TRANSGENIC ROOT CULTURES OF GENTIANA PUNCTATA L.

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ABSTRACT

Shoot cultures of Gentiana punctata L. were inoculated with suspension of Agrobacterium rhizogenes strain A4 M70GUS. Hairy roots which appeared 2-3 weeks later were cultured on hormone-free, liquid, WPM (Lloyd and McCown 1980) basal medium for more than 5 years (60 subcultures). Growth rate of transformed roots was higher than the growth rate of non-transformed roots. Spontaneous shoot regeneration occurred only in three culture vessels in subcultures No. 40 and 42. Plants had phenotype characteristics typical for A. rhizogenes transformed plants including: wrinkled leaves, short internodes, plagiotropic roots and in general their growth rate was reduced. These plants also manifested precocious formation of flower buds without vernalization and flowering under in vitro conditions. Flowers were pale yellow, the same as in the standard phenotype.

KEY WORDS: Agrobacterium rhizogenes, Gentiana punctata L., excised root cultures, shoot regeneration, in vitro flowering.

INTRODUCTION

Gentiana punctata L. together with G. lutea L. is an endangered, nearly extinct species in Yugoslavia. Devastation of both species is caused by destructive harvesting of roots and rhizomes which are used in traditional medicine and for preparation of aperitifs.

The annual requirement of Gentiana lutea roots needed for industrial purposes in Europe has been estimated at 1500-2500t. Gentians for commercial use can be grown from seeds and cultivated in plantations. However plant biotechnology offers an alternative approach founded on the use of excised root culture and aimed at production of root biomass on commercial basis in bioreactors/fermentors. Furthermore, it has been shown that hairy root cultures obtained from plants transformed with Agrobacterium rhizogenes are characterized by very fast growth which often exceeds growth of untransformed root cultures (Tepfer and Casse-Delbart, 1987).

Inoculation of plants with Agrobacterium rhizogenes and first hairy root cultures were obtained and investigated by Tepfer (1983, 1984), later followed by numerous reports of other authors. Transformation of plants with A. rhizogenes was reviewed by Hamill et al. 1987; Birot et al. 1987; Mugnier 1988; Tepfer 1990; Porter 1991 and Doran 1997. Procedures for micropropagation of various Gentians were presented by Wesolowska et al. (1985), Lamproye et al. (1987), Voila and Franz (1989), Skrzypczak et al. (1993), Momčilović et al. (1997b) and Vinterhalter and Vinterhalter (1998). Transformation of Gentians and related species with Agrobacterium rhizogenes was reported by Ishimaru et al., 1990 (Swertia japonica), Handa et al., 1992 (Eustoma grandiflorum), Sugino and Akihama, 1995 (Gentiana scabra) and Momčilović et al., 1997a (Gentiana acaulis, G. cruciata, G. lutea and G. purpurea). In Gentiana cruciata, Mugnies (1988) reported only formation of tumor callus cultures following inoculation.

Agrobacterium mediated transformation of G. punctata was for the first time reported by Vinterhalter et al. (1993) and the presence of bitter secoiridoid glycosides and xanthones in transformed root cultures by Vinterhalter et al. (1997).

The goal of this work was to obtain stable root cultures of G. punctata transformed with Agrobacterium rhizogenes and evaluate its potential use for biomass production.

MATERIAL AND METHODS

Plant material, culture media and culture conditions

Shoot cultures of Gentiana punctata L. were obtained from embryos excised from aseptically germinated seeds which were collected on Šara mountain (2300 m). Procedure for establishment and maintenance of cultures was the same as previously published by Vinterhalter and Vinterhalter (1998).

Basal medium (BM) contained WPM (Lloyd and McCown, 1980) macro salts; MS (Murashige and Skoog, 1962), micro-nutrient salts and iron stock solution and LS (Linsmaier and Skoog, 1965) vitamins, 2% sucrose and 0.62% agar. Medium pH was adjusted to 5.8 prior to autoclaving which lasted for 20 minutes at 114°C. Prior to inoculation cultures were subcultured on media with 0.25 mg l⁻¹ BA and 0.1 mg l⁻¹ IAA.

Conditions of the growth room were: temperature 25 ± 2°C, photoperiod: 16h light/8h dark, light intensity 45 μmol m⁻² s⁻¹ as measured by Li Cor 190SA quantum sensor coupled with Li-1000 Datalogger (Lincoln, Ne., USA).
Fig. 1. Appearance of A4M70GUS transformed roots on shoots 20 days after inoculation with bacterial suspension.
Fig. 2. Transformed roots on hormone-free liquid medium.
Fig. 3. Transformed roots on hormone-free agar solidified medium (47-th subculture ≈ 4.5 years).
Fig. 4. Shoot cultures regenerated from transformed roots with in vitro formed flowers.
Fig. 5. Expression of GUS gene demonstrated with colour reaction following treatment with X-gluc. Left – staining of terminal bud and adjacent leaflets; right – strong staining of transformed root with regenerated shoots.
Fig. 6. Transection of transformed root stained with X-gluc. Intensive colour develops in epidermal cells and in vascular tissues. Bar = 60 μm.
Biomass corresponds to fresh weight of the explant at the
beginning of the experiment or the weight of the whole cul-
ture at the end of subculture calculated per culture vessel.
Index of biomass production is calculated as final tissue
weight divided by initial weight for a pre-defined vessel and
medium volume.

**Bacterial strain**
In this work we used *Agrobacterium rhizogenes* non dis-
armed agropine-type strain A4M70GUS constructed by Dr.
M. M. Tepfer (INRA, Versailles, France). This strain is har
bouring co-integrative plasmid with GUS construct integrated into
GUS construct contains uidA sequence under the 70S pro-
moter (enhancer-doubled 3SS CaMV promoter), followed by
NOS polyadenylation sequence. Bacterial strains were main-
tained on agar (1.5%) supplemented YEB medium (Van Lare-
bake et al., 1977) with addition of 100 mg l⁻¹ neomycin sul-
phate (ICN-Galenika). Plants were inoculated with 24 h old
bacterial culture grown at 28°C on a shaker adjusted to 220
rev. min⁻¹. Suspension density was ρ = 10⁶ cells/ml.

**Inoculation, hairy-root culture and plant regeneration**
Shoots 2 cm in length consisting of 3-4 nodes were inocu-
lated by puncturing nodal regions with a hypodermic needle
dipped into bacterial suspension. Explants were placed on
hormone-free BM from which 4-5 days later they were trans-
ferred to hormone-free BM supplemented with 200 mg l⁻¹
cefotaxime-Na (Jugoremedica, Zrenjanin, Yugoslavia). When
roots reached 20 mm in length they were excised and trans-
ferred to the cefotaxime supplemented medium in Petri dis-
ches. Concentration of the antibiotic was gradually decreased so the
fifth subculture was made on antibiotic-free medium follow-
ing a thorough test for microorganism contamination.

For root elongation and branching experiments, ten 15 mm
long apical root explants were used, and placed in either Petri
dishes with 25 ml agar solidified medium or 100 ml wide
neck Erlenmayer flasks with 40 ml liquid medium. Flasks
with liquid medium were maintained on shakers.

The effect of 0.1-8% sucrose was also investigated in liquid
medium using explants consisting of 400 mg of root biomass.

Shoot regeneration from transformed root cultures was in-
vestedigated on cytokinin supplemented medium containing
0.01-10 µM BA, kinetin, CPPU or TDZ. Regenerated shoots
were cultured on medium with 0.5 mg l⁻¹ BA and 0.2 mg l⁻¹
GA₃.

**Histochemical GUS assays**
Histochemical localization of GUS expression was per-
formed as described by Jefferson (1987). Tissue samples were
incubated overnight at 37°C in 0.05% (w/v) solution of X-
gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) in 50 mM
sodium phosphate buffer, pH 7.0. Reaction of X-gluc with
substrate gives indigo-blue colour. For histochemical investi-
gation longitudinal and cross sections were made from material
which was fixed in FAA and embedded in parafin. Sections
5-10 µ thick were photographed on Leica, DMRB micro-
scope.

**Plant DNA isolation and PCR analysis**
DNA was isolated from 50 mg fresh tissue with 1 ml DNA-
zole™ (Molecular Research Center Inc.; Cincinnati, Oh.,
USA) according to manufacturer protocol. The success of
transformation was confirmed with PCR method in which
amplification of part of theUidA gen (168bp) was performed.
Following primers were used: 5' AGCTGACGCGCTGTGGG-
CAT-3' and 5'-CTGATACCAACGCTGGCCCGATAA-3'.
Every PCR reaction mixture was 50 µl in volume containing
1 x PCR bufer, 10 µM dNTPs, 20 µM MgCl₂, 5 µM of each
primer and 0.5 U Taq DNA polymerase (Perkin Elmer).
Before amplification, samples were denatured at 94°C for 4 min.
Each of 25 PCR cycles consisted of denaturation step at 94°C
lasting for 30' followed by annealing at 65°C lasting for 45'
and a polymerisation reaction at 72°C lasting for 45'. PCR
reaction was performed on DNA Thermal Cycler-Perkin Elmer
9600.

**RESULTS**
Transformation and culture of transgenic roots
Three weeks after the inoculation adventitious roots ap-
peared in 32 out of 188 (17%) of shoots which were treated
with bacterial suspension. Roots were tender and fragile and
it was better to allow them to elongate for one more subcul-
ture prior to excision (Fig. 1). After isolation, roots started to
elongate faster whilst branching was poor. There was no ap-
parent difference among individually established root clones
therefore all regenerated roots were considered to belong to
the same clone. These A4GUS transformed root cultures have
been so far maintained in our laboratory for more than 5
years (over 60 subcultures). During this period occasionally
some roots formed callus. This callus could not be maintained
as a separate culture (clone) since it became necrotic and
perished after subculturing on fresh medium.

If the excised roots are cultured on liquid (Fig. 2) instead of
agar solidified medium (Fig. 3) then the increase of their bio-
mass expressed as weight of a single root is much higher in
liquid medium. This increase results not only from better root
elongation but also from root branching which occurs in li-
quid medium (Table 1). Comparison of A4GUS transformed
with non-transformed roots shows that former have higher
elongation and biomass production whilst latter have superior
branching. It is important to notice here that non-transformed

<table>
<thead>
<tr>
<th>Culture type</th>
<th>Medium</th>
<th>No. of excised roots</th>
<th>The increase in root length, mm ± SE</th>
<th>No. of lateral roots per root ± SE</th>
<th>Fresh weight per root, mg ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-transformed</td>
<td>agar</td>
<td>50</td>
<td>0.34 ± 0.2</td>
<td>3.2 ± 0.3</td>
<td>4.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>liquid</td>
<td></td>
<td>15.7 ± 1.2</td>
<td>7.8 ± 0.4</td>
<td>17.9 ± 2.5</td>
</tr>
<tr>
<td>A4GUS transformed</td>
<td>agar</td>
<td>59</td>
<td>38.8 ± 1.4</td>
<td>0</td>
<td>23.2 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>liquid</td>
<td>99</td>
<td>45.2 ± 1.9</td>
<td>1.54 ± 0.2</td>
<td>36.8 ± 4.8</td>
</tr>
</tbody>
</table>

**TABLE 1.** Effect of agar solidified versus liquid medium on root elongation and biomass production of non-transformed and A4GUS transformed roots after 35 days.
TABLE 2. Effect of sucrose concentration on the biomass increase of A4M70 GUS transformed roots.

<table>
<thead>
<tr>
<th>% sucrose</th>
<th>No. explants</th>
<th>Initial root clump weight, mg ± SE</th>
<th>Final root clump weight, mg ± SE</th>
<th>Index of biomass increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>5</td>
<td>401.6 ± 59.9</td>
<td>583.6 ± 52.9</td>
<td>x 1.5</td>
</tr>
<tr>
<td>2</td>
<td>53</td>
<td>487.7 ± 18.4</td>
<td>1660.0 ± 100.0</td>
<td>x 3.4</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>388.8 ± 11.7</td>
<td>929.7 ± 55.5</td>
<td>x 2.4</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>432.2 ± 20.7</td>
<td>759.7 ± 46.2</td>
<td>x 1.7</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>400.9 ± 17.1</td>
<td>691.6 ± 26.3</td>
<td>x 1.7</td>
</tr>
</tbody>
</table>

root culture medium was supplemented with 0.2 mg l⁻¹ IBA and 0.1 mg l⁻¹ GA₃ which was previously found to support highest root elongation and branching (Vintealhter et al. 1993).

If explants for subculturings instead of single apical root segments consisted of clumps of A4GUS transformed roots then their growth was equally good on agar solidified and liquid media. Maximum growth of such root cultures in liquid medium was registered at 2% sucrose (Table 2). Higher sucrose concentrations not only decreased the biomass of culture but also induced start of tissue necrosis.

Regeneration of shoots

Attempts to obtain shoot regeneration from transformed roots on cytokinin (0.01-5.0 mg l⁻¹ BA, kinetin, CPPU and TDZ) supplemented media were not successful. However in the 40-th subculture spontaneous shoot regeneration was observed on a single root and in 42-nd subculture on two more roots. These shoots belonged to a phenotype which differed in comparison with the phenotype of shoots from non-transformed plants. They were characterized by short internodes, leaf epinastis, formation of callus on the cut surface of the explant and tendency to become hyperhydric.

Regenerated shoots were transferred on hormone-free basal medium but shoot multiplication was absent. Shoot were then transferred to medium with BA 0.5 + GA₃ 0.1 mg l⁻¹ on which multiplication occurred at a low rate for more than a year. After a 12 months in culture, one clone formed three flower buds. All three flowers opened in vitro (Fig. 4), they were yellow in colour and appeared normal except of somewhat deformed pistils. In vitro fertilization was performed but seeds failed to develop since cultures which formed flowers persisted.

Histochemical localization of GUS activity in leaf and root of transgenic plants

Activity of the A4M70GUS gene in the transformed tissue was demonstrated with histochemical tests in which X-gluc was used as substrate. Since transformation comprises incorporation of an active β-glucuronidase gene, action of this gene can be monitored through its enzyme reaction during which intensively blue colour develops. This reaction was demonstrated in situ in apical root segments and leaves of transformed plants (Fig. 5). As a control non-transformed plants were used.

Hystological analysis of sections enabled more accurate localization of enzyme reaction. In cross sections of roots, blue colour indicating GUS activity was observed in epidermal cells, trichomes and cells of root cap especially in meristematic and vascular cells (Fig. 6).

PCR analysis

Results of the PCR reaction further supported our suggestion that we established transgenic root culture of G. punctata. Appearance of 168 bp amplification product in the lane 2 of the figure 2 confirmed the presence of GUS gene in the genome of cells making up our root cultures. The PCR product of the same size was obtained in the reaction were the DNA template was genomic DNA isolated from the Arabidopsis mutant (Orbović et al., 1995) which was previously shown to carry the GUS gene (Fig. 7 lane 5). On the other hand, when the tissue sample from the non-transformed roots was tested under the same conditions, no amplification product was found (Fig. 7 lane 1). To prove that the PCR product from the lane 2 was not an artifact we ran two additional reactions where we omitted DNA (Fig. 7 lane 3) or Taq enzyme (Fig. 7 lane 4) and they yielded no product.

DISCUSSION

Plants transformed with A. rhizogenes exhibit a distinct set of features which according to Sugimura and Akihama (1995) and literature cited therein include: wrinkled leaves, reduced apical dominance, short internodes, plagiotropic roots and in some species early flowering and formation of flower buds without vernalization. We observed all mentioned features in G. punctata plants which spontaneously regenerated from hairy root cultures. This observations together with results obtained with GUS reaction and PCR analysis provide sufficient evidence that hairy root cultures of G. punctata contain functional foreign genes.

Fig. 7. PCR analysis: 0 – standard DNA marker, 1 – non-transformed roots, 2 – A4 M70 GUS transformed roots, 3 and 4 – negative controls, 5 – positive control.
The growth rate of _G. punctata_ hairy root cultures was much higher than in excised root cultures of non-transformed plants. Since we previously showed (Vinterhalter et al. 1997) that hairy root cultures produce much more gentiopicroin than non-transformed root culture (0.57% vs. 0.02%) we believe that the idea to use hairy root cultures of _G. punctata_ for commercial production of secondary metabolites in bioreactors has so far been justified. Next step in this research would be to investigate which of the several bioreactor types (Whitney, 1992) would be the best choice for hairy root cultures of Gentians.

It is important to note that hairy root cultures of _G. punctata_ did not contain amarogentin found as 0.02% in non-transformed root cultures (Vinterhalter et al. 1997). Similarily, Ishimaru et al. (1999) analysing secondary metabolites in hairy root cultures of _Swericia japonica_ (Gentianaceae) found two bitter compounds amarogentin and amarosorin but could not detect all xanthones which were present in _in vitro_ cultured plants.

Studies dedicated to the clonal propagation of 5 Gentian species performed in our laboratory ( _G. aculis, G. cruciata, G. lutea, G. purpurea_ – Momčilović et al. 1987b) and _G. punctata_ – Vinterhalter and Vinterhalter (1998) showed a marked similarity between _G. punctata_ and _G. lutea_ specially in the rooting stage since both species require auxins for root initiation. Following transformation with A4M7O GUS hairy root cultures of all 5 Gentian species grew in absence of exogenous auxin. According to (Momčilović et al. 1997a) spontaneous shoot regeneration was observed only in _G. cruciata_ whilst in _G. purpurea_ it could be induced indirectly via regeneration of organogenic callus which developed in 5% cultures cultivated on media with 0.1-4.0 mg/l Kin. The other two species, _G. aculis_ and _G. lutea_ failed to regenerate shoot either spontaneously or upon cultivation on cytokinin supplemented media same as _G. punctata_. Although we obtained shoot regeneration of _G. punctata_ the low frequency of this event can be attributed to chance alone and not to the regeneration ability of this species. Among other Gentians regeneration ability was demonstrated in _G. scabra_ (Suginuma and Akihama, 1995) and in _Eustoma grandiflorum_ (Gentianaceae) Handa (1992).

**LITERATURE CITED**


TRANSGENICZNE KULTURY KORZENI GENTIANA PUNCTATA L.

STRESZCZENIE

Z kultur tkankowych pędów Gentiana punctata L. inokulowanych zawiesiną szczepu A4M70GUS Agrobacterium rhizogenes, po 2-3 tygodniach wzrostu izolowano włośniki korzeniowe, które następnie hodowano w płynnym podstawowym roztworze WPM (Lloyd i McCown 1980) pozbawionym hormonów, przez ponad 5 lat (60 subkultur). Szybkość wzrostu transformowanych korzeni przewyższała szybkość wzrostu korzeni nie transformowanych. Spontaniczna regeneracja pędów wystąpiła tylko w trzech naczyniach hodowlanych, w subkulturach nr 40 i 42, a otrzymane rośliny wykazywały cechy fenotypowe charakterystyczne dla roślin transformowanych A. Rhizogenes, takie jak: pomarszczone liście, krótkie międzywęźla, plagiotropowe korzenie i obniżone tempo wzrostu. Rośliny te charakteryzowały także przedwczesne związywanie pączków kwiatowych nie poprzedzone wermalizacją i zdolność do zakwitania w kulturach in vitro. Podobnie jak w standardowym fenotypie, kwiaty roślin transformowanych były bladożółte.