BIOLOGIA PLANTARUM 55 (3): 414-422, 2011

Variability in shoot cultures regenerated from hairy roots of *Gentiana punctata*

B. VINTERHALTER[1], S. ZDRAVKOVIĆ-KORAĆ[1], S. NINKOVIĆ[1], N. MITIĆ[1], T. JANKOVIĆ[2], J. MILJUŠ-DJUKIĆ[3] and D. VINTERHALTER[1]*

Plant Physiology Department, Institute for Biological Research "S. Stanković", Belgrade University, Bulevard despota Stefana 142, 11000 Belgrade, Serbia[1]
Institute for Medicinal Plants Research "Josif Pančić", Tadeuša Košćuška 1, 11000 Belgrade, Serbia[2]
Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 444 a, 11000 Belgrade, Serbia[3]

Abstract

Differences among three clones of *Gentiana punctata* L. hairy root shoot regenerants were investigated in relation to their growth patterns, production of secondary metabolites and 2D protein profiles. Prominent differences in growth parameters were stable thus qualifying regenerant clones as true somaclones. Marked differences in protein spots were registered among the regenerant clones but not in comparison with the non-transformed control. Southern blot hybridization of regenerants showed the absence of *rolA, B* and *C* genes, initially present in the main hairy root lines. *Orf13* and *rolD* were present and *orf8* was missing in all three regenerant clones whereas *orf3* was missing only in clone 2. Although lacking the three major *rol* genes, plants of regenerant clones retained characteristics of the hairy root phenotype.

Additional key words: *Agrobacterium rhizogenes*, 2D-electrophoresis, *rol* genes, secondary metabolites, somaclonal variation.

Introduction

Gentiana punctata L. is a sub-alpine species of south-east Europe, known as an endangered medicinal plant. Its propagation *in vitro* was reported by Vinterhalter and Vinterhalter 1998, and *A. rhizogenes* (A4M70GUS) mediated transformation by Vinterhalter *et al.* 1999. Secondary metabolite production of excised and hairy root cultures of *G. punctata* was studied by Menković *et al.* 1998, 2000.

Agrobacterium rhizogenes-mediated transformation provides means for fast and efficient transformation of plants from various taxonomic groups (Birot *et al.* 1987, Tepfer 1990, Christey 1997, 2001). The presence and activity of bacterial genes in transformed plant tissues makes *A. rhizogenes*-mediated transformation a method unsuitable for improvement of horticultural species grown for use as food and feed. However, it is still recommended and used for transformation of ornamentals (Teixeira da Silva 2003, Mishiba *et al.* 2006), and species producing secondary metabolites (Sevón and Oksman-Caldentey 2002, Rosić *et al.* 2006, Tiwari *et al*. 2008, Bauer *et al.* 2009) including some important medicinal plants (Batra *et al.* 2004, Chaudhury *et al.* 2006, Tiwari *et al.* 2007, Gangopadhyay *et al*. 2008). It is also widely used to study functioning of plant genes and for developing new applications such as the use of disarmed *A. rhizogenes* strains (Veena and Taylor 2007).

A. rhizogenes-mediated transformation has been amply studied in diverse plant species. The position, nucleotide sequence and transcripts of genes within the T-DNA are well known (White *et al.* 1985, Slightom *et al.* 1986) but the functional characterization of the proteins which they code is still inadequate (Nillson and Ollson 1997, Veena and Taylor 2007).

The A4 strain of *A. rhizogenes* harbours agropine

Received 18 March 2010, *accepted* 9 June 2010.
Abbreviations: BA - 6-benzyladenine; GA_3 - gibberellic acid; HR - hairy root phenotype; IAA - indole-3-acetic acid; NAA - α-naphthaleneacetic acid; SRC - shoot regenerant clone; TDZ - thidiazuron; WPM - woody plant medium.
Acknowledgements: Research was funded by the Serbian Ministry of Education and Science, through the project No. 173015.
* Corresponding author, fax (+381) 11 2761 428, e-mail: dvinterhalter@yahoo.com

type plasmid with two distinct and well separated T-DNA regions,TL-DNA and TR-DNA (White *et al.* 1985). Both T-DNA regions are involved in hairy root formation, but improved rhizogenesis is attributed to the presence of TL-DNA located *rol* genes (White *et al.* 1985, David and Tempe 1988). In the early studies of the bacterial T-DNA structure, White *et al.* 1985 pointed out at four TL-DNA located genes naming them *rolA, B, C* and *D* as the major genes responsible for development of altered phenotype in plants transformed with *A. rhizogenes*. Phenotype attained by transformed plants (HR or Ri phenotype) is characterized by decreased shoot development (stunted plants with short internodes), production of malformed leaves (wrinkled, epinastic), improved rooting ability (higher rooting percentage and branching, roots often plagiotropic), reduced fertility and in some biannual species precocious flowering (Tepfer and Casee-Delbart 1987, Tepfer 1990, Christey 1997).

Although there are actually 18 open reading frames (ORF) present on the TL-DNA of agropine type strains (Slightom *et al.* 1986), phenotypic effects of *rol* gene expression in transgenic plants are dramatic. Each one of them was assigned a more or less independent function (for review see Nillson and Olsson 1997, Veena and Taylor 2007) which is nevertheless modified by the individual or combined presence of other *rol* and *orf* genes. Unfortunately, their roles seem to differ in certain species depending mostly on the hormonal balance required for regeneration. From the studies of Levesque *et al.* 1988 it is known that TL-DNA bacterial genes are related and redundant, indicating common origin.

A number of early reports like those of Spena *et al.* 1987, Spano *et al.* 1988 showed that the presence and expression of *rolA, B* and *C* is sufficient for plants to exhibit the hairy root phenotype. Therefore, the role and importance of *rolA, B* and *C* was overrated while *rolD* was far less investigated as noted by Mauro *et al.* 1996. Other *orf* genes were also largely neglected. Lemke and Schmülling 1998 re-examined the individual effects of non-*rol* genes in tobacco using leaf transformation tests and showed that strong morphogenetic effects can be attributed to *orf3, 8* and *13*.

The first report of *A. rhizogenes* (A4M70GUS) mediated transformation of *G. punctata* (Vinterhalter *et al.* 1999) provided a transformation protocol, GUS histochemical assay and PCR amplification of the *uidA* fragments. Spontaneous shoot regeneration in hairy root cultures was observed only twice over a long cultivation period (1992 - 2007) and from each of the three regeneration spots a separate shoot regenerant clone (SRC clones, here designated as SRC 1 - 3) was established. SRC clones display the hairy root phenotype, stunted growth, short internodes, decreased shoot multiplication *via* axillary bud formation and increased rooting ability. Apparent differences among certain SRC clones were stable, appearing in each subculture. We therefore considered them to be true somaclonal variants and aimed our study at factors imposing the registered differences. Apart from the comparison of standard growth parameters and secondary metabolite production, attempts were made to further characterize SRC clones by using proteomic analysis. 2D-electrophoresis offered a valuable insight into their active protein patterns. Southern hybridization showed absence of the three important *rol* genes *A*, *B* and *C* in all three SRC clones. Further PCR testing enabled us to obtain accurate picture of TL-DNA genes of SRC clones, which is presented and discussed in details.

Materials and methods

Initial shoot cultures of *Gentiana punctata* L. were established from epicotyls of aseptically germinated seeds collected at Šar-Planina Mt. (2300 m). Shoot cultures derived from its A4M70GUS hairy root regenerants (Fig. 2) were maintained on a modified Woody plant medium (WPM; Lloyd and McCown 1980) supplemented with 0.25 mg dm^{-3} 6-benzyladenine (BA) and 0.2 mg dm^{-3} gibberellic acid (GA_3) as previously described (Vinterhalter *et al.* 1999). Medium pH was adjusted to 5.8 prior to autoclaving lasting 20 min at 114 °C. Conditions of the growth room were: temperature 25 ± 2 °C, 16-h photo-period and irradiance 33 - 45 μmol m^{-2} s^{-1} produced by cool white *Philips TLD* 58W/54 neon lamps and measured with *Li Cor 190SA* quantum sensor coupled with *Li-1000* datalogger (Lincoln, NE, USA).

For every clone 24 - 30 shoot or root explants were replicated at least three times. Significant differences between means of each individual treatment were determined using Fisher's least significant difference (LSD) test at $P < 0.05$.

Air-dried samples from shoot and root tissue were extracted with methanol for 30 min in a water bath and filtrated. Ratio between drug and solvent was 1:10. TLC and HPLC analyses of methanol extracts were performed according to Menković *et al.* 2000.

For protein isolation 150 mg of shoot tissue was ground to powder in liquid NO_2, dissolved in extraction buffer (285 mM Tris, 1 % *Triton X 100*, 20 % glycerol) and centrifuged for 20 min at 13 000 *g* (4 °C). Supernatant was used for protein separation. Total protein concentration was measured using Bradford kit (*Bio-Rad*, Hercules, CA, USA,) according to the manufacturer's instructions.

For the first dimension protein separation, the *Immobiline* dry strip gels (11 cm) with a pH gradient (pH 3 - 10) were loaded on *Protean* isoelectric focusing

(IEF) cell (*Bio-Rad*). About 50 μg of protein was mixed with rehydration buffer (7 M urea, 2 M thiourea, 2 % *CHAPS*, 40 mM dithiotreitol, DTT, 0.2 % ampholyte) to give a total volume of 0.35 cm^3. The following IEF cell running program was used: 250 V 15 min, 8000 V 2 h, 500 V 30 min hold.

For the second dimension, strips were first equilibrated for 15 min in sodiumdodecyl sulphate (SDS) buffer (6 M urea, 30 % glycerol, 2 % SDS, 0.05 M Tris, pH 8.8, 15 mM DTT, bromphenol blue, BPB), then mounted on 12 % SDS-PAGE gels. Electrophoresis was carried out on vertical gel slab units (*Hoefer Scientific Instruments*, Holliston, MA, USA) at 25 mA, 150 V, until the bromophenol blue front reached the bottom of the gel. Staining was done with *SilverXpress* silver staining kit (*Invitrogen*, San Diego, CA, USA).

Polymerase chain reaction: Genomic DNA was extracted from both non-transformed and A4M70GUS transformed gentian roots following the procedure of Murray and Thompson (1980) and subjected, together with a positive control (pRiA4M70GUS), to PCR amplification specific to the *rolA, B, C*, *uid A* and *virD1*. PCR was performed in a 0.03 cm^3 reaction mixture containing 250 ng dm^{-3} DNA, 200 μM dNTP, 200 nM primers, 2.5 U *TrueStart* Taq DNA polymerase (*Fermentas*, Vilnius, Lithuania). Genomic DNA extracted from the three SRC clones was also PCR analyzed for the presence of the *rolD*, *orf3*, *orf8*, *orf13* and *virD1* (Fig. 1). All primer sequences (Table 1) were purchased from *Metabion* (Martinsried, Germany).

Table 1. Primer sequence and size of amplified products (A.p.).

	Primer sequence 5'→3'	A.p. [bp]
ORF 3	ATCATGCCCGCCAACGACGCGAC	1353
	ACTAGTTAAGACAAAACCCAAA	
ORF 8	GTGATGGATCTTCCATATCAGCG	2289
	CTATTTTTTATTCCAGTCCACCG	
ORF 10	GTTAGGCGTGCAAAGGCCAAG	203
(*rol A)*	GCGTATTAATCCCGTAGGTC	
ORF 11	AAAGTCTGCTATCATCCTCCTATG	348
(*rol B*)	AAAGAAGGTGCAAGCTACCTCTCT	
rol AB	ATGGAATTAGCCGGACTAAACG	1541
	ATGGATCCCAAATTGCTATTCC	
ORF 12	TACGTCGACTGCCCGACGATGATG	342
(*rol C*)	AAACTTGCACTCGCCATGCCTCAC	
ORF 13	TGAATGGCTCGTTATTGCAGTGG	606
	CTATTCCAGCAGCAGCACCTTGC	
ORF 15	CCTTACGAATTCTCTTAGCGGCACC	477
(*rol D*)	GAGGTACACTGGACTGAATCTGCAC	
uid A	CCTGTAGAAACCCCAACCCGTG	366
	CCCGGCAATAACATACGGCGTG	
vir D1	ATGTCGCAAGGCAGTAAG	441
	CAAGGAGTCTTTCAGCATG	

The amplification protocol for all genes was: initial 5 min denaturation at 95 °C, followed by 36 cycles of a 1 min denaturation at 94 °C, a 1 min annealing at 61 °C, a 1 min elongation at 72 °C (2 min for *ORF3* and *ORF8*), and final extension at 72 °C for 5 min. The amplified fragments were electrophoretically separated on 1.5 % agarose gel (*Sigma*, St. Louis, Missouri) and scanned by *Gel-Doc™ EQ* system (*Life Science Research*, *Bio-Rad*).

Genomic DNA of three SRC clones was analyzed by Southern hybridization to check for the presence of the *rolA, B, C* and *uidA* genes. DNA was extracted using the method of Murray and Thompson (1980). The horse chestnut hairy root line No 36, transformed with the same construct, was used as a positive control (Zdravković-Korać *et al.* 2004). 40 μg of DNA per clone was digested with *Bam*HI endonuclease (*Fermentas*), separated electrophoretically on 0.8 % agarose gel (*Sigma*, St. Louis, MI, USA) and blotted onto a positively charged nylon membrane (*Roche*, Mannheim, Germany) by capillary transfer for 24 h. The membrane was first probed with *rolAB* probe (1541 bp), and then stripped and reprobed with both *rolC* (342 bp) and *uidA* (366 bp) probes in separate hybridizations. The probes were labeled with digoxigenin *DIG-dUTP* (*Roche*) by PCR, using the pRiA4M70GUS DNA as a template. The *rolC* and *uidA* probes were amplified under conditions specified in the previous section, and the *rolAB* was amplified under conditions described by Tiwari *et al.* (2007) using DIG-dUTP : dTTP (1:9) in the reaction mixture. The hybridizations were performed in *DIG Easy Hyb* buffer (*Roche*) at 42 °C (for the *rolAB*) and 45 °C (for the *rolC* and *uidA*) for 16 h. The membrane was then washed 2 × 5 min with the following buffers: *1*) 2× SSC + 0.1 % SDS and *2*) 1× SSC + 0.1 % SDS at a hybridization temperature and *3*) 0.5× SSC + 0.1 % SDS and *4*) 0.1× SSC + 0.1 % SDS at 65 °C for the *rolAB* and 68 °C for the *rolC* and *uidA*. The hybrids were detected with anti-digoxigenin antibody (*Roche*), visualized with chemiluminescent substrate *CDP-Star* (*Roche*) and recorded with X-ray film (*Kodak*, Paris, France).

Results

Agrobacterium rhizogenes A4M70GUS transformed hairy root clone of *Gentiana punctata* established in 1992 was cultured until 2007. Over this time period only three shoots appearing spontaneously were recovered and maintained as regenerant clones. Attempts to promote shoot regeneration by cytokinin supplementation were not successful indicating spontaneous shoot regeneration in *G. punctata* to be a low probability event.

Each of the three regenerant clones manifested stable growth parameters and phenotype characteristics in many

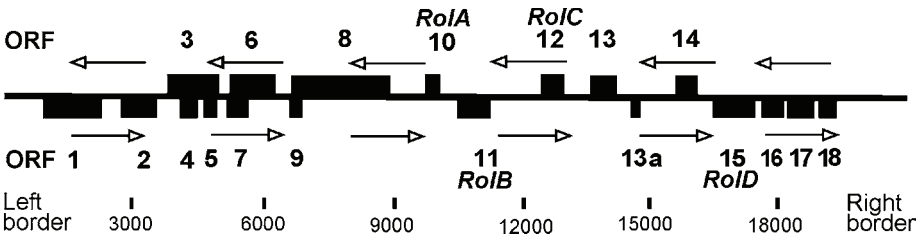

Fig. 1 TL-DNA sequence of *A. rhizogenes* agropine type plasmids (after Slightom *et al.* 1986 and Lemke and Schmülling 1998).

Fig. 2. Shoots of SRC 1 - 3 (I, II and III) on medium with 0.25 mg dm^{-3} BA and 0.2 mg dm^{-3} GA_3 and control non-transformed shoot culture (C) on medium with 0.25 mg dm^{-3} BA and 0.1 mg dm^{-3} IAA.

subsequent subcultures. Their common phenotype characteristics were stunted growth, short internodes, large leaves and poor shoot multiplication, requiring addition of BA and GA_3 to the maintenance medium. However, the BA/GA_3 supplementation could only partly compensate for their poor growth characteristics since the control, non-transformed plants were superior in all aspects of shoot development.

Hairy root origin of the SRC clones is evident from their increased rooting ability on the maintenance medium containing of 0.25 mg dm^{-3} BA on which the non-transformed plants are unable to produce roots. SRC 1 was prone to fasciation which when combined with short internode lengths further decreases shoot multiplication. SRC 2 manifested high rooting ability (rooting percentage) and root elongation while SRC 3 had higher shoot multiplication than the other two clones. However, shoot multiplication of all three regenerant clones was always much lower than in non-transformed control plants. The excised root cultures of SRC clones 1 - 3 were established on several occasions but they could not be maintained for more than a year.

Gentiopicrine, the main secondary metabolite of *G. punctata* was found to accumulate in vegetative tissues of all SRC clones but its concentrations was lower than in non-transformed shoots. Gentopicrine content in SRC 3 was more than two times higher than in SRC 1 and 2 but still reaching only 81.6 % of the gentiopicrine found in the control. SRC clones apparently cannot compete with hairy root clones as a source for production of gentiopicrine. Swertiamarine, another less abundant secondary metabolite was present in traces in shoot tissues of SRC regenerant clones. In plants from nature gentiopicrine is the dominant metabolite in roots while swertiamarine is dominant in shoots. Swertiamarine was absent in tissues of excised root and hairy root cultures but it appeared in shoot tissue of SRC clones (Menković *et al.* 1998, 2000).

Protein separation by 2D-electrophoresis of SRC 1 - 3 samples resolved more than 100 proteins over a pI range 3 - 10 (Fig. 3). Multiple 2D-E gels were acquired, and the best gels were selected to serve as the reference maps. Visual comparison of the 2D-E gels revealed that most of the protein spots were shared, *i.e.* present in all three SRC clones but at least 10 spots were truly specific. Some of the spots, common to all extracts, expressed abundance differences (spots No. 11 - 14; Fig. 3).

A single protein spot shared by all three SRC clones but missing in control can be considered as GUS protein, expected to be expressed from the bacterial TL-DNA integrated in the plant genome. It is a protein of Mr 76 kDa, and pI about 9. It is expressed quite well in SRC 1 and 2, but very weakly in SRC 3. No other spots shared by the SRC clones but absent from the control were registered. The first two spots (No. 1 and 2; pI 6.5 and 6.8 and Mr 40 and 35, respectively), are proteins unique to non-transformed shoots, but missing in SRC clones. The SRC 1 is lacking six protein spots present in the control, SRC 2 and 3 (No. 4, 6, 7, 8, 9 and 10). These proteins are most abundant in clone 2, except for proteins 4, 9 and 10. As SRC clone 1 regenerates poorly, these missing proteins might be involved in organogenesis

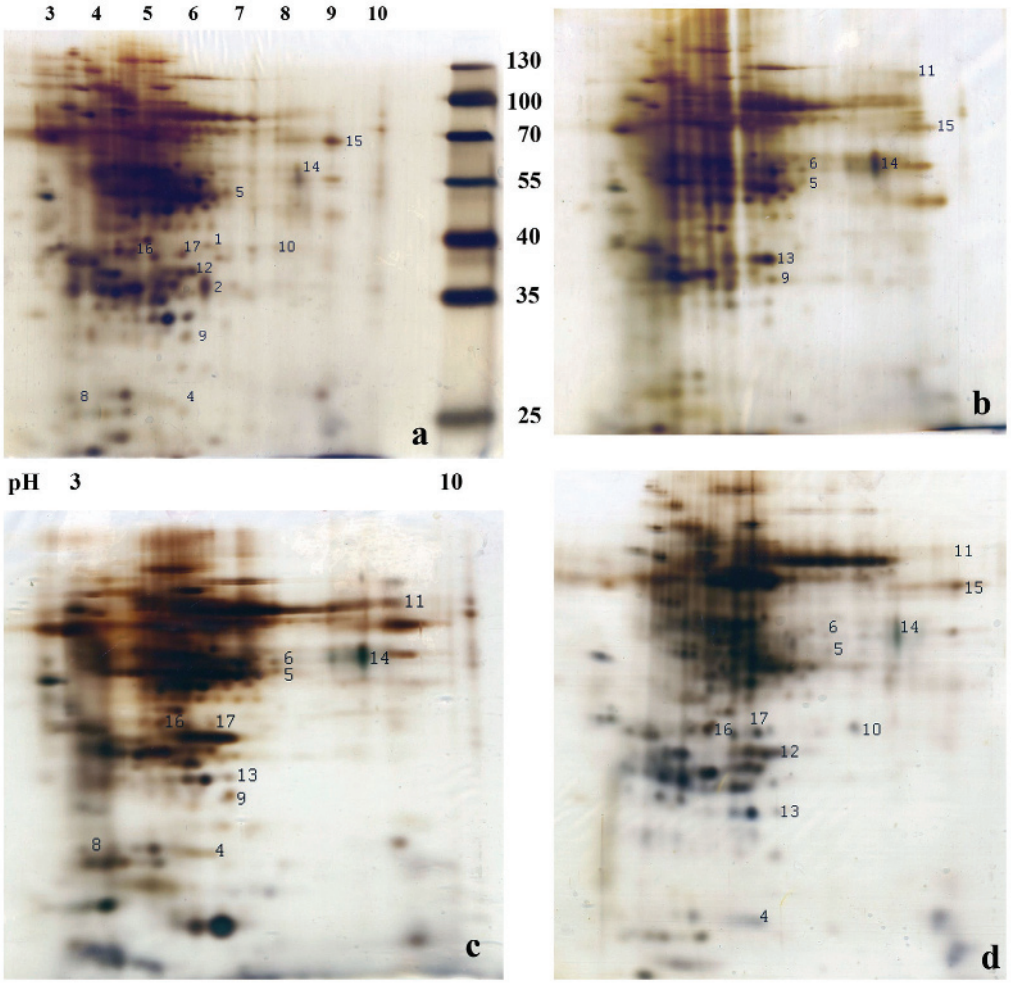

Fig. 3. 2D-protein electrophoresis of SRC clones and untransformed control shoot tissues: *a* - SRC 1, *b* - SRC 2, *c* - SRC 3, *d* - control.

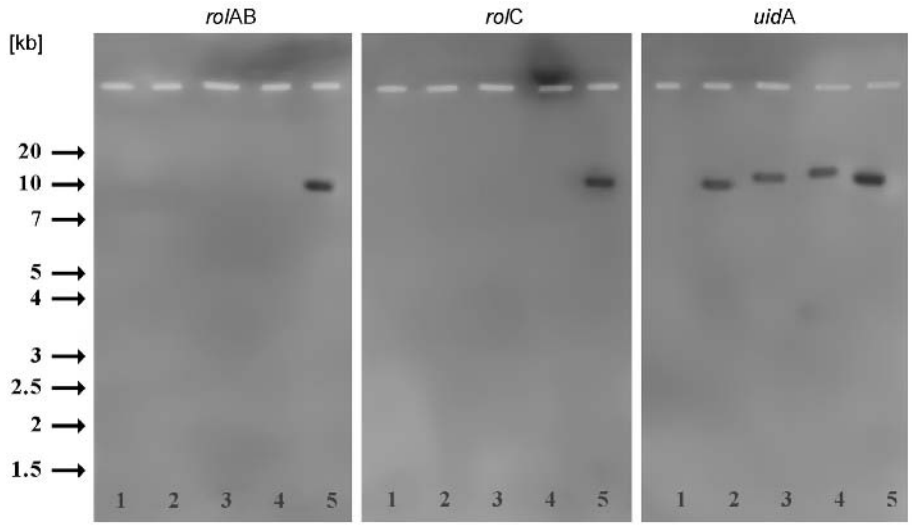

Fig. 4. Southern analysis of *Gentiana punctata* genomic DNA digested with *Bam*HI and hybridized to the probe *rolAB*, *rolC* and *uidA*. *Lane* 1 - non-transformed shoots, *lanes* 2, 3, 4 - transformed shoots SRC 1 - 3, *lane* 5 - positive control, *Aesculus hypocastanum* hairy root clone No. 36.

bearing in mind that SRC 2 has a well developed root system. SRC 2 also shares one spot with the control, which is missing in SRC 1 and 3 (No. 6, Mr 27, pI 3.3). Furthermore, also protein No. 4 (spot No. 10), abundant in SRC 3, is lacking in SRC 2. This protein missing also in SRC 1, may have a role in organogenesis, since both SRC 1 and 2 exhibit poor shoot multiplication.

It is noteworthy that SRC 3 is the clone most closely resembling the phenotype of control, untransformed plants. Besides the above mentioned protein differences, it shares one more common spot (No. 10) with the control while spots No. 8 and 9 are missing. Spots numbered 5, 6, 13, 14 and 15 are common to all clones but with the different abundance.

The single protein spot found in all three SRC clones but missing in the non-transformed control, was somewhat disappointing since we expected to detect some more differences imposed by the presence of *rol* and other bacterial genes. We therefore performed the Southern hybridization of SRC 1 - 3 samples compared to non-transformed plants as a control. Since the samples of the original main hairy root clones were not available we used the A4M70GUS-transformed horse chestnut hairy root clone No 36 as a positive control (Zdravković-Korać *et al.* 2004).

Table 2. Presence and activity of TL-DNA genes in hairy roots and SRC 1 - 3. Testing was done using Southern blotting (s) and PCR amplification (p); nt - not tested

Genes	Hairy roots	SRC 1	SRC 2	SRC 3	Control plants	Testing method
orf3	nt	+	-	+	-	p
orf8	nt	-	-	-	-	p
orf10 (*rolA*)	+	-	-	-	-	p, s
orf11 (*rolB*)	+	-	-	-	-	p, s
orf12 (*rolC*)	+	-	-	-	-	p, s
orf13	+	+	+	+	-	p
orf15 (*rolD*)	nt	+	+	+	-	p
virD	-	-	-	-	-	p
uidA	+	+	+	+	-	p, s

Following the hybridization with *rolAB* probe, genomic DNA of the three SRC clones produced no signal (Fig. 4). As the *rolAB* probe covers the entire coding sequence of both *rolA* and *rolB*, this finding indicates the absence of *rolA* and *rolB* from the genomes of SRC shoot cultures. In the positive control, a signal of approximately 10 kb was produced, indicating the presence of a 8a BamHI fragment of full size 9.8 kb, according to the nomenclature of Jouanin (1984). The same membrane re-probed with *rolC* showed this gene was absent from all three SRC clones (Fig. 4). In the positive control, two fragments of predicted sizes (10 and 1.5 kb) were present (the 8a and 30a BamHI fragments). Finally, following hybridization with *uidA* probe all SRC clones produced a signal of variable length of approximately 9 - 10 kb, thus confirming the presence of GUS gene sequence (Fig. 4).

This analysis actually revealed that some of the bacterial genes supposed to be present in the TL-DNA of A4M70GUS were absent from tissues of SRC 1 - 3 lines. In our previous studies the presence of *rol* genes was taken for granted and it was not analyzed in the original hairy root clone. In the absence of fresh material the analysis of stored samples was performed.

Genomic DNA of original gentian hairy roots analyzed by PCR revealed the presence of genes *rolA, B* and *C* and *uidA* (Fig. 5 *A-D*). PCR specific for the *virD1* sequence of *A. rhizogenes* resulted in no amplification,

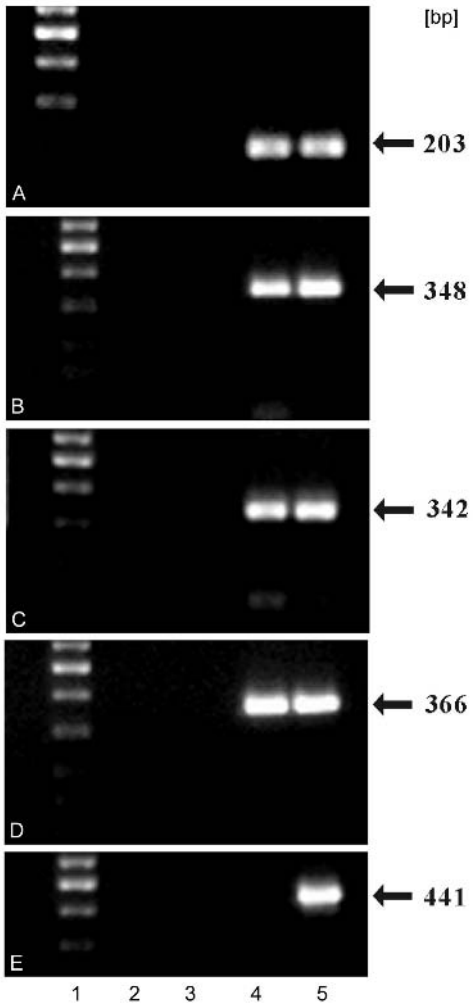

Fig. 5. PCR analysis of genomic DNA isolated from *Gentiana punctata* main hairy root clone: *A - rolA, B - rolB, C - rolC, D - uidA,* ***E -*** *virD1*. *Lane* 1 - molecular size marker, *lane* 2 - blank control, *lane* 3 - non-transformed roots, *lane* 4 - transformed roots, *lane* 5 - positive control (pRiA4M70GUS).

confirming the absence of residual agrobacteria in the sample (prolonged bacterial contamination). This analysis confirmed that the *rolA, B* and *C* genes were present in the original main hairy root clone from which SRC clones regenerated. SRC clones were actually obtained a number of years before hairy root samples were collected for phytochemical analyses.

To clarify the presence of TL-DNA genes SRC clones were additionally tested for the presence of other bacterial genes known to induce morphogenetic effects. The analysis showed that apart from *rolA, B* and *C* all SRC clones are also missing *orf8*, while *orf*13 and *orf*15 (*rolD)* are present (Table 2). Additionally *orf*3 is missing in clone SRC 2. It is apparent that a large group of TL-DNA genes with prominent morphogenetic effects is missing in SRC clones.

Discussion

Expression of HR phenotype in many *A. rhizogenes* transformed plant species can be highly variable and diverse as demonstrated in studies of *Brassica oleracea* and *B. rapa* (Christey *et al.* 1999). There is a number of variability-generating mechanisms active at certain sub-cellular genome related positions. First, the natural *Agrobacterium*-mediated insertion of DNA is of mutagenic nature resulting in null, loss of function, gain of function and other possible phenotypes depending on the region targeted by the insert and discrete element structure within the T-DNA like promoters (Filipecki and Malepszy 2006). Introgression of bacterial T-DNA in the genome of host plants although highly efficient generates DNA alterations including base substitutions, insertions, deletions and duplications at the insertion site (Nacry *et al.* 1998). In *Arabidopsis* they demonstrated chromosomal changes and rearrangements, reciprocal translocations and small inversions and deletions. Similarly, Tax and Vernon (2001) showed that trans-formants may contain unexpectedly high frequency of rearrangements consisting of duplication/rearrangement events. In plants regenerated from agropine type pRi plasmids TL and TR-DNA can be either linked or independent or present as inverted tandems (Jouanin *et al.* 1989). Rearrangements, which they observed at the insertion site, resulted in the appearance of a new Eco RI site on the right of the TR-DNA/plant DNA junction. Rearrangements at the insertion sites were also observed by Gheysen *et al.* 1987.

The changes in the expression of T-DNA genes integrated in the plant genome have been well documented specially for marker genes. The variability in the expression of the GUS histochemical marker can be easily demonstrated in various tissues of the same plant. In some of the introgressed genes, expression can stop after some time. This phenomenon, gene silencing, attributed to the existence of multiple copies of the same gene has been demonstrated also in genus *Gentiana* (Mishiba *et al.* 2005). Also genes that were silent for some time renewed their expression later (Guivarc'h *et al.* 1999).

However, there are only few reports showing the absence of *rol* genes in regenerants originating from *rol* gene-containing hairy roots. Sevón *et al.* 1997 showed that three out of six hairy root regenerated clones of *Hyosciamus muticus* contain plants in which PCR analysis could not confirm presence of *rolA*, *B* and *C* genes. Similarly, in hairy root regenerants of silver birch, Piispanen *et al.* (2003) described a large group of regenerant plants with no *aux* or *rol* genes. Finally among 250 shoot regenerants obtained in a study of *A. rhizogenes*-mediated transformation of *Catharanthus roseus*, Batra *et al.* (2004) found two slow growing clones in which *rolA* and *B* were absent. These reports are similar to our findings that the SRC clones of *G. punctata* contain no *rolA, B* and *C* genes, originally present in the hairy roots.

Based primarily on the results of Southern blotting we may assume that the omission of bacterial genes from SRC clones results from a single or repeated deletions in the TL-DNA region. On the right side of the TL-DNA fragment situation is clear with the DNA rupture occurring between *orf12* and *orf13*. On the left side, *orf3* is present in SRC 1 and SRC 3 limiting this deletion to include *orf6* to *orf12* and genes in between or in the worst case to include even *orf4* and *orf5* which were not analyzed in this study. In the SRC 2, *orf3* is missing also increasing the length of the deleted TL-DNA segment.

Today we can not determine the exact time point at which the group of *rol* and *orf* genes slipped out of the genome of SRC regenerants but we can assume that it was a crucial moment for the successful shoot regeneration. This assumption is based mainly on the extremely low frequency of shoot regeneration in *G. punctata* hairy root cultures. It is also possible that the *rol* and *orf* genes omission is a much more frequent (widespread) event. Since the resulting cells and their lineage are not competent for growth as the cells containing the full complement of TL-DNA genes, they are likely to perish in the auxin-free medium of hairy root cultures.

Among the five south-European *Gentiana* species included in the *A. rhizogenes* mediated transformation studies only *G. cruciata* regenerated shoots spontaneously and *G. purpure*a upon cytokinin supplementation (Momčilović *et al.* 1997). Other three species G. *lutea, G, acaulis* and *G. punctata* do not regenerate shoots on regular basis (Momčilović *et al.* 2001, Vinterhalter *et al.* 1999). In *G. scabra* var. *buergeri* and *G. triflora* × *G. scabra* (cv. Polarno white) shoot

regeneration from hairy root cultures can be induced by cytokinin supplementation (Suginuma and Akihara 1995, Hosokawa *et al.* 1997). In hairy root cultures of *G. macrophylla* shoot regeneration was not mentioned (Tiwari *et al.* 2007).

Survey of the shoot regeneration ability of *A. rhizogenes* A4M70GUS hairy root clones shows fast and frequent spontaneous shoot regeneration in many species including *Lotus corniculaturs* (Nikolić *et al.* 2003/4), *Aesculus hippocastanum* (Zdravković-Korać *et al.* 2004), *Hypericum perforatum* (Vinterhalter *et al.* 2006), *Brassica oleracea* var. *sabauda* and *capitata* (Sretenović-Rajičić *et al.* 2006), *Centaurium erithrea* (Subotić *et al.* 2003) and *Alyssum murale* (Vinterhalter *et al.* 2008). Some species like *Chenopodium rubrum* although generaly recalcitrant to transformation produced hairy roots upon inoculation with A4M70GUS (Dmitrović *et al*. 2010).

Our results showed the existence of sufficient proteomic differences between the three regenerant clones and the control that could be expected as the background of the manifested phenotype differences.

References

Batra, J., Dutta, A., Singh, D., Kumar, S., Sen, J.: Growth and terpenoid indole alkaloid production in *Catharanthus roseus* hairy root clones in relation to left- and right-termini-linked Ri T-DNA gene integration. - Plant Cell Rep. **23**: 148-154**,** 2004.

Bauer, N., Kiseljak, D., Jelaska, S.: The effect of yeast extract and methyl jasmonate on rosmarinic acid accumulation in *Colleus blumei* hairy roots. - Biol. Plant. **53**: 650-656, 2009.

Birot, A.-M., Bouchez, D., Casse-Delbart, F., Durand-Tardif, M., Jouanin, L., Pautot, V., Robaglia, C., Tepfer, D., Tepfer, M., Tourneur, J., Vilaine, F.: Studies and uses of the rRi plasmids of *Agrobacterium rhizogenes*. - Plant Physiol. Biochem. **25**: 323-335, 1987.

Chaudhury, K.N., Ghosh, B., Tepfer, D., Jha, S.: Spontaneous plant regeneration in transformed roots and calli from *Tylophora indica*: changes in morphological phenotype and tylophorine accumulation associated with transformation by *Agrobacterium rhizogenes*. - Plant Cell Rep. **25**: 1059-1066, 2006.

Christey, M.C.: Transgenic crop plants using *Agrobacterium rhizogenes* mediated transformation. - In: Doran, P.M. (ed.): Hairy Roots: Culture and Applications. Pp. 99-111. Harwood Academic Publishers, Amsterdam 1997.

Christey, M.C.: Use of Ri mediated transformation for production of transgenic plants. - *In Vitro* cell. dev. Biol. Plant. **36**: 687- 700, 2001.

Christey, M.C., Braun, R.H., Reader, J.K.: Field performance of transgenic vegetable Brassicas (*Brassica oleracea* and *B. rapa*) transformed with *Agrobacterium rhizogenes*. - Sabrao J. Breed. Genet. **31**: 93-108, 1999.

David, C., Tempe, J.: Genetic transformation of cauliflower (*Brassica oleracea* L. var. *botrytis*) by *Agrobacterium rhizogenes*. - Plant Cell Rep. **7**: 88-91, 1988.

Dmitrović, S., Mitić, N., Zdravković-Korać, S., Vinterhalter, B., Ninković, S., Ćulafić Lj.: Hairy roots formation in recalcitrant-to-transform plant *Chenopodium rubrum*. - Biol. Plant. **54**: 566-570, 2010.

Filipecki, M., Malepszy, S.: Unintended consequences of plant transformation: a molecular insight. - J. appl. Genet. **47**: 277-286, 2006.

Gangopadhyay, M., Sircar, D., Mitra, A., Bhattacharya, S.: Hairy root culture of *Plumbago indica* as a potential source for plumbagin. - Biol. Plant. **52**: 533-537, 2008.

Gheysen, G., Dhaese, P., Van Montagu, M., Zambrzsky, P.: Integration of *Agrobacterium tumefaciens* transfer DNA (T-DNA) involves rearangements of target plant DNA sequences. - Proc. nat. Acad. Sci. USA **84**: 6169-6173, 1987.

Guivarc'h, A., Boccara, M., Prouteau, M., Chriqui, D.: Instability of phenotype and gene expression in long term culture of carrot hairy root clones. - Plant Cell Rep. **19**: 43-50, 1999.

Hosokawa, K., Matsuki, R., Oikawa, Y., Yamamura, S.: Genetic transformation using wild-type *Agrobacterium rhizogenes*. - Plant Cell Tissue Organ Cult. **51**: 137-140, 1997.

Jouanin, L.: Restriction map of an agropine-type Ri plasmid and its homologies with Ti plasmid. - Plasmid **12**: 92-102, 1984.

Jouanin, L., Bouchez, D., Drong, R.F., Tepfer, D., Slightom, J.L.: Analysis of TR-DNA / plant junctions in the genome of *Concolvulus arvensis* clone transformed with *Agrobacterium rhizogenes* strain A4. - Plant mol. Biol. **12**: 75-85, 1989.

Lemke, K., Schmülling, T.: Gain of function assays identify non-*rol* genes from *Agrobacterium rhizogenes* TL-DNA that alter plant morphogenesis or hormone sensitivity. - Plant J. **15**: 423-433, 1998.

Levesque, H., Delepelaire, P., Rouzé, P., Slightom, J., Tepfer, D.: Common evolution origin of the central portions of the Ri TL-DNA of *Agrobacterium rhizogenes* and Ti T-DNAs *Agrobacterium tumefaciens.* - Plant mol. Biol. **11**: 731-744, 1988.

Lloyd, G.B., McCown, B.H.: Commercially feasible micro-propagation of mountain laurel - *Kalmia latifolia* by use of shoot-tip culture. - Proc. int. Plant Propag. Soc. **30**: 421-427, 1980.

Mauro, M.L., Trovato, M., De Paolis, A., Gallelli, A., Costantino, P., Altamura, M.M.: The plant oncogene *rolD* stimulates flowering in transgenic tobacco plants. - Dev. Biol. **180**: 693-700, 1996.

Menković, N.**,** Šavikin-Fodulović, K., Vinterhalter, B., Vinterhalter, D., Grubišić, D.: Secoiridoid content of naturally grown and *in vitro* cultured *Gentiana punctata*. - Pharm. Pharmacol. Lett. **8**: 110-111, 1998.

Menković, N., Šavikin-Fodulović, K., Vinterhalter, B., Vinterhalter, D., Janković, T., Krstić, D.: Secoiridoid content in hairy roots of *Gentiana punctata*. - Pharm. Pharmacol. Lett. **10**: 73-75, 2000.

Mishiba, K.-I., Nishihara, M., Abe, Y., Nakatsuka. T., Kawamura, H., Kodama, K., Takesawa, T., Abe, J., Yamamura, S.: Production of dwarf potted gentian using wild-type *Agrobacterium rhizogenes*. - Plant Biotechnol.

23: 33-38, 2006.

Mishiba, K.-I., Nishihara, M., Nakatsuka, T., Abe, Y., Hirano, H., Yokoi, T., Kikuchi, A., Yamamura, S.: Consistent transcriptional silencing of 35S' driven transgenes in gentian. - Plant J. **44**: 541-556, 2005.

Momčilović, I., Grubišić, D., Kojić, M., Nešković, M.: *Agrobacterium rhizogenes*-mediated transformation and plant regeneration of four *Gentiana* species. - Plant Cell Tissue Organ Cult. **50**: 1-6, 1997.

Momčilović, I., Grubišić, D., Nešković, M.: Transgenic Gentiana species (Gentian). - In: Bajaj, Y.P.S. (ed.): Biotechnology in Agriculture and Forestry. Vol. 48. Transgenic crops III. Pp. 123-138. Springer-Verlag, Berlin - Heidelberg - New York 2001.

Murray, M.G., Thompson, W.F.: Rapid isolation of high molecular weight plant DNA. - Nucl. Acids Res. **8**: 4321-4325, 1980.

Nacry, P., Camilleri, C., Courtial, B., Caboche, M., Bouchey, D.: Major chromosomal rearangements induced by T-DNA transformation in *Arabidopsis*. - Genetics **149**: 641-650, 1998.

Nillson, O., Olsson, O.: Getting to the root: The role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. - Physiol. Plant. **100**: 463-473, 1997.

Nikolić, R., Mitić, N., Ninković, S., Miljuš-Đukić, J., Nešković, M.: Efficient genetic transformation of *Lotus corniculatus* L. and growth of transformed plants in field. - Biol. Plant. **47**: 137-140, 2003/4.

Piispanen, R., Aronen, T., Chen, X., Saranpää, P., Häaggman, H.: Silver birch (*Betula pendula*) plants with *aux* and *rol* genes show consistent changes in morphology, xylem structure and chemistry. - Tree Physiol. **23**: 721-733, 2003.

Rosić, N., Momčilović, I., Kovačević, N., Grubišić D.: Genetic transformation of *Rhamnus fallax* and hairy roots as a source of anthraquinones. - Biol. Plant. **50**: 514-518, 2006.

Sevón, N., Dräger, B., Hiltunen, R., Oksman-Caldentey, K.M.: Characterization of transgenic plants derived from hairy roots of *Hyosciamus muticus.* - Plant Cell Rep. **16**: 605-611, 1997.

Sevón, N., Oksman-Caldentey, K.M.: *Agrobacterium rhizogenes*-mediated transformation: root cultures as a source of alkaloids. - Planta med. **68**: 859-868, 2002.

Slightom, J.L., Durand-Tardif, M., Jouanin, L., Tepfer, D.: Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. - J. biol. Chem. **261**: 108-121, 1986.

Spano, L., Mariotti, D., Cardarelli, M., Branca, C., Constantino, P.: Morphologenesis and auxin sensitivity of transgenic tobacco with different complements of Ri T-DNA. - Plant Physiol. **87**: 479-483, 1988.

Spena, A., Schmulling, T., Koncz, C., Schell, J.S.: Independent and synergistic activity of *rolA, B* and *C* loci in stimulating abnormal growth in plants. - EMBO J. **6**: 3891-3899, 1987.

Sretenović-Rajičić, T., Ninković, S., Miljuš-Đukić, J., Vinterhalter, B., Vinterhalter, D.: *Agrobacterium rhizogenes*-mediated transformation of *Brassica oleracea* var. *sabauda* and *B. oleracea* var. *capitata*. - Biol. Plant. **50**: 525-530, 2006.

Subotić, A., Budimir, S., Grubišić, D., Momčilović, I.: Direct regeneration of shoots from hairy root cultures of *Centaurium erythraea* inoculated with *Agrobacterium rhizogenes*. - Biol. Plant. **47**: 617-619, 2003.

Suginuma, C., Akihama, T.: Transformation of Gentian with *Agrobacterium rhizogenes.* - Acta Hort. **392**: 153-160, 1995.

Tax, F.E., Vernon, D.M.: T-DNA-associated duplication/ translocation in *Arabidopsis*. Implications for mutant analysis and functional genomics. - Plant Physiol. **126**: 1527-1538, 2001.

Teixeira da Silva, J.A.: *Chrysanthemum*: advances in tissue culture, cryopreservation, postharvest technology, genetics and transgenic biotechnology. - Biotechnol. Adv. **21**:715-766, 2003.

Tepfer, D.: Genetic transformation using *Agrobacterium rhizogenes*. - Physiol. Plant. **79**: 140-146, 1990.

Tepfer, M., Casse-Delbart, F.: *Agrobacterium rhizogenes* as a vector for transforming higher plants. -Microbiol. Sci. **4**: 24-28, 1987.

Tiwari, R.K., Trivedi, M., Guang, Z.-C., Guo, G.-Q., Yang, G.-C.: Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes*: growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. - Plant Cell Rep. **26**: 199-210 2007.

Tiwari, R.K., Trivedi, M., Guang, Z.-C., Guo, G.-Q., Zheng, G-C.: *Agrobacterium rhizogenes* mediated transformation of *Scutellaria baicalensis* and production of flavonoids in hairy roots. - Biol. Plant. **52**: 26-35, 2008.

Veena, V., Taylor, G.C.: *Agrobacterium rhizogenes*: recent developments and promissing applications. - *In Vitro* cell. Dev. Biol. Plant **43**: 383-403, 2007.

Vinterhalter, B., Momčilović, I., Vinterhalter, D.: High biomass producing root cultures of *Gentiana punctata* L. transformed with *Agrobacterium tumefaciens* C58C1 (pArA4b). - Arch. Biol. Sci. Belgrade **52**: 85-90, 2000.

Vinterhalter, B., Ninković, S., Cingel, A., Vinterhalter D.: Shoot and root culture of *Hypericum perforatum* L. transformed with *Agrobacterium rhizogenes* A4M70GUS. - Biol. Plant. **50**: 767-770, 2006.

Vinterhalter, B., Orbović, V., Vinterhalter, D.: Transgenic root cultures of *Gentiana punctata* L. - Acta Soc. Bot. Polon. **68**: 275-280, 1999.

Vinterhalter, B., Savić, J., Platiša, J., Raspor, M., Ninković, S., Mitić, N., Vinterhalter, D.: Nickel tolerance and hyperaccumulation in shoot cultures regenerated from hairy root cultures of *Alyssum murale* Waldst et Kit. - Plant Cell Tissue Organ Cult. **94**: 299-303, 2008.

Vinterhalter, B., Vinterhalter, D.: *In vitro* propagation of spotted gentian *Gentiana punctata* L. - Arch. Biol. Sci. Belgrade **50**: 177-182, 1998.

White, F.F., Taylor, B.H., Huffman, A., Gordon, M.P., Nester, E.W.: Molecular and genetic analysis of the transferred DNA regions of the root inducing plasmid of *Agrobacterium rhizogenes*. - J. Bacteriol. **164**: 33-44, 1985.

Zdravković-Korać, S., Muhovski, Y., Druart, P., Ćalić, D., Radojević, L.: *Agrobacterium rhizogenes*-mediated DNA transfer to *Aesculus hippocastamum* L. and the regeneration of transformed plants. - Plant Cell Rep. **22**: 698-704, 2004.