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Genetic Transformation of *Coleus blumei* Benth. Using *Agrobacterium*

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Summary

Efficiency in transformation of three hybrids of *Coleus blumei* using wild and mutant strains of *Agrobacterium* sp. was evaluated. Successful transformation depended on specific bacterial strain-plant genotype combination and co-cultivation treatment. The wild type B6S3 of the bacterial strains employed gave the maximum efficiency. Strains C58C1(pArA4abc), GV3101(pGV2215), 8196 and A281 were also effective. Among several co-cultivation conditions tested, the most efficient treatment was as follows: excised leaf explants were incubated immeadiately in bacterial suspension for 5 min and were co-cultured with *Nicotiana tabacum* crown gall callus for two days. Plant hormone autotrophy, PCR analyses and hybridization analysis confirmed genetic transformation of the cultures. Sixteen different transgenic callus and cell cultures were maintained for more than four years in the absence of exogenous growth regulators and antibiotics without deterioration in the growth rate.

Key words: callus culture, *Coleus blumei*, transformation, *Agrobacterium*

Introduction

Coleus blumei Benth. (Lamiaceae) is an ornamental plant, growing all over the world in an enormous number of different cultivars that vary in colour and shape of the leaves. It is used in India, Indonesia and Mexico as a medical plant. One of the most prominent secondary compounds in *C. blumei* is rosmarinic acid (RA), which is believed to be a part of the plant´s defense system against fungal and bacterial infections and predators (*1*). Steps in RA biosynthesis originating from phenylalanine and tyrosine have been characterised (*2*). *C. blumei* plants are not used for the isolation of RA, although their cell cultures have been investigated for the purpose of a commercial production of RA (*3*). The establishment of non-transformed tissue and cell cultures of *C. blumei* has already been reported (*3–7*).

Crown galls and hairy roots, induced by agrobacteria, of several plant species have been shown to synthesise secondary metabolites such as pharmaceuticals and fine chemicals including RA in higher amount than non-transformed tissue does (*8–16*). Successful infection of a host by *Agrobacterium* requires a compatible reaction between the host plant and bacterial strain. The ability of the bacterium to form a compatible reaction varies widely among species, therefore, evaluation of strain-cultivar compatibility is an important step in the

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Abbreviations: MS: Murashige and Skoog medium (1962), BA: 6-Benzylaminopurine, NAA: α-Naphthaleneacetic acid, RA: Rosmarinic acid, PCR: Polymerase chain reaction

establishment of a transformation protocol. The transformation of *C. blumei*, to the best of our knowledge, except for our preliminary results (*17*), has not yet been reported. Thus, the aim of the present investigation was to establish the conditions for the transformation of *C. blumei* tissues with several strains of *Agrobacterium* and further to analyse whether the transformation process itself would have any effects on the expression of RA pathway.

In addition, the influence of different co-cultivation conditions on the transformation efficiency was tested. The major goal of this study was to establish *C. blumei* as a compatible host for *Agrobacterium* infection and to demonstrate fast growth of callus tissue on growth regulator-free medium.

Materials and Methods

Plant material and bacterial strains

Shoots of the three different genotypes of *Coleus blumei* Benth. hybrids established *in vitro* were used in the experiments. Donor cultures were maintained from nodal segments by routine subculture in 300 mL Erlenmeyer flasks containing 50 mL 1 x MS medium (*18*) with 3 % (w/v) sucrose and 0.8 % agar every ten weeks.

The green hybrid possessed green leaves with white spots and did not synthesise anthocyanins. The variegated hybrid had dark red leaves with a thin green border, and the red hybrid had white, pink, dark red and green spots differently distributed on a leaf surface.

Five *Agrobacterium tumefaciens* strains, B6S3(pTiB6S3) (*19*), GV3101(pGV2215), GV3101(pGV2255) (*20*), A281(pTiBo542) (*21*), C58C1(pArA4abc), and *Agrobacterium rhizogenes* strain 8196(pRi8196) (*22*) were used for transformation of *C. blumei* leaf explants.

Bacteria were grown in liquid medium with 0.8 % beef extract, 0.7 % yeast extract and 0.5 % sucrose (pH= 7.2). Two-day-old bacterial cultures, grown at 24 °C on rotary shaker, were used for leaf explant inoculations.

Leaf explant and transformation procedure

The leaves used for transformation experiments, excised from *in vitro* growing *Coleus* plants, were cut into 1 cm^2 squares and incubated with agrobacteria according to Horsch *et al*. (*23*). Next, co-cultivation conditions were evaluated to determine the most efficient procedure for *C. blumei* leaf explant transformation: (*i*) one day long pre-treatment of leaf explants on MS medium supplemented with 8.9 μM BA and 5.4 μM NAA, (*ii*) different duration of incubation with bacteria (5 or 15 min), and (*iii*) co-cultivation of inoculated leaf explants with the established *Nicotiana tabacum* crown gall callus (five leaf explants placed alternately with 6 callus pieces on hormone-free MS (MS0) medium in ø 9 cm Petri dishes). Leaf explants were inoculated upside-down. The influence of different conditions was tested on the variegated *Coleus* explants inoculated with *A. tumefaciens* B6S3.

Afterwards, the efficiency of *Agrobacterium* strains (B6S3, A281, GV3101(pGV2215), GV3101(pGV2255), C58C1(pArA4abc), and 8196) in the transformation of *Coleus* was evaluated using the most efficient co-cultivation condition: the leaf explants were incubated immediately for 5 min in bacterial suspension after cutting and they were then co-cultured with *Nicotiana tabacum* crown gall callus on MS0 medium for two days. Afterwards the leaf explants were subcultured to MS0 medium with 500 mg/L of carbenicillin.

Leaf explants, infected with 18 min autoclaved (121 °C, 1.5 psi) agrobacterial cultures and cultivated in the same way as treated explants, were used as control. The efficiency of transformation was expressed as a percentage of the responded leaf explants (explant that 14 days after bacterial inoculation develops at least one callus clump) and as a mean number of callus clumps per responded leaf explant. Each treatment consisted of 30 explants.

Establishment of tumour tissue culture

Two weeks after agrobacterial inoculation tumour clumps were excised from the leaf explants. Transformed callus cultures were established by routine subculturing every four weeks. The first three subcultures were incubated on solidified hormone-free MS medium containing 500 mg/L of carbenicillin, and the next were cultured on MS0 medium, at 24 °C under fluorescent tube light (16 h photoperiod). The growth index of transformed callus lines were calculated according to formula:

$$\text{growth index} = (\text{FW}^* \text{ (g) at day 32} - \text{FW (g) at day 0}) / \text{FW (g) at day 0}$$

* FW = fresh weight

DNA isolation, PCR and hybridization analysis

Plant DNA was extracted from *in vitro* cultured tumour tissue (approximately 200 mg of fresh tissue) according to the procedure of Edwards *et al*. (*24*). The presence of T-DNA in callus lines was detected by the polymerase chain reaction (PCR, PERKIN ELMER, GeneAmp PCR System 2400 instrument). The PCR amplification of the target sequences was performed in 25 μL of the following reaction mixture: 0.2 mM of each dNTP, *Taq* pol buffer, 3 mM $MgCl_2$, 10 pmol of each primer, 1.0 U *Taq* polymerase (Finnzymes) and 50 ng of plant DNA. In the calli, transformed with strain B6S3 and A281, the part of *6a* gene was amplified. In the tumours induced with the strain 8196 the amplification of the *mas1* gene was obtained. No amplification of the *virB10* gene confirmed sterility of transgenic callus lines. Table 1 shows the genes, the primers and the expected length of amplified fragments.

The PCR conditions for amplification of *6a* and *virB10* genes were as follows: 30 cycles, 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1.5 min, while for the amplification of the *mas1* gene the annealing temperature was 55 °C.

The identity of the PCR products was confirmed by hybridization analysis. After denaturation, the DNA was transferred onto positively charged Boehringer Mannheim Nylon Membran using 10 x SSC buffer. PCR products were hybridized with probes labelled with DIG-dUTP, using DIG Labelling and Detection Kit (Boehringer Mannheim). Prehybridization and hybrid-

Table 1. Amplified genes, primers and expected length of amplified fragments in transformed *Coleus* callus lines

Gene	Primers	Length of amplified fragment	Callus lines
6a	5'-TGCTTCAGATGGATTGCTTGCC-3'	336bp	B6S3
T-DNA	5'-GATAGCACCATCTAACTCCACG-3'		A281
mas1	5'-CGGTCTAAATGAAACCGGCAAACG-3'	970bp	8196
T-DNA	5'-GGCAGATGTCTATCGCTCGCACTCC-3'		C58C1
virB10	5'-CAATCCCGATCAAGTCGTGCGC-3'	644bp	B6S3
outside T-DNA	5'-AGACGCCAACCTCGTGAAACCG-3'		A281
			8196
			C58C1

ization were performed at 65 °C. Hybridization probe was DIG labelled bacterial *6a* gene.

Results

The susceptibility of Coleus leaf to different co-cultivation conditions

Fig. 1 shows the influence of different treatments on the efficiency of variegated leaf explants transformation with *A. tumefaciens* B6S3. The transformation efficiency was determined as the percentage of explants responded to the infection (Fig. 1a) and as the number of callus protuberances that appeared on a reactive explant (Fig. 1b).

In treatment B and C 5 min incubation was better while in A and D 15 min incubation gave higher frequency of transformation.

The best results were obtained when leaf explants were incubated for 5 min with bacterial suspension immediately after excising and subsequently co-cultured with *Nicotiana tabacum* crown gall callus for two days (treatment C, Fig. 1). After such treatment, 100 % of leaf explants responded to the infection with an average number of 6.5 callus clumps per explant. During the treatment, leaves swelled and enlarged in size within 5 days of infection, and the first tumour proliferation occurred on the 8th day. Tiny callus clumps appeared on the control explants co-cultivated with *Nicotiana tabacum* callus. After subcultivation to MS0 medium, the clumps died together with the explant tissue contrary to the clumps of tumour callus, which kept on growing fast on MS0 medium.

Compatibility of Agrobacterium-Coleus infection

Leaf explants of the three hybrids of *Coleus blumei* Benth. (variegated, red and green) were inoculated with bacterial suspension of six strains using the most efficient co-cultivation treatment which ensures the highest number of different callus lines. The efficiency of transformation was determinated two weeks after inoculation.

The responses of the three different genotypes of *Coleus blumei* to the tested strains of agrobacteria are shown in Fig. 2. The three tested genotypes were susceptible to the tested strains (green and red hybrids were not treated with strain C58C1(pArA4abc)). Initiation of tumours started after the 6th day in green hybrid, and after the 10th day in red and variegated hybrids. Callus clumps developed in the green *Coleus* were the biggest and often overgrew the explant edges. Percentage of tumour formation varied according to the genotypes. In the green hybrid, 100 % of explants pro-

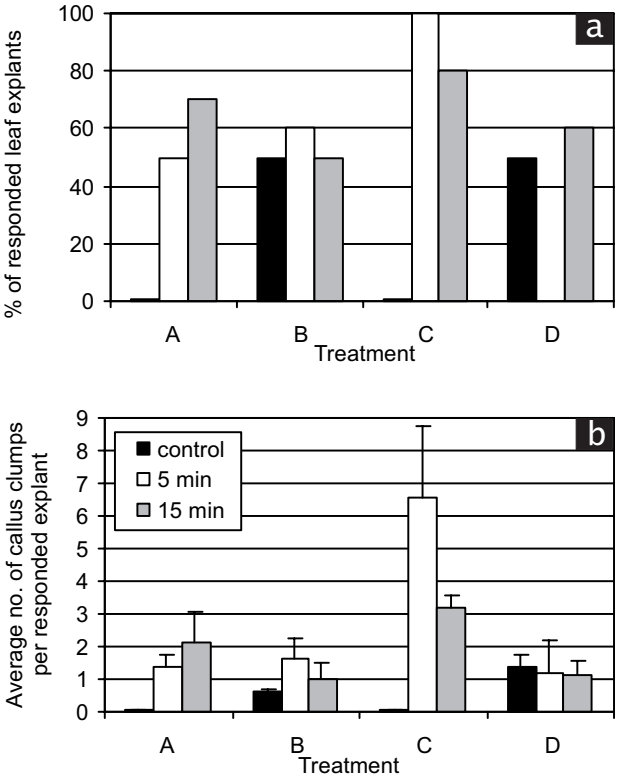

Fig. 1a,b. Transformation efficiency of variegated hybrid of *Coleus blumei* Benth. inoculated with *Agrobacterium tumefaciens* B6S3. Results were scored 14 days after the inoculation. **A** – after infection leaf explants were cultured for two days on MS0 medium, and then transferred to MS0 medium with 500 mg/L carbenicillin; **B** – before bacterial inoculation leaf explants were preincubated for one day on MS with 8.9 μM BA and 5.4 μM NAA and then cultured on MS0 medium with carbenicillin; **C** – after infection, leaf explants were co–cultured for two days with *Nicotiana tabacum* crown gall callus on MS0 medium and then transferred to MS0 with 500 mg/L carbenicillin; **D** – the same as in C except that before bacterial inoculation leaf explants were preincubated for one day on MS with 8.9 μM BA and 5.4 μM NAA. Data are mean values of 30 replicates per treatment. Vertical bars denote standard deviation.

Control:

■ 5 min leaf explants – autoclaved agrobacterium suspension co-cultivation

❑ 5 min leaf explants – agrobacteria co-cultivation

■ 15 min leaf explants – agrobacteria co-cultivation

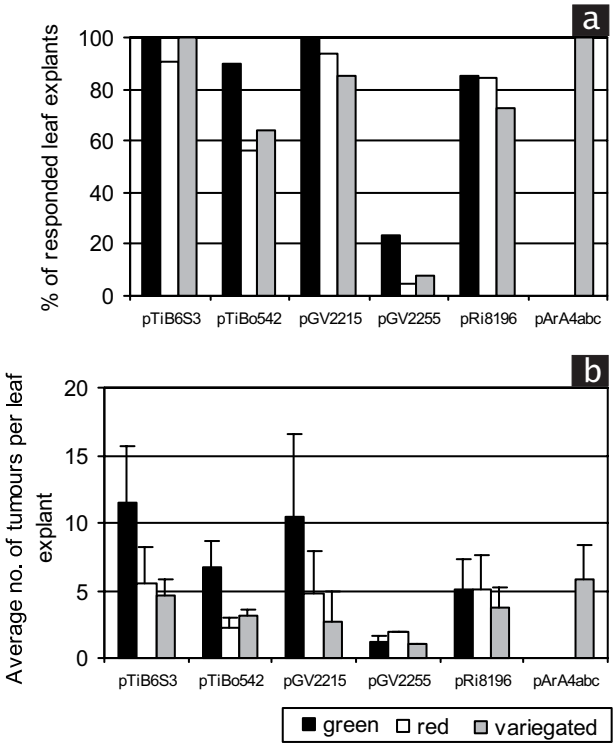

Fig. 2a,b. Compatibility of *Agrobacterium–Coleus* interactions. After infection leaf explants were co-cultured for two days with *Nicotiana tabacum* crown gall callus on MS0 medium and then transferred to MS0 with 500 mg/L carbenicillin. The response was calculated as percentage of responded leaf explants with tumour (a), and a mean number of tumours obtained on the responded explant (b). Results were scored 14 days after inoculation. Data are mean values of 30 replicates per treatment. Vertical bars denote standard deviation.

duced tumours after inoculation with both the strain B6S3 and the strain GV3101(pGV2215). Ninety percent and 85 % explants produced tumours when transformed with the strain A281 and the strain 8196, respectively. The variegated hybrid responded 100 % with both the strain B6S3 and the strain C58C1(pArA4abc). Eighty-five percent, 73 and 64 % explants produced tumours with the strain GV3101(pGV2215), the strain 8196, and the strain A281, respectively. In the red hybrid, the highest percentage of responded leaf explants (94 %) was with the strain GV3101(pGV2215), while 91, 84 and 56 % explants responded with strains B6S3, 8196 and A281, respectively.

The virulent root inducing strains C58C1(pArA4abc) and 8196 were not able to induce root regeneration, but vigorous callus clumps developed on the places of infection. The strain B6S3 showed maximum tumorigenic activity (Fig. 2). All bacterial strains, except the strain GV3101(pGV2255), were able to induce tumorigenesis in the three *Coleus* hybrids. Tumours incited by the GV3101(pGV2255) died after three weeks in culture.

Establishment of tumour tissue culture

Excised tumour clumps, cultured on agar-solidified medium lacking growth regulators (MS0), grew rapidly. According to consistence, growth and RA production

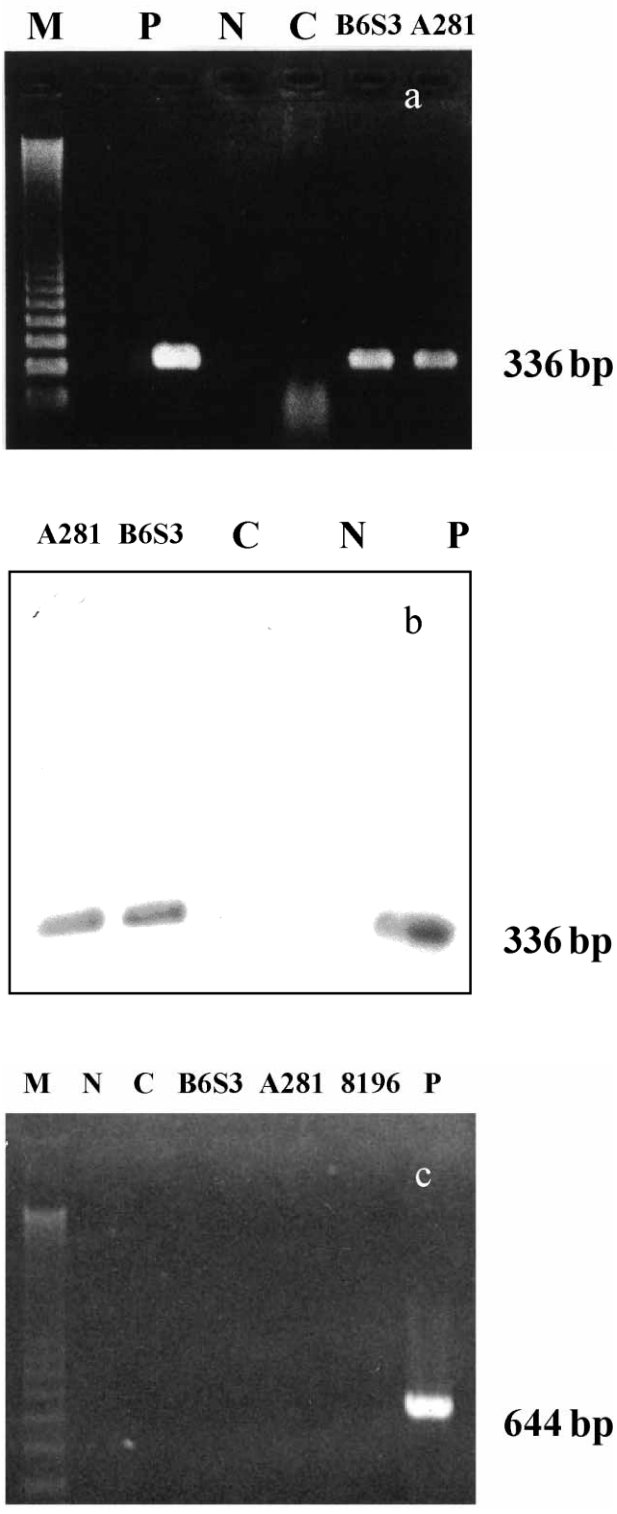

Fig. 3a,b,c. Agarose gel electrophoresis and hybridization analysis of the *6a* gene (a,b) amplified by PCR of genomic DNA from callus lines transformed with strains B6S3 and A281, and PCR of *virB10* gene (c) in DNA isolated from callus lines transformed with strains B6S3, A281 and 8196. Lanes: M, *T. molitor* marker; P, positive control, *Agrobacterium tumefaciens* B6S3; N, negative control (H_2O); C, non-transformed callus tissue; B6S3, tissue transformed with strain B6S3; A281, tissue transformed with strain A281, 8196 tissue transformed with strain 8196.

we selected 16 callus lines for further investigations. Selected lines originated from three *Coleus blumei* hybrids and three *Agrobacterium* strains (A281, B6S3, 8196). Nine of 16 callus lines had friable consistency and they were able to grow as cell suspensions. Seven lines grew in a shape of a hard compact or lumpy callus. We could not find a correlation between a specific phenotype of callus and a bacterial strain or a *Coleus* genotype. The tissue colour varied among lines from pale green to ochre or yellowish-brown. The growth indexes of lines ranged from 3.5 to 28. In general, friable lines grew faster. Sixteen selected transgenic callus lines continued to proliferate after 4 years in culture on MS medium lacking hormones and antibiotics.

Molecular analysis of tumour tissue

All callus lines were proven to be transformed (three years after induction) by the presence of the part of the *6a* gene (for transformation with strains B6S3 and A281), and the *mas1* gene (for strains 8196 and C58C1) in DNA extracts from plant tissues. PCR amplification using appropriate primers generated fragments of 336 bp for the *6a* gene and 970 bp for the *mas1* gene (Table 1, Figs. 3a and 4).

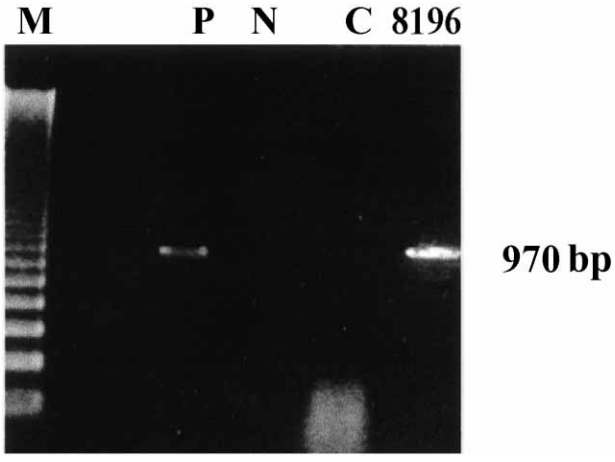

Fig. 4. Agarose gel electrophoresis of the *mas1* gene amplified by PCR in DNA isolated from established callus lines transformed with 8196. Lanes: M, *T. molitor* marker; N, negative control (H_2O); P, positive control, *Agrobacterium rhizogenes* 8196; C, non-transformed callus tissue; 8196, tissue transformed with strain 8196.

DNA from non-transformed callus cultures of *C. blumei* was used as a negative control, and bacterial DNA was used as a positive control. The PCR analysis showed that the *6a* gene was incorporated in the lines transformed with B6S3 and the lines transformed with A281. The *mas1* gene was incorporated in the lines transformed with 8196 and lines transformed with C58C1(pArA4abc). The fragments were not present in the non-transformed tissue. The *virB10* gene was present in the bacterial positive control and it was absent in the transformed lines and control callus (Fig. 3c). These results confirmed that the PCR results were not the consequence of the bacterial contamination.

Hybridization analysis of PCR products confirmed the authenticity of the amplified fragments (Fig. 3b).

Discussion

The primary objective of this study was to determine the favourable conditions necessary for transformation of *Coleus blumei* with the wild and mutant type of agrobacterial strains. Our results provide a good indication that *Coleus blumei* leaf tissues are susceptible to *Agrobacterium* infection. Among six strains tested, only the rooty mutant *A. tumefaciens* GV3101(pGV2255) was unable to induce roots or tumour proliferation. Other *Agrobacterium* strains stimulated callus proliferation in *Coleus blumei* leaf explants. The rooty mutant GV3101(pGV2255) and the shooty mutant GV3101(pGV2215) were derived from the same strain B6S3, however, only the shooty mutant induced tumour on *Coleus* leaf tissues.

We assume that *Coleus blumei* cells were transformed by *A. tumefaciens* GV3101(pGV2255), but they did not have a potential for growth and division without exogenous citokinin (the gene *4* is responsible for citokinin biosynthesis). Besides, the gene *4* strain GV3101(pGV2255) lacks the *6a* and *6b* genes that influence tumour formation (*25*). The gene *6b* has important role in tumour induction; to certain plants the 6b product stimulates tumour formation in the absence of other T-DNA genes (*26*). Some authors have reported higher growth rate of the GV3101(pGV2255) transformed tissue in other plant species (*25,27,28*).

Several co-cultivation conditions were examined for the improvement of transformation. The aim of precultivation of the leaf explants on MS medium, supplemented with 8.9 μM BA and 5.4 μM NAA was to induce proliferation of the leaf parenchyma since the active replication of plant DNA is needed for integration of T-DNA (*29*). Healing, that occurred on the explants' cut ends within 24 h, probably reduced secretion of phenolic compounds required for bacterial attraction (*30,31*). There were no significant differences among leaf explants incubated for 5 or 15 min with agrobacteria, but a longer incubation (one hour) reduced substantially the number of transformed callus clumps on infected leaves (results not shown). Phenolic compounds, released from the leaves that stimulated agrobacteria to fasten on plant cells after longer period diffused in liquid medium together with RA. *Nicotiana tabacum* crown gall callus, inoculated adjacent to the infected explants, amplified plant cell viability and stimulated cell division. The stimulation effect of *Nicotiana tabacum* callus was proven on the control explants that were induced to proliferate on cut ends. Several authors have described *A. tumefaciens* A281 as hypervirulent (*21,32*). In our experiments, *Coleus* was not very susceptible to the strain A281. Red and variegated hybrids showed resistance to *A. tumefaciens* A281 infection, while the green hybrid was resistant to the *A. rhizogenes* 8196 infection. A significant diversity among green and two other hybrids of *Coleus blumei* on agrobacterial infection was shown. Generally, red and variegated hybrids were more resistant to infection then green hybrid. Different responses of the three hybrids to the transformation may have been the effect of genetic heterogeneity common to the members of the family

Lamiaceae (*33*). Could it be related to different levels of RA in leaf tissue? There was 2.4, 5.4 and 6.9 % of RA spectrophotometrically measured in dry leaf tissue of the green, variegated and red hybrid, respectively. Although we do not know the mechanism of antibacterial activity of RA, we can wonder if it inhibits DNA synthesis and prevents T-DNA transport into the plant cells.

Our preliminary investigations show the feasibility of RA production in transgenic callus tissue of *Coleus blumei* (*17*).

After four years in culture, 16 different callus lines are maintained. They differ in colour, morphology, growth index and RA synthesis. After purification, characterisation and cloning of the key enzymes from RA biosynthetic pathway it would be possible, using overexpresion and antisense technology, to investigate regulation of RA biosynthetic pathway and its correlation with primary metabolism (*33*).

In conclusion, we have developed a rapid and efficient protocol for the genetic transformation of *Coleus blumei* using co-cultivation of leaf explants with *Agrobacterium*. The process is simple, and many explants can be inoculated in an experiment. The transformation rate was 90–100 %. Further investigations on the optimal conditions for RA synthesis in *Coleus* transgenic lines are currently in progress.

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Genetička transformacija ukrasne koprive, *Coleus blumei* Benth. s pomoću bakterija *Agrobacterium*

Sažetak

Tri genotipa ukrasne koprive (*Coleus blumei* Benth.) transformirana su s pomoću bakterija *Agrobacterium tumefaciens* i *A. rhizogenes*. Uspješnost transformacije ovisila je o sukultivacijskom postupku lisnih eksplantata i bakterija te o interakciji bakterijskoga soja i biljnoga genotipa. Soj divljeg tipa B6S3 bio je najvirulentniji. Najuspješniji je postupak transformacije bio petminutna inkubacija svježe narezanih lisnih eksplantata s bakterijama te nakon toga dvodnevna sukultivacija s tumorskim tkivom duhana (*Nicotiana tabacum*). Rast kalusnoga tkiva na hranidbenoj podlozi bez regulatora rasta, PCR analiza te hibridizacijska analiza potvrdili su genetičku transformaciju tkiva. Ustanovljeno je 16 transgeničnih kalusnih linija koje su se međusobno razlikovale po fenotipu i stopi rasta. Fenotipske značajke pojedine linije nisu se mogle povezati s bakterijskim sojem upotrijebljenim u poticanju transformiranih linija niti s biljnim genotipom.