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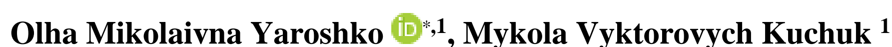
Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

Agrobacterium – caused transformation of cultivars *Amaranthus caudatus* L. and hybrids of *A. caudatus* L. x *A. paniculatus* L.



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Abstract: The procedure for vacuum infiltration of cultivars *A. caudatus* L. and hybrids of *A. caudatus* L. x *A. paniculatus* L. was optimized**.** The functioning of gene construction pCBv19 in the *Amaranthus* leaves was evaluated by the transient expression after vacuum infiltration with *Agrobacterium rhizogenes* A4. After hypocotyl transformation of the varieties of amaranth species *A. caudatus* L.: *Helios*, *Karmin*, *Kremovyi rannii*, and hybrids *A. caudatus* x *A. paniculatus* L. – cv. *Sterkh*, *A. caudatus* x *Sterkh*- cv. *Zhaivir* with the wild strain *A. rhizogenes* A4, the culture of "hairy roots" was obtained. Embedding and transcription of genes in the roots are confirmed by the results of the PCR analysis.

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Amaranthus, *Agrobacterium*, transformation, transgenic roots.

1. INTRODUCTION

Amaranth plants are used in the food industry, medicine, cosmetics and agriculture, they are also a source of biologically active compounds, the most valuable of them are squalene and amarantin. Squalene has anticancer and wound healing properties while amarantin – has an antioxidant activity [1, 2]. The seeds of *Amaranthus* species are rich in methionin and lysine amino acids. Biologically valuable substances can be obtained by using biotechnological methods.

Plants that synthesize alien substances may well be received through genetic transformation by using *Agrobacterium* bacteria. Members of this genus are pathogens. Due to Ti- and Ri - plasmids these bacteria can cause the formation of plants «hairy roots» (*A. rhizogenes*) or tumors (*A. tumefaciens*). The hormonal balance of plants changes (after insertion and expression of bacterial gene), which results in specific phenotype in infected plants [3].

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Initial experiments connected with the transformation of amaranths species with *Agrobacterium* strains were unsuccessful [4]. By then, it was proved that transgenic amaranths plants with *A. rhizogenes* and *A. tumefaciens* could be obtained. But still there are only several works devoted to the transformation of amaranth.

The transgenic roots were obtained for *Amaranthus tricolor* [5] and *A. spinosus* L. [6]. The transgenic plants were obtained for *A. hypochondriacus* L. and *A. tricolor* L. [7, 8], *A. retroflexus*, *A. viridis, A. cruentus* [9]. There is no information about transformation of *A. caudatus*, the varieties of which is also used in agriculture.

The aim of this work was to evaluate the functioning of gene construction pCBv19 of *Agrobacterium rhizogenes* A4 in amaranth tissues and obtain transgenic roots of amaranth after genetic transformation with using the wild strain of *A. rhizogenes* A4.

2. MATERIALS and METHODS

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

The objects of the research were cultivars of species of *Amaranthus caudatus* L.: *Helios*, *Karmin*, *Kremovyi rannii*, hybrids: *A. caudatus* x *A. paniculatus* L. – cv. *Sterkh*, *A. caudatus* x *Sterkh* - cv. *Zhaivir*.

Our objective was to check and evaluate the functioning of gene construction pCBv19 of *A. rhizogenes* A4 (Fig. 1) in transiently transformed amaranth leaves.

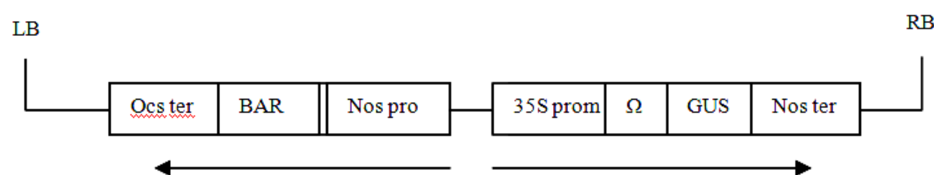

Fig. 1. Schematic representation of the T-DNA site of the pCBv19 construction LB – left border sequence, RB – right border sequence; Nos pro – nopaline synthase promoter, Nos ter - nopaline synthase terminator; 35S prom – 35S promoter; BAR – *bar* gene, GUS – *gus* gene; Ocs - octopine synthase; Ω - regulatory sequence enhancer

For this purpose we used the method of vacuum infiltration [10] and detection of GUS activity [11]. For infiltration of the 2-month-old plant leaves, the varieties and hybrids mentioned above, were used. As an infection agent we used *A. rhizogenes* A4 gene construction pCBv19, which contained *bar* and *gus* genes.

First, *A. rhizogenes* A4 was sown in the liquid LB medium (for 24 h. mixing on shaker). We added 1 ml of *A. rhizogenes* into 50 ml of the medium with 0,2 mM of acetosiringone. Next, *Agrobacterium* was centrifuged during 12 min, 5000 rpm. Then, *Agrobacterium* was resuspended into the medium with sucrose (50 g/l + 0,2 % super wetting agent Silwet).

The next stage was dipping the leaves of amaranth into the flask with the medium containing *A. rhizogenes* (for 5-10 min, 22 - 24C^{0}) in the vacuum chamber. After this operation, leaves were put on the wet filter paper in Petri plates (Fig. 4).

After the leaves had been lying on the wet filter paper for 4 days, β – glucuronidase fluorometric assay (GUS activity) was conducted at 37 ^{0}C according to Jefferson [11]. GUS reactions were stopped in 24 h. of incubation at 37^{0}C. Specific activities were detected visually by the appearance of staining the plant tissues in blue color.

2.2. Transgenic roots obtaining

Seeds of amaranth germinated on the sterile nutrient agar medium Murasige and Skoog (MS_{30}) [12] with 30 g/l sucrose. For transformation we used the hypokotyl segments of 14-day-old seedlings of cultivars of the following species *A. caudatus* L.: *Helios*, *Karmin*, *Kremovyi rannii*, and hybrids: *A. caudatus* x *A. paniculatus* L. - cultivar *Sterkh*, *A. caudatus* x *Sterkh* - cultivar *Zhaivir*, the seeds were obtained from the Botanical Garden of M.M. Grishko NAS of Ukraine. Transformation was carried out by co-cultivating the hypokotyls with the agropine strain of *A. rhizogenes* A4. Transformation of amaranths was carried out according to the modification of techniques proposed by Jofre-Garfias and colleagues [13].

First, *A. rhizogenes* A4 was sown in the liquid LB medium (for 24 h. mixing on shaker). We used 1 ml of *A. rhizogenes* into 50 ml of the medium with 0,2 mM of acetosiringone. Next, *Agrobacterium* was centrifuged during 12 min, 5000 rpm. Then, *Agrobacterium* was resuspended into the medium liquid ½MS_{15}. In this medium explants were soaked for 2 hours.

After 2 hours of incubation, explants were transferred on the solid growth medium ½MS_{15} without antibiotics. Co-cultivation on this medium lasted 1 day and then the hypocotyls transferred on the ½MS_{15} medium with the addition of 500 mg/l of cefotaxime ("Darnitsa", Ukraine).

Every 2 weeks hypocotyls were transferred to the ½MS_{15} medium with a reduced content of cefotaxime (400 mg/l, 300 mg/l, 200 mg/l). In the last transfer we used ½MS_{15} medium without adding of cefotaxime.

Hypocotyls of 14-day-old seedlings of the same varieties were used as a control, which weren't cocultured with *A. rhizogenes* A4, first, they were laid out on ½MS_{15} medium, then on ½MS_{15} with 500 mg/l of cefotaxime. The following sub-cultivation for control samples were not carried out, because after 15 days the hypocotyls died.

2.3. Polymerase chain reaction

Genomic DNA isolated by CTAB method [14]. 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.), 2µl primers, 2µl deoxyribonucleotide triphosphate (dNTP), 0.15µl Dream Taq-polymerase, 2µl DNA (20-30 ng/ml DNA). The volume of the reaction mixture is 20µl).

To identify the gene *rolB*, the primers were used: *5'*-CTCACTCCAGCATGGAGCCA-*3'* as well as *5'*-ATTGTGTGGTGCCGCAAGCTA-*3'*. The expected size of the amplification product for *rolB* gene was 592 bp. Amplification conditions: initial denaturation at 94 ^{0}C for 3 min, annealing at 60 ^{0}C for 30 s, extension at 72 ^{0}C for 30 s for the first cycle followed by 32 cycles each. The duration of the synthesis of the *rolB* was 40 s, at 72 ^{0}C, final polymerization was at 72 ^{0}C for 5 min.

3. RESULTS

For evaluation of functioning of *Agrobacterium rhizogenes* A4 gene construction pCBv19 in *Amaranthus* tissues, we carried out the histochemical reaction [11] (Fig.2, Fig. 3).

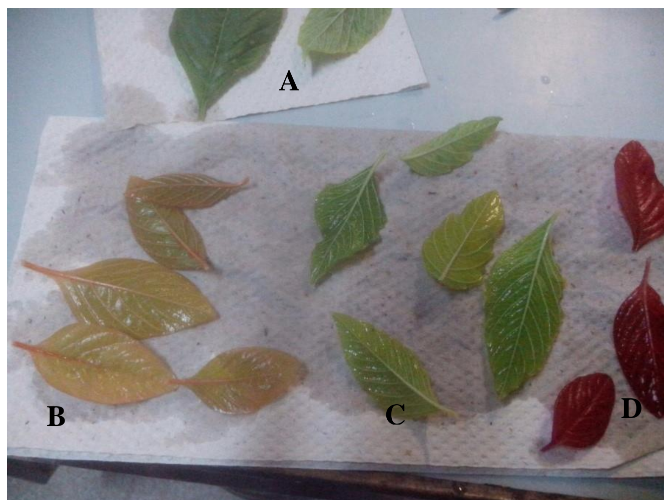

Fig. 2. Leaves of varieties A - *Kremovii ranii*, B – *Helios*, C – *Rushnichok*, D - *Karmin* after infiltration under vacuum.

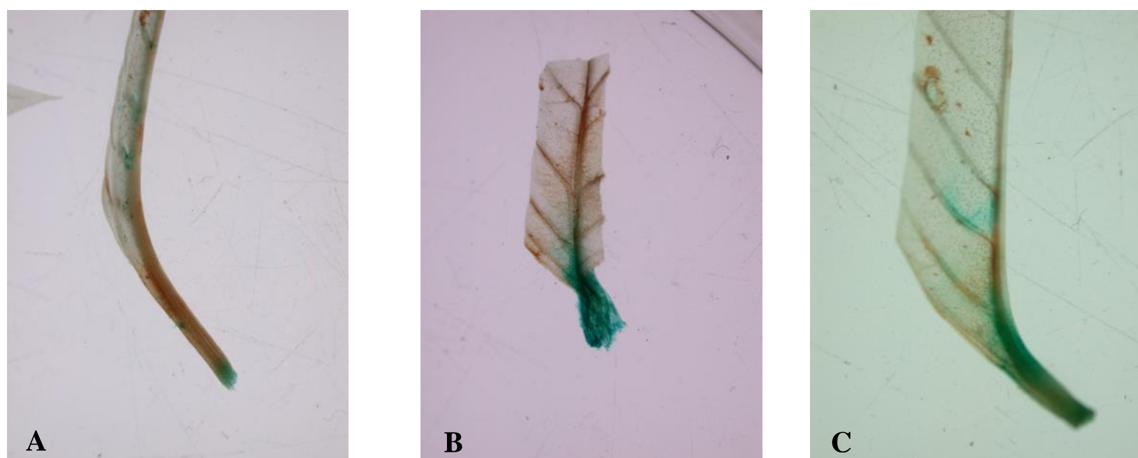

Fig. 3. Leaves of varieties A - *Kremovii ranii*, B – *Helios*, C – *Rushnichok*, D - *Karmin* after a histochemical reaction (detection the activity of GUS).

The GUS activity result was positive for 2 varieties (*Karmin* and *Helios*) (sites that are of blue color). Positive sites were in the area of the midrib (Fig.3). At the next stage transgenic roots were obtained. The growth of "hairy roots" of *Amaranthus* on the hypocotyl explants were observed in 20-25 days after *A. rhizogenes* transformation on the ½MS_{15} medium with cefotaxime. Eight lines of roots were received. When transferred the parts of the roots (~10 mm) on the hormone free medium without growth regulators, their intensive growth was observed. The roots outwardly resembled "hairy roots" (Ri- roots) form (Fig. 4), due to transfer of TL- fragment of the T-DNA of pRi plasmid of the agropine type with gene *rolB*.

Fig. 4. Formation of "hairy roots", after transformating the hypocotyls of *A. caudatus Helios* with the strain *A. rhizogenes* A4

To confirm the presence in the transformed roots TL-fragment of the T-DNA pRi plasmid, the amplification of total DNA with primers, specific to *rolB* gene, was carried out. During the analysis of 8 samples of tested cultivars of species *A. caudatus* L.: *Helios*, *Karmin*, *Kremovyi rannii*, and hybrids: *A. caudatus* x *A. paniculatus* L.- cv. *Sterkh*, *A. caudatus* x *Sterkh* – cv. *Zhaivir*, the presence of the DNA fragment with 592 bp size for 3 samples (№3 – *Helios*; №6, №8 - *Karmin*), was discovered, this confirms the presence of the *rolB* gene in the transformed roots (Fig. 5).

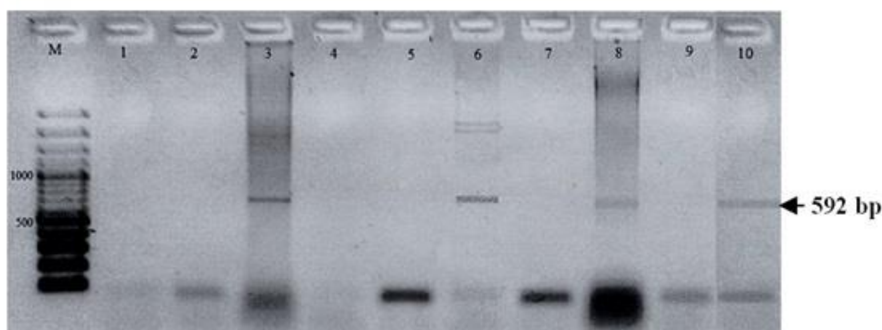

Fig. 5. PCR analysis of amaranth plants using primers for *rolB* gene: M - DNA marker (O'GeneRuler^{TM}1kb DNA Ladder, "Fermentas"), 1-8 total DNA of plants transformed with *A. rhizogenes* A4 (1 - *Sterkh*; 2, 3, 4 - *Helios*; 5- *Kremovyi rannii*; 6, 8 - *Karmin*; 7 - *Zhaivir*), 9 - negative control, DNA non-transformed plants, 10 - positive control, the plasmid DNA of *A. rhizogenes* A4 (592 bp).

4. DISCUSSION

Positive results were obtained in the transformation of *Amaranth tricolor* L. - Swain with colleges [5] and *Amaranth spinosus* L. - Pal and colleges [6] with the wild strains of *Agrobacterium rhizogenesis* A 4. The authors get transgenic roots. Positive results were obtained in the transformation of amaranth species with strains of *Agrobacterium tumefaciens.* Jofre-Garfias with co-authors – *Amaranthus hypochondriacus* L., cv. "Azteca". They used *Agrobacterium* construction with marker genes [13]. Transgenic *Amaranthus tricolor* L. was obtained by two different groups of scientists - Swain with colleges and Pal with co-authors [7, 8]. Pal with co-authors used the construction with marker genes. Also there are 2 works dedicated to the transformation through amaranth inflorescence **-** Umaiyal Munusamy with co-authors. They used the construction with selective genes [15].

Prof. Taipova conducted experiments with *Amaranthus retroflexus*, *A. viridis, A. cruentus* [9]. In both works it is said that positive and promising results were obtained and they get transgenic seeds. The results obtained don't seem convincing. Since in the work by Umaiyal Munusamy it isn't indicated with what kind of amaranth they worked. Prof. Taipova didn't indicate with what kind of bacteria they worked. The results of the biochemical and genetic analysis aren't shown, referring to which it would be possible to state with accuracy that they received transgenic seeds.

We have for the first time obtained transgenic plant parts for the varieties of *Amaranthus caudatus* L. Since, there are still no reports on the transformation of *A. caudatus*. The PCR analysis shows that not all the "hairy root" samples carried the genes of the *Agrobacterium*, although all the variants were phenotypically identical and were similar to typical "hairy roots", grew on the hormone-free MS_{30} medium. The results of the GUS activity were also not positive for all explants. Only 25% of the leaf samples showed a positive result. Positive results for the GUS activity were obtained for the *Karmin* and *Helios* varieties. Transgenic roots were obtained for the same varieties. The reasons for that are still not clear. Perhaps these varieties are more susceptible to *Agrobacterium rhizogenesis* than the other varieties which we tested. To clarify these reasons, further researches will be required.

5. CONCLUSION

So, after the vacuum infiltration of leaves, the GUS activity result was positive for 2 varieties of *A. caudatus*: *Karmin* and *Helios*. After the transformation of hypocotyls of cultivars of amaranth species of *Amaranthus caudatus* L.: *Helios*, *Karmin*, *Kremovyi rannii*, and hybrids: *A. caudatus* x *A. paniculatus* L.- cultivar *Sterkh*, *A. caudatus* x *Sterkh* – cultivar *Zhaivir*, using *A. rhizogenes* A4, were obtained transgenic roots of cultivars *Helios* and *Karmin*. The analyzed samples had gene *rolB* of *A. rhizogenes*.

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