EFFECT OF MEDIUM COMPOSITION AND DATE OF EXPLANT DRAWING ON EFFECTIVENESS OF *Agrobacterium*-MEDIATED TRANSFORMATION IN THE PETUNIA (*Petunia hybrida pendula*)

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A B S T R A C T

An effective technique has been developed for *Agrobacterium tumefaciens*-mediated transformation in the petunia (*Petunia hybrida pendula*). Leaf explants were taken from shoots cultured *in vitro* for at least two years. Transformation was performed on four dates (January 26, March 23, August 3, and November 26). In control explants, kanamycin significantly inhibited organogenesis and rhizogenesis at 25 mg l^{-1}, and totally inhibited organogenesis and rhizogenesis at 100 mg l^{-1}. Explants which regenerated shoots and formed roots in the presence of 100 mg l^{-1} kanamycin were presumed to be transgenic. Only six transgenic plants were obtained from 216 leaf explants inoculated with *Agrobacterium*. Incorporation of the *npt*II gene into the plant genome was confirmed by PCR with specific primers. Expression of the *gus*-intron gene was confirmed histochemically. Transformation date had a significant effect on transformation effectiveness. Transformation was most efficient in November (6.15%) and least efficient in August (0%). As expected, expression of the *gus*-intron gene had no significant effect on the phenotype of transgenic plants.

Key words: *Agrobacterium tumefaciens*, GUS, *in vitro* cultures, marker genes, petunia, genetic transformation

INTRODUCTION

Molecular biology and genetic transformation techniques significantly enhance genetic variability, which can be useful in plant breeding. The first successful plant transformation took place in 1982 (Krens et al., 1982). Genes from various organisms can be introduced into plant genomes. Some of these genes can confer resistance to pests, pathogens, and

abiotic stresses. Others can alter plant morphology, physiology, and chemical composition. Experiments with model plants like *Arabidopsis* or *Tradescantia* help elucidate methodological details important for the process of transformation and for studying the expression of transgenes in plants (Klein, 1996).

In early studies on plant transformation, selectable and reporter genes were used to develop effective transformation and selection protocols for specific plant genotypes. Reporter genes enable the rapid identification of transgenic cells and tissues by histochemical, spectrophotometric or fluorometric assays. Commonly used selectable and reporter genes are presented in Table 1. More information can be found in Guivarch et al. (1996).

The most common mediators of gene transfer in dicotyledonous plants are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. With these mediators, a single copy of a gene can be transferred into a plant genome, producing stable transgenic plants. Special bacterial strains are used for transformation. They exhibit varying levels of virulence toward various plant species. Nopaline and octopine strains of *A. tumefaciens* and agropine strains of *A. rhizogenes* are highly virulent and very efficiently transfer chimeric genes from Ti and Ri plasmids into plant genomes (Klein and Korzonek, 2000). Transformation protocols vary widely, depending on the bacterial strain, plant species, and type of explant used. Transformation protocols have to be specifically designed for each plant species, and sometimes even for each cultivar.

The aim of this study was to develop an efficient protocol for *Agrobacterium*-mediated transformation in the petunia (*Petunia hybrida pendula*) using leaf explants from *in vitro* shoot cultures.

MATERIAL AND METHODS

Plant material

Cultures of petunia shoots were maintained *in vitro* on MS0 medium (Table 2, Murashige and Skoog, 1962). The cultures were maintained for at least two years, and were subcultured every eight weeks.

Bacterial strain and transformation vector

Agrobacterium tumefaciens strain LBA 4404 has been developed for binary plasmid transformation. Virulence genes are located on the pAL 4404 helper plasmid, a disarmed octopine pTI-Ach5 plasmid.

The binary vector pBin19-*gus*-intron was obtained from the Department of Fruit and Vegetable Plants Breeding in Gembloux, Belgium. This vector is based on the pBin19 binary plasmid (Bevan, 1984). Its T-DNA region contains the *npt*II selectable gene and the *gus*-intron reportable gene. The *npt*II gene is under the control of the promoter and terminator of the nopaline synthase gene (*nos*). The *gus*-intron gene is under the control of the 35S promoter and terminator

T a b l e 1 . Marker genes commonly used in transformation

Selectable genes	*npt*II	kanamycin resistance
	hpt	hygromycin resistance
	*aph*IV	hygromycin resistance
	cat	chloramphenicol resistance
	bar, *aro*A	phosphinotricine resistance glyphosate resistance
Reporter genes	*gus*	β-glucuronidase
	luc	luciferase
	β-*gal*	β-galactosidase

from *Cauliflower Mosaic Virus* (CaMV). The *npt*II gene codes for neomycin phosphotransferase, which confers kanamycin resistance and enables selection of transformed cells on kanamycin-containing media. The *gus*-intron gene codes for β-glucuronidase, enabling identification of transformed cells.

The plasmid contains a second kanamycin resistance gene elsewhere in its genome. This gene is expressed in prokaryotes and enables the selection of successfully transformed bacteria.

The plasmid was proliferated in *Escherichia coli*, isolated using the method of Skoneczny (1995), and introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation (Krzymowska, 1995). Successful uptake of the pBin19-*gus*-intron plasmid was confirmed by PCR with primers complementary to the *npt*II and *gus*-intron genes (Tab. 3). Gene fragments were amplified according to the thermal profile proposed by Yang et al. (1999) and Griesbach (1994). PCR products were separated by electrophoresis in 1% agarose gel and visualized with ethidium bromide (Sambrook et al., 1989). A 1 kb DNA ladder (Gibco BRL) was used as the standard.

Preparation of bacteria for transformation

The plasmid-bearing *A. tumefaciens* were grown on an oscillatory shaker at 200 rpm at 28°C in kanamycin-containing LB medium (1% tryptone, 0.5% NaCl, 0.5% yeast extract, pH 7.15, supplemented with 50 mg l^{-1} kanamycin and 25 mg l^{-1} rifampicin). After about 24 hours, when the bacteria had reached the log phase, the culture was centrifuged at 4000 rpm for 15 minutes at room temperature. The pellet was resuspended in fresh, antibiotic-free LB medium.

Transformation and regeneration procedure

A preliminary experiment was conducted to study how kanamycin affects organogenesis in wild-type petunias. Leaf laminae were excised from *in vitro* shoots with a scalpel and placed on MS1 medium (Tab. 2) supplemented with 0, 25, 50, 75 and 100 mg l^{-1} kanamycin. The formation of callus and adventitious buds was observed.

For transformation, well-developed leaves were taken from the lower parts of eight-week-old micropropagated shoots. Transformation was performed on four dates: January 26, March 23, August 3, and November 26. Leaf laminae were incised with a scalpel, dipped into the bacterial suspension for 10-15 minutes, and blotted on filter paper. The inocultated leaf laminae were cultured in the dark for three or four days at 24°C in antibiotic-free MS1 medium (Tab. 2). The explants were washed in sterile MS basal medium containing 500 mg l^{-1} of carbenicillin to remove bacteria, blotted on filter paper, and placed on MS2 selective regeneration medium (Tab. 2) containing 250 mg l^{-1} carbenicillin and 25 mg l^{-1} kanamycin. Explants which had not been inocultated served as the control.

The explants were maintained at 21-23°C, first for ten days in the dark, and then for four to six weeks in 20 μmol m^{-2} s^{-1} of white light with a sixteen-hour photoperiod. The light intensity was then increased to 79 μmol m^{-2} s^{-1}. The light source was a 36W type 'TL'D fluorescent lamp manufactured by Phillips.

Explants were transferred to antibiotic containing MS3, MS4, MS5 and MS6 media (Tab. 2) every two to six weeks during organgenesis. Shoots produced by explants that were cultured on selective medium were rooted on MS0 medium supplemented with 100 mg l^{-1} kanamycin. Explants which produced well-developed roots and grew nor-mally *in vitro* were presumed to be transgenic.

Molecular confirmation of transformation

Incorporation of the *npt*II gene into the petunia genome was confirmed by polymerase chain reaction (PCR). DNA was extracted from leaves using the method of Edwards et al. (1991). DNA concentration was determined with a spectrophotometer. About 10 ng was used as the matrix for PCR. The primers used for amplification of the *npt*II gene were those recommended by Yang et al. (1999) and are presented in Table 3.

β-glucuronidase activity in transgenic explants was determined by the histochemical assay described by Jefferson et al. (1987). Plant tissues were incubated with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) overnight at 37°C. Chlorophyll was removed with 96% ethanol.

Biometric analysis of transgenic plants

To determine the effect of kanamycin on rhizogenesis in the transgenic petunia explants, roots were counted after 7, 21 and 35 days of culture on MS0 medium supplemented with varying concentrations of kanamycin (10, 25, 50, 75 and 100 mg l^{-1}). Shoot length, leaf area, shoot dry weight, and root dry weight were also recorded. Leaf area was measured with a computerized planimeter (Leaf Area Meter – ADC).

The effect of kanamycin on rhizogenesis was determined in a completely randomized two-factor experiment. Other effects were determined in completely randomized one-

Table 2. Media used for *in vitro* culture and transformation of petunias

Component	Medium						
	MSO	MS1	MS2	MS3	MS4	MS5	MS6
Minerals	MS*	MS	MS	MS	MS	MS	MS
$NaH_2PO_4H_2O$ [mg l^{-1}]	-	75	75	75	75	75	75
Vitamins	MS	MS	MS	MS	MS	MS	MS
Sucrose [%]	3	3	3	3	3	3	3
NAA [mg l^{-1}]	-	0.1	0.1	-	-	-	0.2
BAP [mg l^{-1}]	-	1	1	1.5	1.5	0.25	0.5
IBA [mg l^{-1}]	-	-	-	0.2	0.2	0.5	-
Agar [%]	0.8	-	-	-	0.8	0.8	0.8
Phytagel [%]	-	0.2	0.2	0.2	-	-	-
Casein hydrolysate [mg l^{-1}]	-	400	400	400	-	-	-
Kanamycin [mg l^{-1}]	-	-	25	50	50	100	100
Carbenicillin [mg l^{-1}]	-	-	250	250	250	250	250
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8

*after Murashige and Skoog (1962)

Table 3. PCR primers complementary to *npt*II and *gus*-intron genes

Primers for 252 bp fragment of *gus*-intron (5' → 3') (Griesbach, 1994)	
Reverse primer–GUS1	CTT TAA CTA TGC CGG AAT CAA TCG
Forward primer–GUS2	TAA CCT TCA CCC GGT TGC CAG AGG
Primers for amplifying 772 bp fragment of *npt*II (5' → 3') (Yang et al., 1999)	
Reverse primer–NPT1	ATT GCA CGC AGG TTC TCC GG
Forward primer–NPT2	AGA ACT CGT CAA GAA GGC GA

factor experiments. The experiments were carried out in three replicates of fifteen explants in three Erlenmeyer flasks for each transgenic clone. Results were recorded as the mean of all transgenic clones. Results were subjected to variance analysis followed by Duncan's multiple range t-test at P = 0.05.

RESULTS AND DISCUSSION

Colonies of *A. tumefaciens* which grew on selective medium after transformation with pBin19-*gus* intron plasmid were tested by PCR with primers complementary to *npt*II and *gus*-intron genes. All colonies analyzed yielded products of the sizes expected (772 bp for *npt*II, and 252 bp for *gus*-intron, Fig. 1). Electrophoresis confirmed the presence of a plasmid of about 13 000 bp (Fig. 2). This proved that the plasmid had been successfully introduced and had replicated in the bacterial cells.

In non-transformed control explants, kanamycin at 25 mg l^{-1} reduced orga-

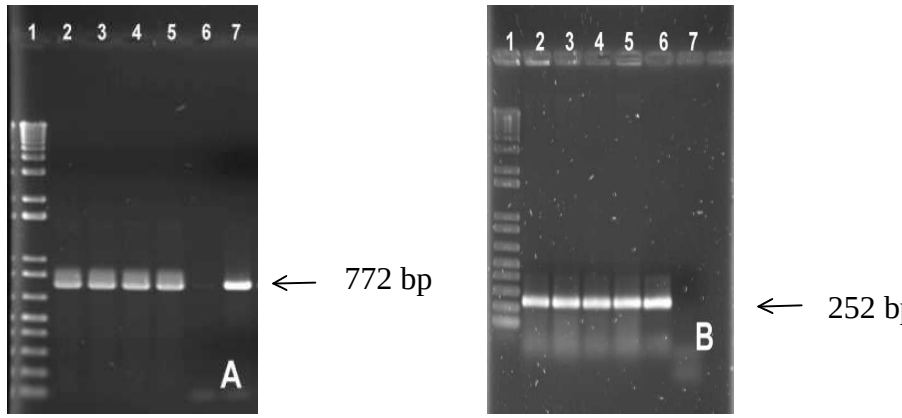

Figure 1. PCR products synthesised with *Agrobacterium tumefaciens* containing pBin19-*gus*-intron as a matrix

A) reaction with *npt*II-specific primers: 1 – 1 kb ladder, 2-5 – reaction with bacteria, 6 – negative control (no DNA), 7 – positive control (reaction with pBin19-*gus*-intron plasmid),
B) reaction with *gus*-intron-specific primers: 1 – 1 kb ladder, 2-5 – reaction with bacteria, 6 – positive control (reaction with pBin19-*gus*-intron plasmid), 7 – negative control (no DNA).

Table 4. Effect of kanamycin on organogenesis in petunias

Kanamycin concentration [mg l^{-1}]	Percentage of explants forming callus and shoots		
	3 weeks	6 weeks	9 weeks
0	98.6 d*	100 d	100 b
25	41.4 c	22.3 c	0 a
50	2.6 b	1.3 b	0 a
75	1.4 a	0 a	0 a
100	0 a	0 a	0 a

*Means with the same letter do not differ significantly at P = 0.05 (Duncan's t-test)

nogenesis by 58.6% after three weeks, and by 77.7% after six weeks (Tab. 4). Kanamycin at 50 mg l^{-1} reduced organogenesis by 97.4% after three weeks, and by 98.7% after six weeks. When 75 mg l^{-1} kanamycin was used, only 1.4% of explants formed shoot primordia after three weeks. Kanamycin killed non-transformed petunia explants after nine weeks of treatment at all concentrations tested.

Both transformed and control explants started to form roots after about seven weeks on kanamycin-free MS0 medium. There were no differences between transformed and control explants either in the number of roots formed after 7, 21 and 35 days of culture (Tab. 6) or in root morphology (Fig. 5). In control explants, kanamycin drastically reduced rhizogenesis at 25, 50 and

Table 5. Date of treatment and efficiency of transformation

Type of explant	Date of transformation			
	Jan. 26	Mar. 23	Aug. 3	Nov. 26
Explants inoculated	50	46	55	65
Callus-forming explants	8 (16%)c*	5 (10.8%)b	3 (5.45%)a	15 (23.3%)d
Shoot-forming explants	3 (6%)c	2 (4.34%)b	1 (1.8%)a	5 (7.69%)d
Root-forming explants	1 (2%)b	1 (2.1%)b	0 (0%)a	4 (6.15%)c

*For explanation, see Table 4

Table 6. Effect of kanamycin on rhizogenesis of petunia shoots

Kanamycin concentration [mg l^{-1}]		Mean number of roots per shoot		
		7 days	21 days	35 days
0	**TRANSFORMED**	**1.99 ef**	**9.93 d**	**13.93 d**
	CONTROL	2.00 f*	10.06 d	14.11 d
25	**TRANSFORMED**	**1.97 def**	**9.22 cd**	**13.80 d**
	CONTROL	0.89 c	1.55 b	2.03 c
50	**TRANSFORMED**	**1.98 ef**	**9.54 cd**	**13.81 d**
	CONTROL	0.43 b	0.74 ab	0.67 b
75	**TRANSFORMED**	**1.98 ef**	**9.96 d**	**14.16 d**
	CONTROL	0.09 a	0.13 a	0.09 a
100	**TRANSFORMED**	**2.00 f**	**9.99 d**	**14.17 d**
	CONTROL	0 a	0 a	0 a

*For explanation, see Table 4

75 mg l^{-1}, and completely inhibited rhizogenesis at 100 mg l^{-1} (Tab. 5). In transformed explants, kanamycin did not affect rhizogenesis at any of the concentrations tested. Explants forming normal shoots and roots in the presence of 100 mg l^{-1} kanamycin were presumed to be transgenic.

Of a total of 216 explants subjected to transformation on all dates, only six formed roots on kanamycin-containing medium. Four root-forming explants were obtained in November, and one each in January and March. No root-forming explants were obtained in August. The numbers of callus-forming and shoot-forming explants were also very low in August (Tab. 5).

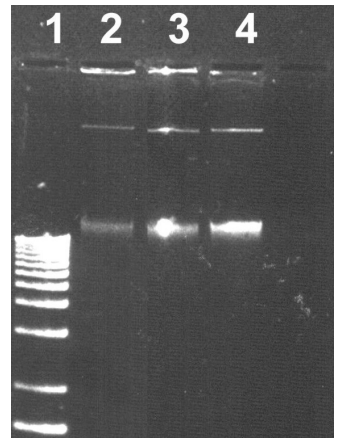

Figure 2. Electrphoresis of plasmid DNA isolated from *Agrobacterium tumefaciens* transformed with pBin19-*gus*-intron: 1 – 1kb ladder, 2-4 plasmid DNA isolated from bacteria

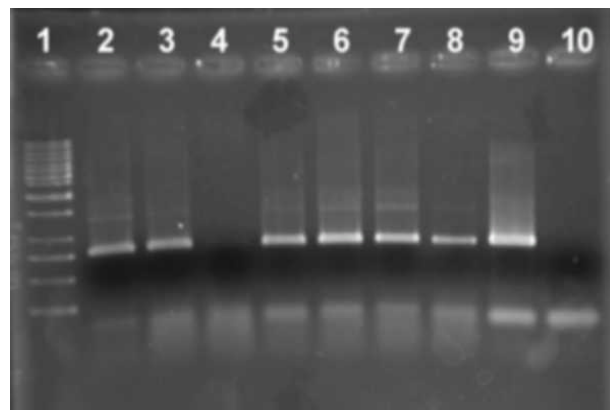

Figure 3. Products of PCR with *npt*II specific primers: 1 – 1 kb ladder, 2-8 – reaction with genomic DNA isolated from transgenic plants, 9 – positive control (reaction with pBin19-*gus*-intron plasmid), 8 – reaction with genomic DNA isolated from control plant

PCR confirmed the successful incorporation of *npt*II into the genomes of all six root-forming explants (Fig. 3). Kanamycin resistance confirmed the proper expression of the *npt*II gene. Histochemical testing with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) confirmed the expression of the *gus*-intron gene. The formation of a blue color in the calli and laminae of transformed explants confirmed β-glucuronidase activity (Fig. 4).

There were no differences in shoot length, leaf area, shoot dry weight and

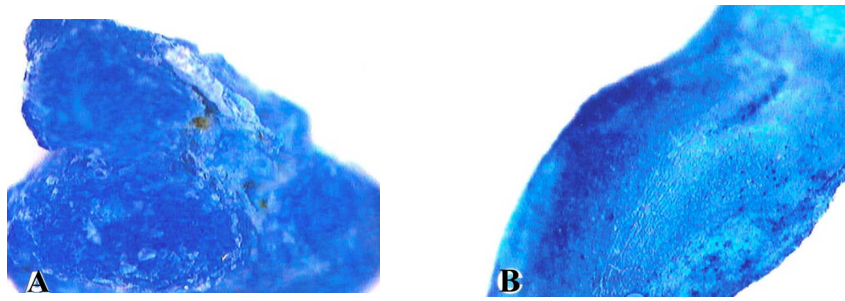

Figure 4. β-glucuronidase activity in tissues of transgenic petunias
A) callus, B) fragment of leaf blade

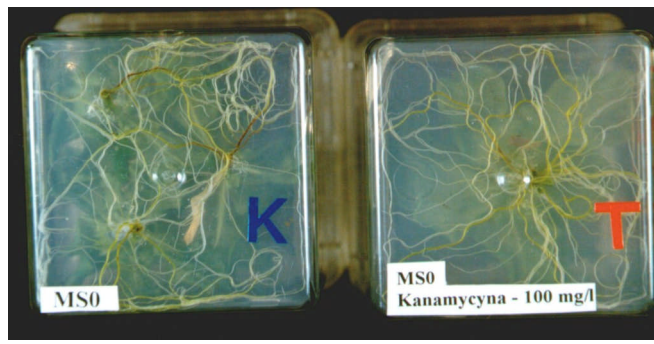

Figure 5. Rhizogenesis of control petunia shoots (K) on MSO medium and transgenic shoots (T) on MSO medium supplemented with 100 mg l^{-1} kanamycin

Table 7. Morphology of transgenic and control petunia plantlets rooted *in vitro*

Type of plant	Shoot length [cm]	Leaf area [cm²]	Shoot dry weight [%]	Root dry weight [%]
TRANSFORMED	6.97 a*	2.28 a	7.62 a	8.21 a
CONTROL	7.37 a*	2.74 a	7.09 a	7.96 a

*For explanation, see Table 4

root dry weight between transgenic and control plants (Tab. 7). This proves that the technique used for selection and organogenesis did not induce visible phenotypic changes which can occur during regeneration from dedifferentiated callus tissue.

Agrobacterium tumefaciens is an efficient vector for transformation in petunias. The *npt*II gene is a highly

effective selectable marker gene. The low transformation efficiency in this study is consistent with those reported for other plant species when kanamycin is used for selection.

In this study, there was considerable seasonal variation in transformation efficiency even though the explants were taken from shoot cultures which had been maintained *in vitro* under stable light and temperature conditions for at least two years. Plants preserve their annual cycle for a long time even without environmental stimuli. Seasonal variation in transformation efficiency had not been reported in the literature before now. Further studies are needed to confirm and explain this phenomenon.

Efficient techniques for the regeneration of transformed tissues are essential. Petunias easily regenerate shoots from various tissues (George et al., 1987; Michalczuk, 2000). In this study, transgenic petunia shoots were regenerated from dedifferentiated callus tissue.

According to Martinelli et al. (1997), low selection pressure increases the risk of chimeric plants. Kanamycin totally inhibited organogenesis in control explants only after prolonged treatment at very high concentrations. To eliminate non-transformed cells and reduce the risk of chimeric plants, calli were subcultured several times on selective media before being transferred to the regeneration medium. The uniform expression of *gus*-intron gene both in transgenic calli and in the tissues of transgenic plants proves that this selection method was effective. In a previous study, the type of light used affected organogenesis in transgenic petunias (Michalczuk and Michalczuk, 2000).

The method presented here will be used to introduce IAA-glucose synthase gene from maize (*Zea mays* L.) into petunias.

CONCLUSIONS

Kanamycin singnificantly inhibits organogenesis in non-transformed petunias at concentrations as low as 25 mg l^{-1}. Kanamycin totally inhibits organogenesis only at 100 mg l^{-1}, or after prolonged treatment at lower concentrations. To prevent chimeric plants, media for selection should contain 100 mg l^{-1} kanamycin.

There is considerable seasonal variation in transformation efficiency in petunias.

Biometric analysis of transgenic showed that the incorporated transgenes and the regeneration technique did not affect the phenotype of transgenic petunia explants.

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WPŁYW SKŁADU POŻYWKI I TERMINU POBIERANIA EKSPLANTATÓW NA WYDAJNOŚĆ TRANSFORMACJI GENETYCZNEJ PETUNII (*Petunia hybrida pendula)* ZA POŚREDNICTWEM *Agrobacterium tumefaciens*

Barbara Michalczuk i Danuta Wawrzyńczak

S T R E A S Z C Z E N I E

Opracowano wydajną technikę transformacji genetycznej petunii (*Petunia hybrida pendula*) metodą wektorową z wykorzystaniem bakterii *Agrobacterium tumefaciens* zawierających plazmid pBin19-*gus*-intron z genem odporności na kanamycynę – *npt*II oraz genem reporterowym *gus*-intron. Eksplantaty liściowe pobierano z pędów petunii utrzymywanych w kulturach *in vitro* przez okres co najmniej 2 lat. Transformację przeprowadzono w czterech terminach (26 I, 23 III, 3 VIII, 26 XI). U eksplantatów kontrolnych kanamycyna w stężeniu 25 mg l^{-1} istotnie ograniczała organogenezę i ryzogenezę, a stężenie 100 mg l^{-1} antybiotyku całkowicie ją hamowało. Za rośliny transgeniczne uznano te, które zregenerowały pędy z eksplantatów i wytworzyły korzenie w obecności 100 mgl^{-1} kanamycyny. Z 216 eksplantatów liściowych poddanych transformacji we wszystkich terminach otrzymano 6 roślin transgenicznych. Wprowadzenie genu markerowego *npt*II do genomu tych roślin potwierdzono w reakcji PCR ze specyficznymi starterami. Ekspresja genu reporterowego *gus*-intron została potwierdzona metodą histochemiczną. Stwierdzono istotny wpływ terminu przeprowadzenia transformacji na jej wydajność. Najwyższą wydajność transformacji (6,15%) uzyskano w listopadzie a najniższą (0%) w sierpniu. Zgodnie z oczekiwaniami, ekspresja genu *gus*-intron nie miała istotnego wpływu na fenotyp transgenicznej petunii.

Słowa kluczowe: *Agrobacterium tumefaciens*, geny markerowe, GUS, kultury *in vitro*, petunia, transformacja genetyczna