (screening for the resistant plants holding a *bar* gene).

The next step was detection of the *gus* gene presence in selected resistant plants by histochemical assay (GUS activity) (Jefferson, 1987). The surviving seedlings at the age of three weeks were used for the conduction of this experimental step.

After performing these stages of the experiment, a statistical analysis of the *bar*- and *gus*-positive plants was carried out.

Genomic DNA was isolated by the CTAB method (Stewart & Via, 1993). For the PCR analysis, we used the reaction mixture of the

following composition: 2 µl single PCR buffer with ammonium sulphate (Dream Taq Green Buf.), 1 µl primers, 2 µl deoxyribonucleotide triphosphate (dNTP), 0.15 µl FirePol DNA Polymerase, 1,5 µl DNA (20–30 ng/ml DNA). The volume of the reaction mixture was 20 µl.

To identify the *bar* gene, primers 5'-CATCGAGACAAGCACGGTCA-3' and 5'-GAAACCCACGTCATGCCAGT-3' were used. The expected size of the amplification product for *bar* gene was 405 bp. Amplification conditions: initial denaturation at 94°C for 4 min, denaturation at 94°C for 30 sec, annealing at 65°C for 30 s, extension at 72°C for 1 min for the first cycle followed by 34 cycles each. Final polymerization was at 72°C for 5 min. The size of the expected amplicon was 405 base pairs (b.p.).

RESULTS AND DISCUSSION

Usually achievements of plant genetic engineering connect with the optimization of methods of agrotransformation and factors influencing the transfer of a selective marker and/or reporter gene via *Agrobacterium* to the plant cell. This is followed by the alien gene integration in the recipient plant genome and subsequently its expression and stability in the transgenic plants. Considering the useful properties of *Amaranthus* plants, the present work was conducted with an aim of identifying the functioning of pCBv19 gene vector of *Agrobacterium tumefaciens* in amaranth tissues, detecting the lowest lethal dose of PPT herbicide on amaranth seedlings, and obtaining transgenic amaranth plants after genetic transformation with use of strain GV3101 of *A. tumefaciens* (pCBV19).

The first step of the experiment was detection of the functioning of pCBV19 gene vector of *Agrobacterium tumefaciens* in amaranth tissues. For this purpose, *gus* reaction was conducted. It is known that a correct interpretation of the obtained results of *gus* reaction can face several problems.

For example, residual *Agrobacterium* adhering to non-transformed plants in culture might leads to false *gus* positive results in a standard histochemical assay and, thus, may complicate analysis of results of transformation.

To avoid such a situation, a portable plant intron was introduced into the coding sequence of β-glucuronidase gene (*gus*) to interrupt the open reading frame (Vancanneyt et al., 1990). Gus-intron chimeric gene constructs were safely and successfully used in *Agrobacterium*-mediated genetic transformation of several plants: spinach (Knoll et al., 1997), cabbage (Sparrow et al., 2004), spine gourd (Thiruvengadam, Chung, 2011), and *Spinacia oleracea* L. (Zhang, Zeevart, 1999).

Sometimes the main problem of transformation system lies in the non-predictability of the pattern of integration of transgenes in the host genome and their expression. The causes of variability of transgene expression in plants may be the copy number (Hobbs et al., 1993; Koprek et al., 2001; Kohli et al., 2003), tandem/inverted repeat organization (Muskens et al., 2000; Wang, Waterhouse, 2000), the site of transgene integration into the plant genome (position effect), methylation of transgenes (Matzke et al., 1996; Meyer et al., 1996), and integration of vector backbone sequences (Kononov et al., 1997; De Buck et al., 2000).

One obvious complication arises due to differences in the copy number of transgenes present in independent transgenic plants. A positive correlation between high copy number and expression of transgenes was reported in potato, tobacco, and rice (Gendloff et al., 1990; van der Hoeven et al., 1994). However, a negative correlation was reported for petunia, maize, and tobacco (Hobbs et al., 1990; Matzke et al., 1996; Koprek et al., 2001; Kohli et al., 2003; Tenea, Cucu, 2006; Donnarumma et al., 2011).

Transgene copy numbers were shown to have varied with the method employed for transformation. Transgenic plants obtained by a direct DNA transfer method (biolistics or electroporation) were found to contain a large number of transgene copies (up to 100), whereas *Agrobacterium*-mediated transformation revealed the insertion of fewer transgene copies (<10) with a more frequent occurrence of

single-copy integrations (Koprek et al., 2001; Gelvin, 2003; Reddy et al., 2003).

Our results of transient GUS activity after infiltration were not positive for all plants either.

The GUS activity result was positive for two varieties ("Karmin" and "Helios") (the sites featuring blue colour). Positive sites were in the area of midrib and lateral vein (Figs. 2, 3).

Histochemical reaction with the GUS enzyme was performed with X-gluc. In leaves the *35S* promoter was strong in veins (mainly in midrib). *Gus* expression was prominent in hypocotyl and cotyledon vascular bundles of seedlings. High expression of the *gus* gene was detected in the root tip. Thus it is obvious that expression of the *gus* gene was high in the areas of meristematic tissues. Our results of the localization of *gus* genes in plants with transient expression are similar to those which were obtained by Jan Jasic (Jasic et al., 2011).

Due to a possible escape rate (obtaining of false positive results) of non-transformed plants, it was necessary to make a compromise with the survival percentage of the putative transformed and non-transformed plants.

That is why the second step of the experiment was determination of the lowest lethal dose of PPT herbicide on non-transformed amaranth seedlings. There were no significant differences between plants treated with 10 and 20 mg/l herbicide concentrations. The lowest lethal dose was the 40 mg/l concentration of phosphinothricin (Fig. 4).

This dose of herbicide (40 mg/l) was lethal for 100% of non-transformed *Amaranthus* plants, but a certain quantity of assumedly transformed plants remained alive which may indicate they were resistant and it can be assumed that they had a built-in *bar* gene (Harrison et al., 2006).

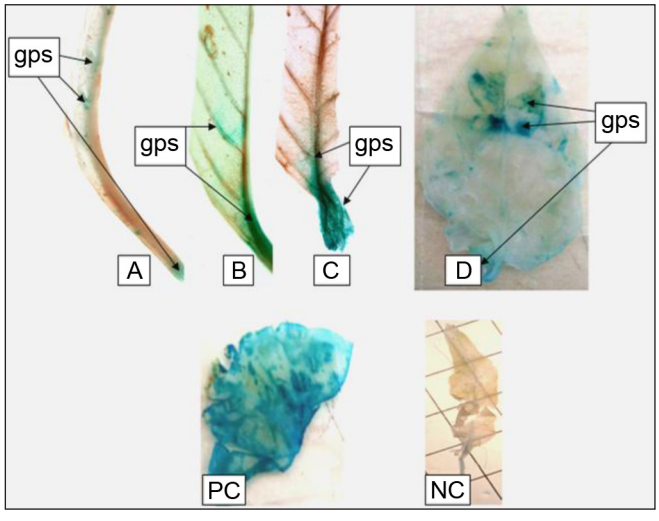

Fig. 2. Leaves of the cultivars "Karmin" (A, B) and "Helios" (C, D) after a histochemical reaction (detection the *gus* gene presence), PC – positive control (*Nicotiana tabaccum* with *gus* gene), NC – negative control (non-transformed cv. "Karmin" *Amaranthus caudatus*), gps – *gus* positive sites

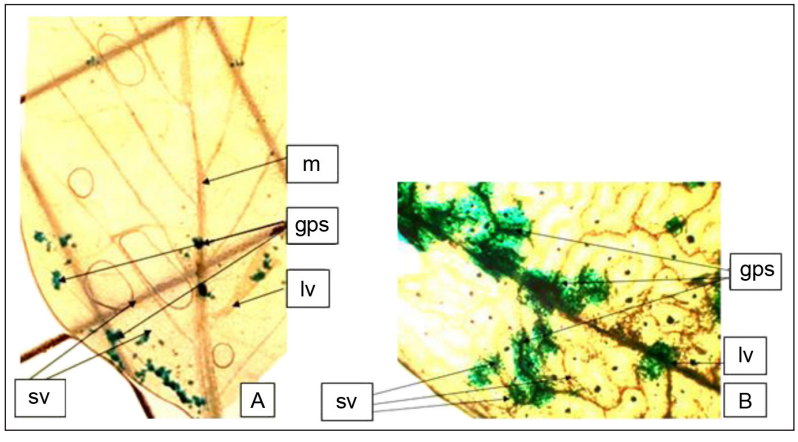

Fig. 3. Leaves of the varieties "Karmin" (A, B). m – midrib, lv – lateral leaf vein, sv – small vein, gps – *gus* positive sites

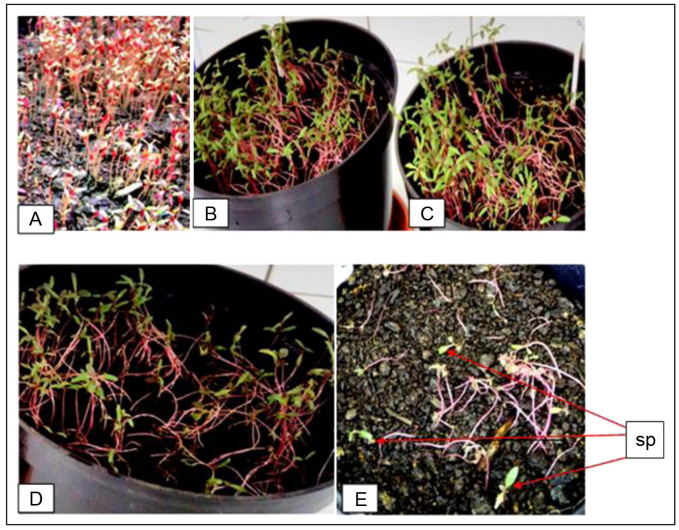

Fig. 4. The effect of different concentrations of herbicide PPT on the seedlings of non-transformed *Amaranthus caudatus* cv. "Kremoviy rannii". A – 0 mg/l (control), B – 10 mg/l, C – 20 mg/l, D – plants before spraying of PPT herbicide, E – plants spraying with PPT herbicide (40 mg/l) after seven, days sp – seedlings which survived

First experiments connected with the transformation of amaranth species with *Agrobacterium* strains were unsuccessful (De Cleene, De Ley, 1976). At the moment, there exists a proof that it is possible to get transgenic amaranth plants with *A. rhizogenes* and *A. tumefaciens*. However, there are only several works dealing with the transformation of amaranth. Positive results were obtained in the transformation of *Amaranth tricolor* L. – Swain with colleges (Swain et al., 2010) and *Amaranth spinosus* L. – Pal with colleges (Pal et al., 2013) with wild strains of *Agrobacterium rhizogenesis* A 4. The authors obtained transgenic roots.

Positive results were obtained in the transformation of amaranth species with strains of *Agrobacterium tumefaciens.* Jofre-Garfias with co-authors received transgenic plants of *Amaranthus hypochondriacus* L., cv. "Azteca". They used *Agrobacterium* construction with marker genes (Jofre-Garfias et al., 1997). Transgenic *Amaranthus tricolor* L. was obtained by two different groups of scientists – Swain with colleges and Pal with co-authors (Swain et al., 2009; Pal et al., 2013). Pal with co-authors used construction with marker genes.

Also there are two studies dedicated to the transformation through amaranth inflorescence. Umaiyal Munusamy with co-authors used the construction with selective genes (Munusamy et al., 2013), and Taipova conducted experiments with *Amaranthus retroflexus*, *A. viridis*, *A. cruentus* (Taipova, Kuluev, 2015).

In both works asserted that positive and promising results were obtained and they obtained transgenic seeds. Their results do not seem convincing, though. Umaiyal Munusamy and his colleagues did not indicate the species of the amaranth they worked with, while Taipova did not indicate the kind of bacteria they worked with. The results of the biochemical and genetic analysis, referring to which it would be possible to state with accuracy that they received transgenic seeds, are not shown.

For the first time, we have the obtained transient expression and transgenic seeds for the cultivars of *Amaranthus caudatus* L.

After spraying with herbicide, resistant plants were obtained for two cultivars: "Kremoviy rannii" and "Karmin". The percentage of *A. caudatus* cv. "Kremoviy rannii" plants resistant to the effect of the herbicide PPT (40 mg/l) was 21% (68 plants out of 320), and of cv. "Karmin" 20% (137 plants out of 688) (Fig. 5).

Positive results for GUS activity after "floral-dip" infiltration with *A. tumefaciens* were obtained for the "Kremoviy rannii" and "Helios" cultivars (Fig. 6).

The frequency of obtaining plants with *gus*-positive sites (sites featuring blue colour) was 1% for cv. "Kremoviy rannii" (1 plant from 68), and 2% for cv. "Karmin" about (3 plants out of 137) (Fig. 6).

It is not yet clear why positive result were obtained for only these varieties. Perhaps they

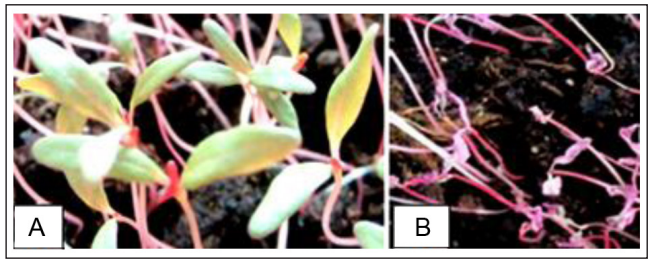

Fig. 5. The effect of herbicide PPT on assumedly transformed seedlings of *Amaranthus caudatus*, cv. "Kremoviy rannii". A – 0 mg/l (control), B – plants after spraying of PPT herbicide (40 mg/l) after 7 days

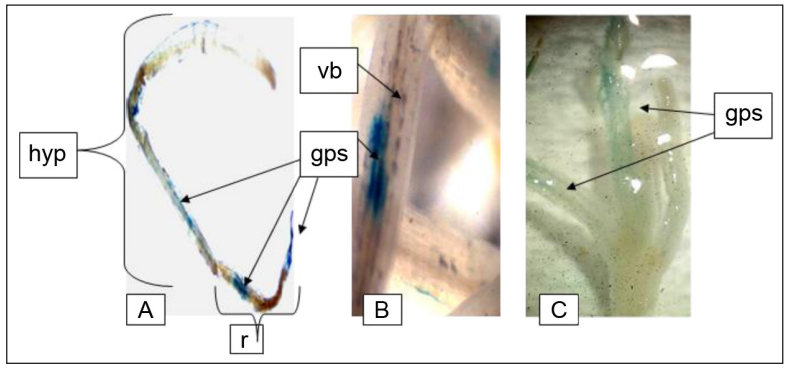

Fig. 6. Variety "Helios" (A, B, C) after a histochemical reaction (detection of the *gus* gene presence). A – part of the seedling with root and hypocotyl, B – part of hypocotyls, C – part of the epicotyl with cotyledons and the pair of first leaves, hyp – hypocotyl, r – root, vb – vascular bundle, gps – *gus* positive sites

are more susceptible to *Agrobacterium tumefaciens* than the other varieties we tested. To clarify these reasons, further researches will be required. Actually, the percentage of obtained *gus*-positive plants corresponds to the results reported by Clough and Bent (Clough, Bent, 1998). The transformation frequency for "floral-dip transformed" *Arabidopsis thaliana* was in the range of 0.5 to 3%.

During the analysis of 39 samples of the tested cultivars of species *A. caudatus* L.: "Helios", "Karmin", "Kremoviy rannii", and "Rushnichok", and hybrids: *A. caudatus* × *A. paniculatus* L. – cv. "Sterkh", *A. caudatus* × "Sterkh" – cv. "Zhaivir", the presence of the DNA fragment with 405 bp size for four cultivars "Sterkch", "Kremoviy rannii", "Rushnichok", and "Helios" was discovered. This confirms the presence of the *bar* gene in the transformed plant (Fig. 7). The percentage of *bar*-positive plants was 0.3% ("Helios"); 0.26% ("Sterkch"); 0.06% ("Kremoviy rannii"); 0.3% ("Rushnichok") from the total initial quantity of plants.

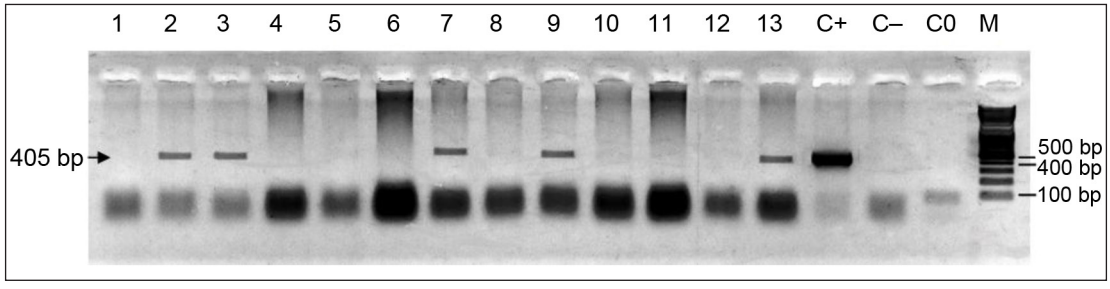

Fig. 7. PCR analysis of amaranth plants using primers for the *bar* gene: lane M – Fisher Thermo Scientific GeneRuler™ DNA Ladder Mix, lanes 1–13 – total DNA of experimental samples (1–2 – "Helios", 3, 11–13 – "Rushnichok"; 4 – "Karmin"; 5–10 – "Sterkch"), C– – negative control, DNA non-transformed plants, C+ – positive control, plant DNA of transgenic *N. tabacum*, C_0 – no-DNA control. 2, 3, 7, 9, 13 – *bar* positive plants; 405 bp – the size of the expected amplicon, specifying *bar* gene in the leaf genomic DNA of putative transgenic lines

CONCLUSIONS

The functioning of pCBV19 gene vector of *Agrobacterium tumefaciens* was evaluated in amaranth tissues. After vacuum infiltration of *Amaranthus* plants, the result of *gus* activity was positive for two cultivars of *A. caudatus*, "Karmin" and "Helios".

The seeds of six varieties of amaranths were obained after treatment with *A. tumefaciens* by the "floral-dip" method. The lowest lethal dose of herbicide PPT was established at 40 mg/l.

After spraying with the herbicide, resistant plants were obtained for two cultivars: "Kremoviy rannii" and "Karmin". Percentage of resistant plants *A. caudatus* cv. "Kremoviy rannii" to the effect of the herbicide PPT (40 mg/l) was 21% (68 plants out of 320). Percentage of resistant plants *A. caudatus* cv. "Karmin" was 20% (137 plants out of 688).

After a histochemical reaction for detection of *gus* genes in amaranth plants, a positive result was obtained for cultivars: "Kremoviy rannii" and "Karmin". The frequency of obtaining plants with *gus* positive sites was for cv. "Kremoviy rannii" 1%, for cv. "Karmin" 2.2%.

After conduction of PCR analysis, were obtained positive results for 4 cultivars. The percentage of *bar* positive plants was 0.3% ("Helios"); 0.26% ("Sterkch"); 0.06% ("Kremoviy rannii"); 0.3% ("Rushnichok") from total initial quantity of plants.

ACKNOWLEDGEMENTS

Part of the work was funded by a grant from the National Scholarship Programme of the Slovak Republic (SAIA) and project VEGA 2/0109/19. The study was supported by a project (State Registration No. 0116U000173) granted by the National Academy of Sciences of Ukraine.

Received 23 September 2018
Accepted 27 November 2018

References

1. Zheleznov AV, Zheleznova NB, Burmakina NV, Yudina RS. Amarant: nauchnyie osnovyi introduktsii. Novosibirsk: Akademicheskoe izdatelstvo "Geo" 2009, 236 p. Russian.
2. Martins PK, Nakayama TJ, Ribeiro AP, da Cunha BADB, Nepomuceno AL, Harmon FG, Kobayashi AK, Molinari HBC. *Setaria viridis* floral-dip: a simple and rapid *Agrobacterium*-mediated transformation method. Biotechnol Rep. 2015; 6: 61–3.
3. Stewart CN, Via LE. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Biotechniques. 1993; 14(5): 748–50.
4. Jefferson RA. Assaying chimeric genes in plants: the *GUS* gene fusion system. Plant Mol Biol Rep. 1987; 5: 387–405.
5. Munusamy U, Abdullah S, Aziz M, Khazaai H. Female reproductive system of *Amaranthus* as the target for *Agrobacterium*-mediated transformation. Adv Biosci Biotechnol. 2013; 4(2): 188–92.
6. Zhang X, Henriques R, Lin SS, Niu Q, Chua NH. *Agrobacterium* mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nat Protoc. 2006; 1(2): 641–6.
7. Curtis IS. Protocols of transgenic crops by floral-dip method. Methods Mol Biol. 2004; 286: 103–9.
8. Bent A. *Arabidopsis thaliana* floral dip transformation method. Methods Mol Biol. 2006; 343: 87–103.
9. Vancanneyt G, Schmidt R, O'Connor-Shanchez A, Willmitzer L, Rocha-Sosa M. Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol Gen Genet. 1990; 220(2): 245–50.
10. Knoll KA, Short KC, Curtis IS, Power JB, Davey MR. Shoot regeneration from cultured root explants of spinach (*Spinacia oleracea* L.):

a system for *Agrobacterium* transformation. Plant Cell Rep. 1997; 17(2): 96–101.

11. Sparrow PAC, Dale PJ, Irwin JA. The use of phenotypic markers to identify *Brassica oleracea* genotypes for routine high throughput *Agrobacterium*-mediated transformation. Plant Cell Rep. 2004; 23(1–2): 64–70.
12. Thiruvengadam M, Chung IM. Establishment of an efficient *Agrobacterium tumefaciens* mediated leaf disc transformation of spine gourd (*Momordica dioica* Roxb. exWilld). Afr J Biotechnol. 2011; 10(83): 19337–45.
13. Zhang HX, Zeevart JAD. An efficient *Agrobacterium tumefaciens*- mediated transformation and regeneration system for cotyledons of spinach (*Spinacia oleracea* L.). Plant Cell Rep. 1999; 18(7–8): 640–5.
14. Hobbs SLA, Kpodar P, DeLong CMO. The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformation. Plant Mol Biol. 1990; 15(6): 851–64.
15. Koprek T, Rangel S, McElroy D, Louwerse JD, Williams-Carrier RE, Lemaux PG. Transposon-mediated single-copy gene delivery leads to increased transgene expression in barley. Plant Physiol. 2001; 125: 1354–62.
16. Kohli A, Twyman RM, Abranches R, Wegel E, Stoge E, Christou P. Transgene integration, organization and interaction in plants. Plant Mol Biol. 2003; 52(2): 247–58.
17. Muskens MW, Vissers AP, Mol JN, Kooter JM. Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. Plant Mol Biol. 2000; 43(2–3): 243–60.
18. Wang MB., Waterhouse PM. High-efficiency silencing of a betaglucuronidase gene in rice is correlated with repetitive transgene structure but is independent of DNA methylation. Plant Mol Biol. 2000; 43(1): 67–82.
19. Matzke MA, Matzke AJM, Eggleston WB. Paramutation and transgene silencing: a common response to invasive DNA? Trends Plant Sci. 1996; 1(11): 382–8.
20. Meyer P, Lohuis MT, van Blockland R, Heidmann I, Niedenhof I. The role of DNA methylation in transgene silencing in plants. Mechanisms and applications of gene silencing. Nottingham University Press, Sheffield; 1996: 43–8.
21. Kononov ME, Bassuner B, Gelvin SB. Integration of T-DNA binary vector 'backbone' sequences into the tobacco genome: evidence for multiple complex patterns of integration. Plant J. 1997; 11: 945–57.
22. De Buck S, De Wilde C, Van Montagu M, Depicker A. T-DNA vector backbone sequences are frequently integrated into the genome of transgenic plants obtained by *Agrobacterium*-mediated transformation. Mol Breed. 2000; 6: 459–68.
23. Gendloff EH, Bowen B, Buchholz WG. Quantitation of chloramphenicol acetyl transferase in transgenic tobacco plants by ELISA and correlation with gene copy number. Plant Mol Biol. 1990; 14: 575–83.
24. van der Hoeven C, Dietz A, Landsmann J. Variability in organspecific gene expression in transgenic tobacco plants. Transgenic Res. 1994; 3: 159–65.
25. Tenea G, Cucu N. The influence of T-DNA copy numbers on gene expression in primary tranformants *Atropa belladonna* plants. Roum Biotechnol Lett. 11(2): 2661–7.
26. Donnarumma F, Paffetti D, Fladung M, Biricolti S, Dieter E, Altosaar I, Vettori C. Transgene copy number estimation and analysis of gene expression levels in *Populus* spp. transgenic lines. BMC Proc. 2011; 5(7): 152.
27. Gelvin SB. *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol Mol Biol Rev. 2003; 67(1): 16–37.
28. Reddy MS, Dinkins RD, Collins GB. Gene silencing in transgenic soybean plants transformed via particle bombardment. Plant Cell Rep. 2003; 21(7): 676–83.
29. Jasik J, Schiebold S, Rolletschek H, Denolf P, Van Adenhove K, Altmann T. Subtissue-specific evaluation of promoter efficiency by quantitative fluorometric assay in laser

microdissected tissues of rapeseed. Plant Physiology. 2011; 157: 563–73.

30. Harrison SJ, Mott EK, Parsley K, Aspinall S, Gray JC Cottage. A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. Plant Methods. 2006; 2: 19.
31. De Cleene M, De Ley J. The host range of Crown Gall. Bot Rev. 1976; 42: 389–466.
32. Swain SS, Sahu L, Barik DP, Chand PK. *Agrobacterium* × plant factors influencing transformation of "Joseph's coat" (*Amaranthus tricolor* L.). Scientia Horticulturae. 2010; 125(3): 461–8.
33. Pal A, Swain SS, Mukherjee AK, Chand PK. *Agrobacterium* pRi TL-DNA *rol*B and TR-DNA Opine Genes Transferred to the Spiny Amaranth (*Amaranthus spinosus* L.) – A Nutraceutical Crop. Food Technol Biotechnol. 2013; 51(1): 26–35.
34. Jofre-Garfias AE, Villegas-Sepúlveda, Cabrera-Ponce JL, Adam e-Alvarez RM, Herrera-Estrella L, Simpson J. *Agrobacterium* mediated transformation of *Amaranthus hypochondriacus*: light- and tissue-specific expression of a pea chlorophyll a/b-binding protein promoter. Plant Cell Reports. 1997; 16: 847–52.
35. Swain SS, Sahu L, Barik DP, Chand PK. Genetic transformation of *Amaranthus tricolor* L. using Ri plasmid vectors. In: Bastia AK and Mohapatra UB (eds.) Recent trends in monitoring and bioremediation of mine and industrial environment. Proc Natl Sem, North Orissa University, Orissa; 2009: 109–16.
36. Pal A, Swain S, Das AB, Mukherjee AK, Chand PK. Stable germ line transformation a leafy vegetable crop amaranth (*Amaranthus tricolor* L.) mediated by *Agrobacterium tumefaciens*, *In Vitro* Cell Dev Biol–Plant. 2013; 49(2): 114–28.
37. Taipova RM, Kuluev BR. Amaranth features of culture, prospects of cultivation in Russia and generation of transgenic Russian varieties, Biomica. 2015; 7(4): 284–99.
38. Clough SJ, Bent AF. Floral dip: A simplified method for *Agrobacterium* mediated transformation of *Arabidopsis thaliana*. Plant Journal. 1998; 16: 735–43.

Yaroshko Olha, Vasylenko Maksym, Gajdošová Alena, Morgun Bogdan, Khrystan Olesia, Velykozhon Liudmyla, Kuchuk Mykola

AMARANTHUS CAUDATUS L. IR HIBRIDŲ *A. CAUDATUS* × *A. PANICULATUS* L. TRANSFORMACIJA „FLORAL-DIP" METODU

Santrauka

„Floral-dip" metodu transformavus *Amaranthus* augalus su *Agrobacterium tumefaciens* potipiu GV3101, pernešančiu pCBV19 geno vektorių, turintį *bar* ir *gus* genus, buvo gautos transgeninės sėklos. Perkeltų genų funkcionavimą *Amaranthus* audiniuose patvirtino herbicidų selekcija (herbicidai PPT – *phospinotricin*) ir *gus* geno aktyvumas. Gauti teigiami „Karmin" ir „Kremoviy rannii" kultivarų rezultatai. Iš bendro pradinio augalų, pasirinktų herbicidų selekcijai, kiekio *gus* teigiamų mėginių procentinę dalį sudarė 1 % („Karmin" kultivaras), 2,2 % („Kremoviy rannii" kultivaras). Šešių amarantų kultivarų sėklos buvo gautos apdorojus *A. tumefaciens* „floral-dip" metodu. Nustatyta mažiausia mirtina PPT herbicido dozė – 40 mg/l. Herbicidams atsparūs buvo šių veislių augalai – „Kremoviy rannii" (21 %) ir „Karmin" (20 %). Teigiami PGR analizės rezultatai gauti 4 kultivaruose. Nustatyta *bar* geną turinčių augalų procentinė dalis: 0,3 % („Helios"); 0,26 % („Sterkch"); 0,06 % („Kremoviy rannii"); 0,3 % („Rushnichok").

Raktažodžiai: *Agrobacterium*, transformacija, „floral-dip", transgeninis *Amaranthus*