

TRANSIENT EXPRESSION OF REPORTER GENES IN CULTIVARS OF *Amaranthus caudatus* L.

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Local cultivars of *A. caudatus*: Helios and Karmin were used as plant material. Amaranth is a new pseudocereal introduced in Ukraine. The plant biomass of amaranth is used in medicine, food industry and cosmetology industry.

Aim. The purpose of the work was to identify the optimal conditions for the transient expression of reporter genes in *Amaranthus caudatus* cultivars.

Methods. Biochemical and microscopy methods were used in the following work. Seedlings and adult plants of different age were infiltrated with agrobacterial suspensions separately (genetic vector pCBV19 with a *uidA* gene and genetic vector pNMD2501 with a *gfp* gene in *Agrobacterium tumefaciens* GV3101 strain).

Results. Transient expression of the *uidA* and *gfp* genes was obtained in amaranth plants after conduction series of experiments. The most intensive transient expression of *gfp* and *uidA* genes was observed in seedlings infiltrated at the age of 1 day. The maximum fluorescence of the GFP protein was observed on 5th–6th days.

Conclusions. It was shown that the cultivar Helios was more susceptible to agrobacterial infection than the cultivar Karmin. The effectiveness of *Agrobacterium* mediated transformation was from 16% to 95% for the Helios cultivar and from 12% to 93% for the Karmin cultivar. The obtained results indicate that the studied amaranth cultivars can potentially be used for obtaining transient expression of target genes and synthesizing target proteins in their tissues in the future.

Key words: *Amaranthus*, *uidA*; *gfp*; *Agrobacterium*; transient expression.

The term “transient gene expression” refers to the expression of genes that are expressed shortly after the nucleic acid of bacteria has been introduced into eukaryotic cells. During transient expression, there is no integration of foreign genes into the nuclear genome of plants. In this way the genetic material that has been integrated into plant cells is not inherited by offsprings during the sexual reproduction of plants [2].

Transient gene expression in plant systems has several advantages over stable expression. Transient expression technology does not need the regeneration of transformed tissues or organs, nor does it influence the plant genome stability. This technology allows accelerating the experiments, so the functions of the target genes can be studied

4–10 days after the incorporation of foreign genes in the plant cells. Transient expression allows studying the gene functioning in non-sterile conditions [1, 2]. Transient expression also permits protein interactions to be studied [3, 4].

Transient gene expression can be achieved via several methods of delivering of genetic information. One of which these is agroinfiltration which allows infiltrating many plants at the short time period. Moreover, several genetic vectors (with different genes) can be used for the infiltration of a single plant [5, 6].

Genetic constructs used to obtain transient expression often carry a gene where the target gene is transcriptionally fused to a reporter gene (for example, the *green fluorescent gene (gfp)*).

Reporter genes are those genes that encode proteins, the presence of which can be quickly detected by the appearance of fluorescence or specific staining of transformed tissues when stained with a dye. In turn, reporter proteins encoded by reporter genes can help to detect the localization of target proteins in certain organs, tissues, or organelles of plant cells [2].

Mainly, *gfp* and *uidA* are used as reporter genes. The presence of the *gfp* gene is detected by the appearance of green fluorescence of transformed plant tissues under blue rays. The presence of the *uidA* gene is detected by staining plant tissues in blue color when they come into contact with a specific dye. Genetic vectors with these genes are often used in *Agrobacterium*-mediated transformation, when it is necessary to obtain a transient or stable gene expression [2].

The choice of a particular reporter gene for use in experiments should be based on data from the localization in the plant cell of the product encoded by the reporter gene. Thus, the GFP protein encoded by the *gfp* gene is an effective reporter protein in experiments where the localization of the target protein is in the nucleus [7, 8], cytoplasm [9, 10], plasma membrane [10], Golgi apparatus [11], endoplasmic reticulum [9, 11], tonoplasts [12], mitochondria [13] and chloroplasts [11], while reporter yellow fluorescent protein (YFP) and mCherry are used to assess the localization of target protein in peroxisomes [6, 14].

Representatives of the *Amaranthus* genus were the objects of our investigation. The choice is due to the wide use of amaranth plant raw materials in various industries: food industry; pharmaceuticals, agriculture. Improving the quality of amaranth using genetic engineering methods offers considerable potential.

Representatives of *Amaranthus* genus have unique amino acid composition and are rich in biologically active compounds (squalene and amarantin). Squalene has anticancer and wound healing properties. Amarantin has an antioxidant effect [16]. The properties of *Amaranthus* can be improved using biotechnological methods to produce biologically valuable substances (for example, squalene and amarantin).

The possibility of transient expression of the *gus* gene was shown in our previous work for adult *A. caudatus* plants [16, 48]. Yet, there has been no information about obtaining the transient expression of the *gfp* gene in representatives of the *Amaranthus* genus. We show here for the first time the results of the

transient expression of the *gfp* gene for the *Amaranthus* genus.

Materials and Methods

The objects of the research were cultivars of *Amaranthus caudatus*: Helios and Karmin. The seeds were obtained from the M. M. Grishko Botanical Garden of the National Academy of Sciences of Ukraine.

Plants of different age: 1 day-old seedlings, 10 day-old seedlings, 2 month-old adult plants were used in the experiments. To obtain 1-day-old seedlings, seeds were soaked for one day in water under non-sterile conditions (22–26 °C, 14-hour light period, illumination — 3 000–4 500 lx). To obtain 10-day-old seedlings and 2-month-old plants, seeds were sown in the pots with soil and grown in a greenhouse under the conditions of 22–26 °C, 14-hour light period and illumination — 3 000–4 500 lx.

The aim of the experiments was as follows: to check and evaluate the functioning of the pCBV19 and pNMD2501 genetic vectors of *A. tumefaciens* in *A. caudatus* plant tissues after *Agrobacterium*-mediated transformation; to determine the optimal age of plants for infiltration and to identify the plant's organs and tissues in which the transient gene expression occurs the most intensively.

The vacuum infiltration method [15] and methods for detection of *uidA* [17] and *gfp* genes presence were used to obtain transient gene expression.

Plants of different ages (previously mentioned) were infiltrated with agrobacterial suspensions. The strains GV3101 of *A. tumefaciens* harboring pCBV19 [16] and pNMD2501 genetic vectors separately were used in the work (supplementary material Fig. 1). The genetic vector pNMD2501 was kindly donated by NOMAD Bioscience GmbH (Germany). Genetic vector pCBV19 carried *uidA* gene, genetic vector pNMD2501 carried *gfp* gene.

The steps of preparation of agrobacterial suspension were described in the author's previous article [16].

Plants were infiltrated in a flask with a medium containing the agrobacterial suspension for 5 min, at 22–24 °C in a vacuum chamber under pressure of 0.1 mPa.

Detection of the *uidA* gene (β -glucuronidase activity) was carried out by histochemical assay on the 5th day after infiltration in the presence of substrate, X-gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) [17].

The leaves of the infiltrated plants and control plants (negative control) which were

not infiltrated, were taken and incubated in a 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). The histochemical reaction was stopped after 24h of incubation at 37 °C in the dark, followed by five rinses in 70% ethanol. Leaves of stably transformed *Nicotiana tabacum* plants were used as positive control.

Next, the leaves of adult plants and whole seedlings were placed on microscope slides for observation (Zeiss axiophot fluorescent microscope®, Germany; microscope magnification $\times 100$ and $\times 200$). Beta-glucuronidase protein (GUS) activity was detected visually by the appearance of blue staining of plant tissues. Leaves of stably transformed *Nicotiana tabacum* were used as positive control.

The presence of the GFP protein was detected after 4 days in the seedlings (that were immersed in a suspension of *A. tumefaciens* with genetic vector pNMD2501) and was evaluated visually under light with a wavelength in the range of 365–400 nm (Black ray®, model B 100 AP the ultraviolet lamp.) and a microscope with an attachment with a special filter (Plan-Neofluar). The result was considered as positive by the appearance of green tissue fluorescence. The results were documented by photographing on digital media.

Data collection and statistical analysis

One hundred plants (young seedlings) and 30 plants (2-month-old adult) of each variety were used for each part of the experiment. Namely 100 seedlings of cv. Helios and 100 seedlings of cv. Karmin (1-day-old); 100 seedlings 10-day-old of each cultivar and 30 plants of each cultivar (2-month-old) were infiltrated with suspension of *A. tumefaciens* (harboring pCBV19 genetic vector).

For the experiment of *gfp* expression were used 100 seedlings of cv. Helios and 100 seedlings of cv. Karmin (1-day-old); 100 seedlings 10-day-old of each cultivar; 30 plants of each cultivar (2-month-old) which were infiltrated with suspension of *A. tumefaciens* (harboring pNMD2501 genetic vector). The same quantity of seedlings and adult plants of each variety as mentioned above (for the experiment of transient expression of *uidA* and *gfp* gene) were used as negative control (non-infiltrated with agrobacterial suspension).

The percentage of *uidA*-positive plants for each age group (as a percentage expressed

the number of plants in which were detected the presence of *uidA/gfp* genes from the total quantity of plants, which were infiltrated) was calculated after obtaining the results. The standard error (SE) and the arithmetical mean (M) were calculated using the Excel program 2007 and the *t*-Student criterion was calculated in the program Statistica in order to determine the accuracy of the obtained results.

Results and Discussion

Transient expression of *uidA* gene

The histochemical reaction was performed after conducting a series of experiments with infiltration [17]. Large areas of plant tissues stained in blue color were identified. Such staining occurred in plant tissues where the GUS protein was bound with the specific X-gluc substrate. This may indicate that after infiltration, bacterial genes were incorporated into plant cells, DNA was correctly transcribed and a functional GUS reporter protein was synthesized in plant tissues.

The intensity of blue staining varied among the plant groups of different ages, as well as varied the surface areas that were colored in the plants of different ages. In young seedlings (in most of the seedlings which were infiltrated at the age of one day) all parts of the plant (root, hypocotyl and cotyledons) were stained (supplementary material Fig. 2). The percentage of positive gus-stained plants for the cultivar Helios was 95%, for the cultivar Karmin — 93%. The areas in which the reporter protein GUS was synthesized (in 10-day-old seedlings) were mainly along the midrib and occupied most of the surface area of the leaf blade (more than 80%) (Fig. 1, supplementary material Fig. 3). The percentage of gus-positive plants (which were infiltrated at the age 10 days) for the cultivar Helios was 61.26%, for the cultivar Karmin — 41.55%.

In plants that were infiltrated at the age of 2 months, small areas stained in blue color were revealed only in the region of the midrib. The percentage of gus-positive plants was for the cultivar Helios — 16% and for the cv. Karmin 12% (supplementary material Fig. 4). These results indicate that very young seedlings 1-day-old of both cultivars (Helios and Karmin) were the most susceptible to agrobacterial infection. In seedlings that were infiltrated at the age of 10 days and 2 months, the cv. Helios displayed a higher susceptibility to agrobacterial infection. Perhaps this is due to the peculiarities of the biochemical composition

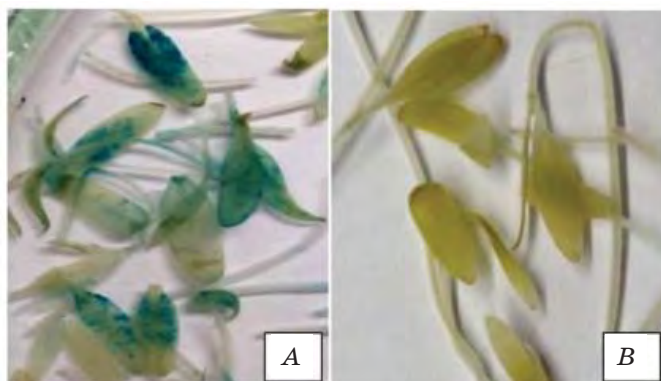


Fig. 1. 15-day-old seedlings of *A. caudatus* cv. Helios after the histochemical reaction:
A — seedlings infiltrated with *A. tumefaciens* harboring genetic vector pCBV19;
B — non-infiltrated control seedlings



Supplementary material Fig. 1. Schematic representation of the T-DNA site of the pNMD2501 genetic vector:

LB — left border sequence; RB — right border sequence; Nos pro — nopaline synthase promoter; Nos ter — nopaline synthase terminator; 35S prom — promoter of cauliflower mosaic virus gene (CaMV); Ocs — octopine synthase terminator; Ω — regulatory sequence enhancer; gfp — green fluorescent protein gene; P19 — gene of protein P19 (suppressor of gene silencing)

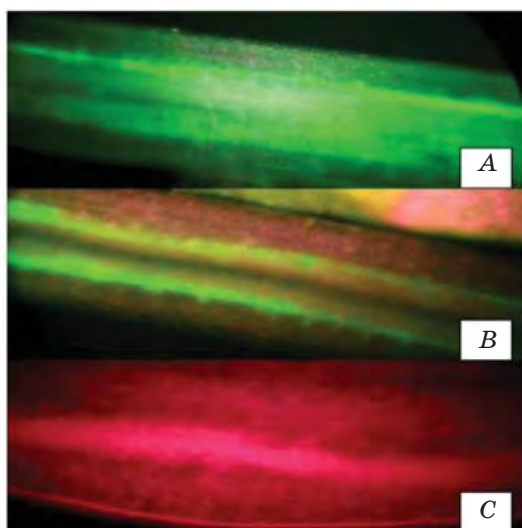
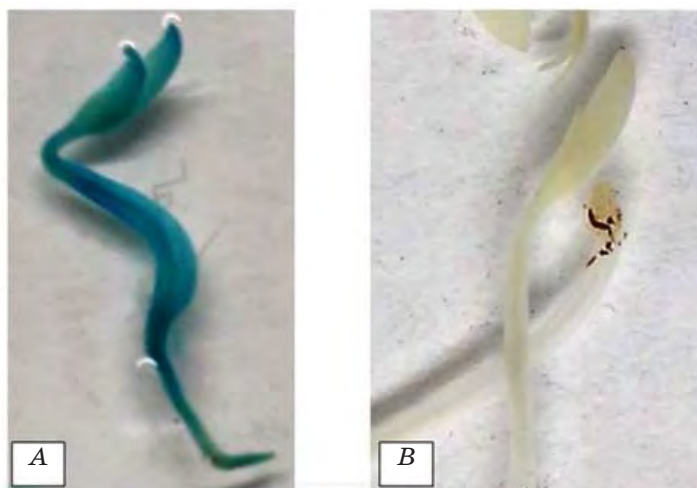


Fig. 2. Hypocotyls of *A. caudatus* seedlings (15-day-old) which were infiltrated with *A. tumefaciens* harboring genetic vector pNMD2501 under UV light (A, B) (magnification ×200)
A — cv. Helios; **B** — cv. Karmin); **C** — hypocotyls of non-infiltrated control plant (cv. Helios) (magnification ×200)



Supplementary material Fig. 2. Seedlings of *A. caudatus* cv. Helios (6-day-old) after the histochemical reaction:
A — seedlings infiltrated with *A. tumefaciens*, genetic vector pCBV19; **B** — non-infiltrated control seedlings of cv. Helios

of plants. The cultivar Karmin has a higher content of betacyanins than the cultivar Helios. Betacyanins can reduce the transformation efficiency of *Agrobacterium* [16].

Transient expression of gfp gene

The next stage of the work was the analysis of plants that were infiltrated via *A. tumefaciens* harboring genetic vector pNMD2501, carrying the *gfp* gene. The results of transient expression of the *gfp* gene were

analyzed visually using an ultraviolet light and were considered as *gfp*-positive when green fluorescence of tissues appeared (Fig. 2–5).

In seedlings of both cultivars (which were infiltrated at the age of 10 days), green fluorescence was observed in hypocotyls and at the edges of leaf blades (Fig. 2, 3).

Microscopic examination revealed that the most intense transient expression of the *gfp* gene occurred in the vascular bundles of the hypocotyl and in the midrib of the leaf blade

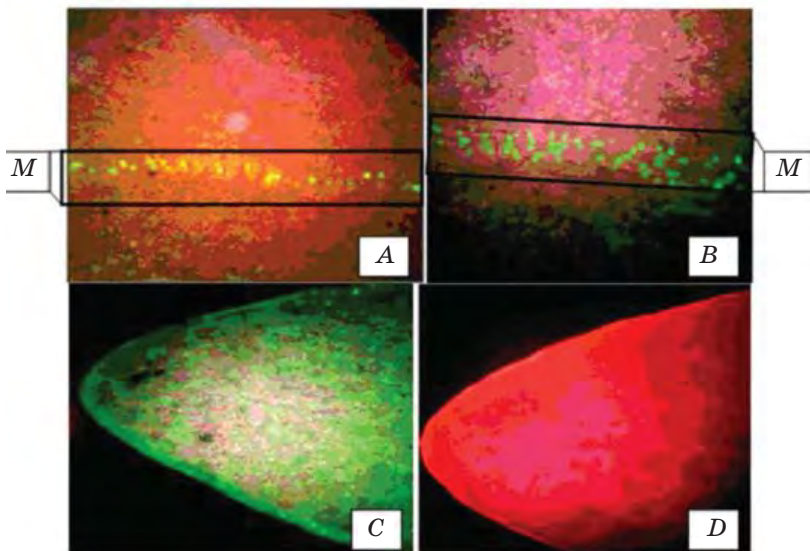
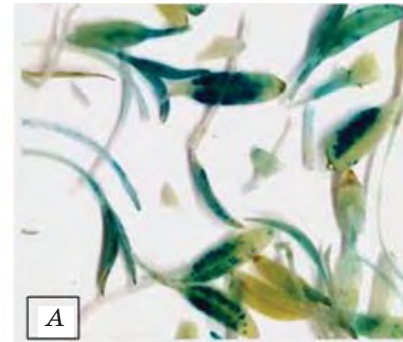


Fig. 3. Cotyledonous leaves of *A. caudatus* seedlings (15-day old) which were infiltrated with *A. tumefaciens*, genetic vector pNMD2501 under UV light (A, B, C): A — cv. Helios (magnification $\times 100$); B — cv. Karmin (magnification $\times 100$); C — top of the cotyledonous leaf cv. Helios (magnification $\times 100$); D — leaf of non-infiltrated control plant (cv. Helios); M — area of midrib



Supplementary material Fig. 3. Seedlings of *A. caudatus* cv. Karmin (15-day-old) after the histochemical reaction: A — seedlings infiltrated with *A. tumefaciens*, genetic vector pCBV19; B — non-infiltrated control seedlings)

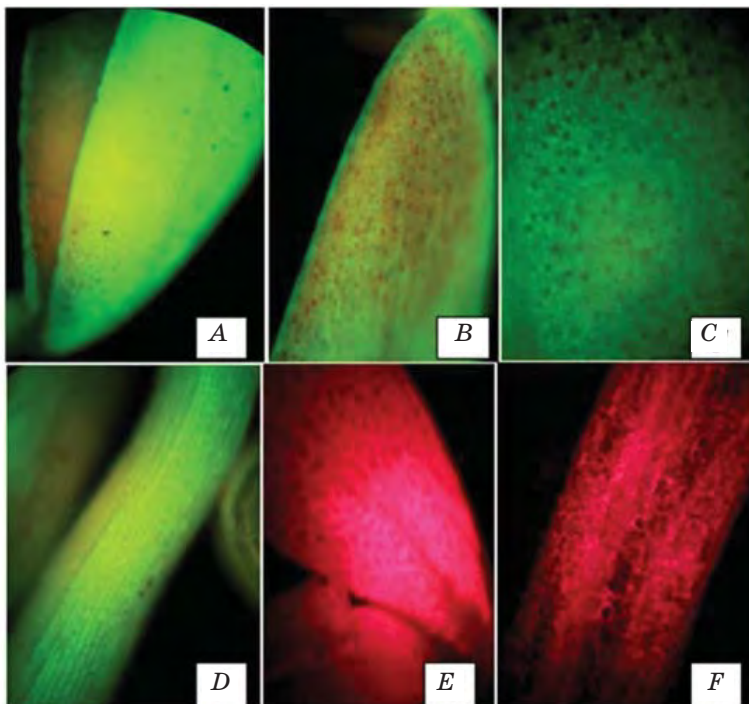
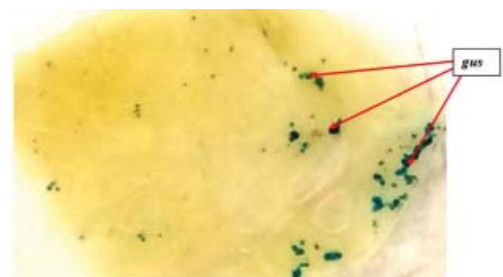


Fig. 4. Seedlings of cv. Helios which were infiltrated with *A. tumefaciens* harboring genetic vector pNMD2501 (6-day old) under UV light (A, B, C, D): A — petiole and lower part of cotyledonous leaves (magnification $\times 100$); B — top of the cotyledonous leaf (magnification $\times 100$); C — hypocotyl (magnification $\times 200$); D — hypocotyl (magnification $\times 100$); E — cotyledonous leaves of non-infiltrated control plant (magnification $\times 100$); F — hypocotyl of non-infiltrated control plant (magnification $\times 200$)



Supplementary material Fig. 4. Leaf of *A. caudatus* variety Karmin (2-month-old) after the histochemical reaction (plant was infiltrated with *A. tumefaciens*, genetic vector pCBV19), gus — areas, where activity of β -glucuronidase was detected

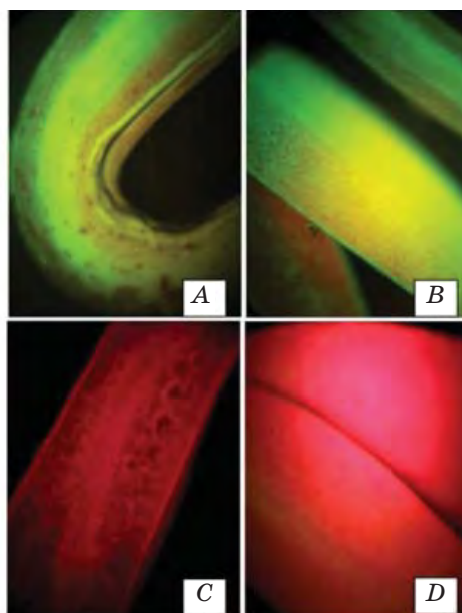


Fig. 5. Seedlings of cv. Karmin which were infiltrated with *A. tumefaciens* harboring genetic vector pNMD2501 (6-day old) under UV light (A, B):

A — hypocotyls (magnification $\times 100$);
 B — hypocotyl and part of cotyledonous leaves (magnification $\times 100$); C — hypocotyl of non-infiltrated control plant (magnification $\times 200$); D — part of non-infiltrated control cotyledonous leaf (magnification $\times 100$)

(Fig. 3).

In seedlings, which were infiltrated at the age of 1 day (both cultivars), intensive green fluorescence was detected in all organs (root, hypocotyl, cotyledonous leaves) (Fig. 4, 5).

Microscopy of the seedlings which were infiltrated at the age of one day, revealed a very intense green glow in all tissues of the aforementioned seedling organs (Fig. 4, 5).

It should be noted that in plants that were infiltrated at the age of 2 months, only a points of green glow were visible on the leaf blades in the region of the central vein. So, we obtained transient expression of the *gus* and the *gfp* genes in all plants of all experimental groups.

Agrobacterial infiltration of the youngest seedlings (1 day-old) turned out to be more effective. Expression was more abundant in young plant tissues which intensively synthesized proteins. In plants that infiltrated at an older age, expression occurred mainly in vascular bundles and leaf midrib (seedlings infiltrated at the age of 10 days), or only in vascular bundles and leaf midrib (plants that infiltrated at the age of 2 months). It was found that amaranth cultivars have different susceptibility to agrobacterial infection. The cultivar Helios was more susceptible to agrobacterial infiltration (Fig. 6).

The number of plants in which were confirmed the expression of the *gus* gene was

significantly or highly significantly different from those group of plants which were not infiltrated with *Agrobacterium*.

So far, transient gene expression has been obtained in the following plants: *Arabidopsis thaliana* [18, 19], *Capsicum annuum* [20, 21]; *Catharanthus roseus* [22, 23]; *Cucumis sativus* [24]; *Fragaria* \times *ananassa* [12], *Fragaria vesca* [25], *Glycine max* [26], *Helianthus annuus* [27], *Juglans regia* [28, 29], *Lactuca sativa* [30], *Fagopyrum esculentum* [31], *Brasica napus* [32].

There is currently a great deal of experimental work on obtaining transient gene expression in *Nicotiana benthamiana* and review articles that mention the successful transient expression of various genes in *Nicotiana benthamiana* [33, 34].

According to the latest literature, the reporter *gfp* gene has been used in *Agrobacterium*-mediated transformation of the following plant species: *Fagopyrum esculentum* [31], *Setaria italic* [35], *Nicotiana tabacum* cv. Bright Yellow 2 [36], *Vigna unguiculata* [37], *A. hypochondriacus* and *A. hybridus* [38], *Oryza sativa* cv. Kitaake [39], *Setaria italica* [40], *Nicotiana benthamiana* [41], *Solanum lycopersicum* [41], *Solanum tuberosum* [41], *Physalis peruviana* [41].

The *uidA* reporter gene was used in *Agrobacterium*-mediated transformation of the

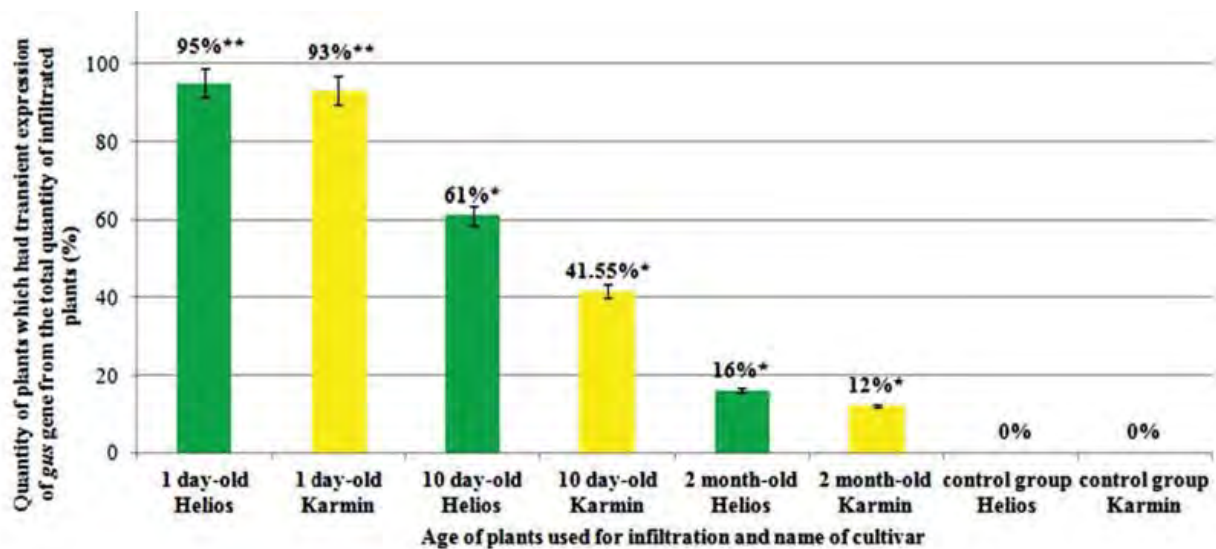


Fig. 6. Effectiveness of vacuum infiltration of different age plants with agrobacterial suspension (*A. tumefaciens* harboring genetic vector pCBV19), expressed as a percentage: values showing significantly differences between the study groups and control groups are marked with asterisks * (* significant ($P < 0,05$); ** — highly significant ($P < 0,01$))

following plant species: *A. hypochondriacus* and *A. hybridus* [38], *Oryza sativa* cv. Kitaake [39], *Setaria italica* [40], *Cannabis sativa* [42].

There is only one report of transient gene expression in representatives of *A. hypochondriacus* and *A. hybridus* [38], indicating insufficient investigation in this sphere.

In our experiments, the most intensive fluorescence of the GFP protein was observed in seedlings infiltrated at the age of one day in all parts of plant. GFP fluorescence was observed also in the hypocotyls (areas of vascular bundles) and in cotyledon leaves (mainly point fluorescence in the area of midrib). In the leaves of 2-month-old plants fluorescence of GFP protein was observed with maximum fluorescence observed on 5th–6th days.

After infiltration of whole amaranth plants under vacuum with a suspension of *Agrobacterium tumefaciens* harboring the genetic vector pCBV19 and histochemical reaction, positive results of β -glucuronidase activity were obtained for two cultivars (Karmin and Helios) (blue areas). Gus-positive areas were located mainly in the middle and lateral veins. This may indicate that the most sensitive tissues to agrobacterial transformation and in which active protein synthesis occurs are the central and lateral veins [43, 44] (supplementary material Fig. 4).

It is known that when interpreting the results of the histochemical reaction, a number of problems may arise. For example, residues of live *Agrobacterium* suspension left on the

surface of untransformed plant tissues can lead to false-positive results in standard histochemical analysis and thus may complicate the analysis of transformation results [16]. Usage of genetic vectors with intron increases the reliability of the histochemical analysis. An intron was presented in the pCBV19 genetic vector, to enable the histochemical reaction to take place only in plant tissues and this ruling out the possibility of a false positive result in the presence of agrobacterial contamination.

Chimeric genetic constructs have been used successfully in the *Agrobacterium*-mediated genetic transformation of several plants: *Spinacia oleracea* [45], *Momordica dioica* [46], *Spinacia oleracea* [47].

Our results of transient expression of the *uidA* gene after infiltration were not positive for all cultivars of *Amaranthus caudatus*. This may be due to differences in biochemical composition of the various cultivars, which in turn may affect susceptibility to *Agrobacterium* infection. In the leaves, β -glucuronidase activity was detected in the central vein. Our results of localization of the *gus* gene in plant tissues and organs during transient expression are similar to those obtained by Jun Jasic [44].

Conclusions

The optimal conditions for the transient expression of reporter genes in *Amaranthus caudatus* cultivars were determined. The most intensive transient expression of *gfp* and *gus*

genes was observed in seedlings which were infiltrated with agrobacterial suspensions at the age of one day. Maximum fluorescence of GFP protein was observed on 5th–6th days. It was shown that cultivar Helios was more susceptible to agrobacterial infection than the cultivar Karmin. The effectiveness of agrobacterial transformation was from 16% to 95% for the Helios cultivar and from 12% to 93% for the cultivar Karmin.

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**ТРАНЗИЄНТНА ЕКСПРЕСІЯ
РЕПОРТЕРНИХ ГЕНІВ
У СОРТАХ *Amaranthus caudatus* L.**

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Як рослинний матеріал для дослідів використовували місцеві сорти *A. caudatus*: Геліос і Кармін. Амарант — нова сільськогосподарська культура для України. Рослинну біомасу, отриману з амаранту використовують у медицині, харчовій промисловості та косметології.

Мета роботи — знайти оптимальні умови для транзійентної експресії репортерних генів у сортах *Amaranthus caudatus*.

Методи. У роботі застосовували біохімічний та мікроскопічний методи. Проростки і дорослі рослини різного віку інфільтрували суспензіями агробактерій окремо (генетичний вектор pCBV19 з геном *uidA* і генетичний вектор pNMD2501 з геном *gfp* у штамі GV3101 *Agrobacterium tumefaciens*).

Результати. Після проведення серії експериментів досягнуто тимчасової експресії гена *uidA* та *gfp* у рослинах амаранту. Найбільш інтенсивна транзійентна експресія генів *gfp* і *uidA* спостерігали у проростків, інфільтрованих у віці 1 дня. Максимум флуоресценції протеїну GFP спостерігали на 5–6 добу.

Висновки. Показано, що сорт Геліос більш сприйнятливий до агробактеріальної інфекції, ніж сорт Кармін. Ефективність агробактеріальної трансформації становила від 16% до 95% для сорту Геліос і від 12% до 93% для сорту Кармін. Отримані результати свідчать про те, що досліджувані сорти амаранту, які досліджувалися, потенційно можуть бути використані для отримання в майбутньому транзійентної експресії цільових генів та синтезу цільових протеїнів в їхніх тканинах.

Ключові слова: *Amaranthus*; *uidA*; *gfp*; *Agrobacterium*; транзійентна експресія.

**ТРАНЗИЕНТНАЯ ЭКСПРЕССИЯ
РЕПОРТЕРНЫХ ГЕНОВ
В СОРТАХ *Amaranthus caudatus* L.**

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В качестве растительного материала для исследований использовались местные сорта *A. caudatus*: Гелиос и Кармин. Амарант — новая сельскохозяйственная культура для Украины. Растительная биомасса, полученная из амаранта, используется в медицине, пищевой промышленности и косметологии.

Цель работы — найти оптимальные условия для транзientной экспрессии репортерных генов в сортах *Amaranthus caudatus*.

Методы. В работе были использованы биохимический и микроскопический методы. Проростки и взрослые растения разного возраста инфильтровали суспензиями агробактерий (генетический вектор pCBV19 с геном *uidA* и генетический вектор pNMD2501 с геном *gfp* в штамме *Agrobacterium tumefaciens* GV3101).

Результаты. После проведения серии экспериментов была достигнута транзientная экспрессия генов *uidA* и *gfp* в растениях амаранта. Наиболее интенсивную транзientную экспрессию генов *gfp* и *uidA* наблюдали у проростков, инфильтрованных в возрасте 1 дня. Максимум флуоресценции протеина GFP наблюдали на 5–6 сутки.

Выводы. Было показано, что сорт Гелиос более восприимчив к агробактериальной инфекции, чем сорт Кармин. Эффективность агробактериальной трансформации составила от 16% до 95% для сорта Гелиос и от 12% до 93% для сорта Кармин. Полученные результаты свидетельствуют о том, что изучаемые сорта амаранта потенциально могут быть использованы для получения в будущем транзientной экспрессии целевых генов и синтеза целевых протеинов в их тканях.

Ключевые слова: *Amaranthus*; *uidA*; *gfp*; *Agrobacterium*; транзientная экспрессия.