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The horse chestnut lines harboring the *rol* genes

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Abstract

An *Agrobacterium rhizogenes*-mediated transformation system for *Aesculus hippocastanum* L. has been developed. Wounded androgenic embryos of *A. hippocastanum* were inoculated with bacteria containing the pRiA4 plasmid, with the *uid* A sequence as a reporter gene. The hairy roots emerging from the wounded sites of androgenic embryos were isolated and maintained in Murashige and Skoog's (MS) liquid hormone-free medium. Five hairy root lines have been maintained *in vitro* for 4 years with unchanged growth rate and might be a suitable source for secondary metabolite production. The transformation events have been confirmed by a polymerase chain reaction specific to the *rol* A, B, C and D genes. The absence of residual contaminating bacteria has been shown by a polymerase chain reaction specific to the *vir* D1 sequence.

Additional key words: *Aesculus hippocastanum* L., *Agrobacterium rhizogenes*, genetic transformation, *rol* A, B, C and D.

Introduction

The horse chestnut is largely but not solely a decorative tree for urban areas. Its seeds and bark are also sources of aescin and aesculin, as unique secondary metabolites widely used in the pharmaceutical industry. Profumo *et al.* (1991) showed significantly higher aescin content in embryogenic calli and embryoids grown *in vitro* than in control tissues taken from nature. Aescin content increased with *in vitro* growth rate and as a result of the addition of plant growth regulators, such as 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA), and gibberellic acid (GA_3) (Profumo *et al.* 1992) to the culture medium. Furthermore, the extraction of aesculin from horse chestnut tissue culture has also proved to be less expensive and less laborious (Gastaldo *et al.* 1996) than extraction from natural sources. The aim of the present study has been to obtain new tissue lines with a high growth potential. Hairy roots have a high level of productivity in addition to biochemical stability (Matsumoto and Tanaka 1991). Therefore, the hairy roots could be a suitable source of secondary metabolites. We report here the establishment of five hairy root lines of *A. hippocastanum* growing well *in vitro* for 4 years without losing growth potential.

Materials and methods

Plants and transformation procedure: For inoculation we have used androgenic embryos (Radojević *et al.* 1999) derived from an *A. hippocastanum* donor tree, more than a hundred years old, grown in the Botanical Garden of Belgrade. Wounded androgenic embryos have been incubated in 2-d-old bacteria suspension for 5 min,

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; MS - Murashige and Skoog's mineral solution (1962); NAA - α-naphthaleneacetic acid; PCR - polymerase chain reaction; PGR - plant growth regulator; YEB - yeast extract broth medium.
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blotted dry on sterile filter-paper and placed onto Murashige and Skoog's (1962) plant growth regulator (PGR) free solid medium. Out of 780 inoculated androgenic embryos, 630 were placed on a medium containing 50 μM aceto-syringone and 150 on an acetosyringone-free medium. Control androgenic embryos (170) were wounded in the same manner and incubated in a sterile (YEB) medium for 5 min. After 72 h of co-cultivation, the inoculated and control androgenic embryos were transferred to the MS solid PGR-free medium supplemented with 500 mg dm^{-3} cefotaxime (*Jugoremedia*, Zrenjanin, Yugoslavia).

We used *Agrobacterium rhizogenes*, a wild type strain A4GUS, harbouring the pRiA4 nondisarmed plasmid, containing the *uid* A sequence integrated between the *rol* C and the *rol* D genes in the TL-DNA (Tepfer and Casse-Delbarth 1987). The bacteria were shaken at 28 °C in a liquid YEB medium (5 g dm^{-3} sucrose, 5 g dm^{-3} *Bacto* beef extract, 1 g dm^{-3} yeast extract, 5 g dm^{-3} peptone, 0.5 g dm^{-3} $MgSO_4$. 7 H_2O) containing 100 mg dm^{-3} neomycin (Vervliet *et al.* 1975). The bacterial culture was diluted to 10^8 cell cm^{-3} for cocultivation.

The basal medium contained MS inorganic salts, 20 g dm^{-3} sucrose, 100 mg dm^{-3} myo-inositol, 200 mg dm^{-3} casein hydrolysate, 2 mg dm^{-3} thiamine hydrochloride, 10 mg dm^{-3} panthotenic acid, 5 mg dm^{-3} nicotinic acid and 2 mg dm^{-3} adenine. The solid media were solidified by using 0.7 % (m/v) agar (*Torlak*, Belgrade, Yugoslavia). The pH was adjusted to 5.8 prior to sterilizing the media at 114 °C (0.07 MPa) for 30 min. Acetosyringone was dissolved in dimethyl sulfoxide (DMSO) and added by filter-sterilization. The cultures were maintained at 25 ± 2 °C, for a 16-h photoperiod, under cool white fluorescent tubes, with an irradiance of 50 μmol m^{-2} s^{-1}. The liquid cultures were shaken at 85 rpm and transplanted biweekly.

The hairy roots were isolated and maintained in a MS liquid PGR-free medium supplemented with cefo-taxime 500 mg dm^{-3} to inhibit bacterial growth, and shaken on a platform shaker. The concentration of cefotaxime was gradually lowered and it was finally omitted after 6 months.

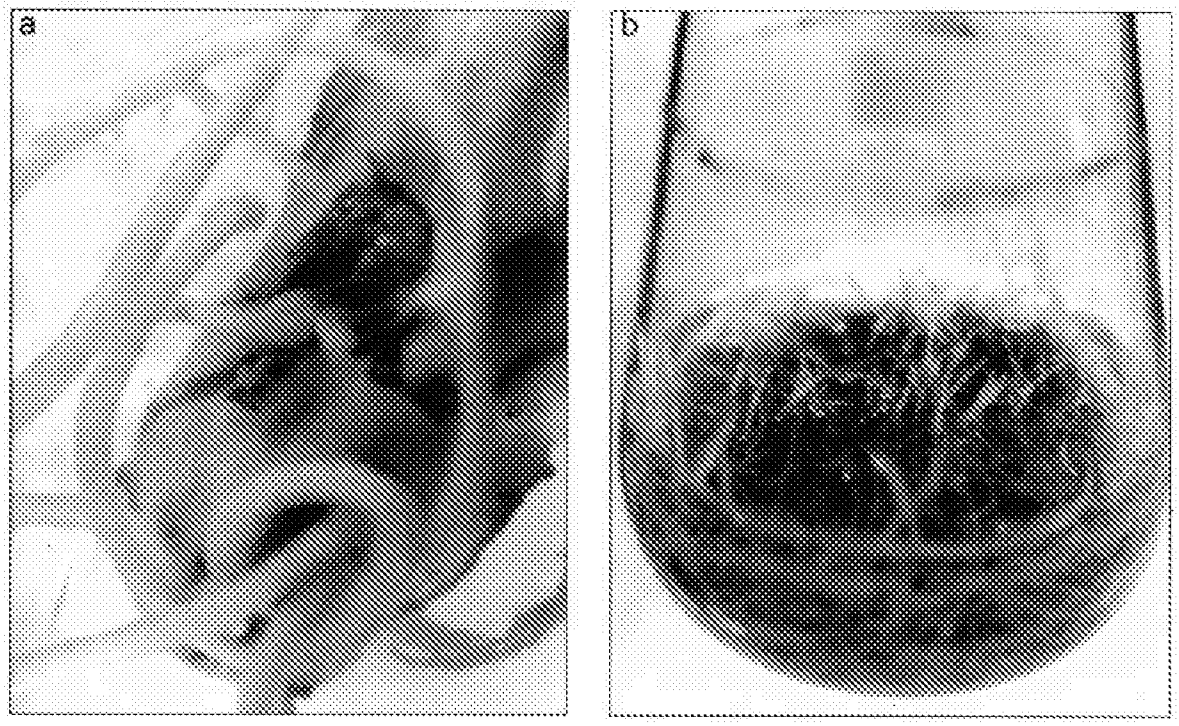

Fig. 1. The hairy roots emerging from radicle of the wounded inoculated androgenic embryo of *A. hippocastanum* (*a*) and established hairy root culture growing autonomously in a MS liquid growth regulator-free medium, showing a loss of gravity response (*b*).

Molecular analysis: Five of the longest growing hairy root lines were chosen for molecular analysis. Negative control was DNA isolated from untransformed androgenic embryos while positive control was pLJ1 cosmid DNA of the pRiHRI plasmid. The T-DNA regions of the pRiA4 and the pRiHRI appear to be identical (Jouanin 1984). Genomic DNAs were extracted according to Murray and Thompson (1980). The *rol* A, B, C, D sequences were detected in the five hairy root lines by a PCR analysis. As a template for PCR, 250 ng of genomic DNAs were used. DNA fragments were amplified using 1 unit of Taq DNA polymerase (*Amersham Pharmacia Biotech*, Little Chalfont, England) in a final volume of 0.03 cm^3. The pairs of primers used were: for the *rol* A (5'-GTT-AGG-CGT-GCA-AAG-GCC-AAG-3' and 5'-TGC-GTA-TTA-ATC-CCG-TAG-

GTC-3'), for the *rol* B (5'-AAA-GTC-TGC-TAT-CAT-CCT-CCT-ATG-3' and 5'-AAA-GAA-GGT-GCA-AGC-TAC-CTC-TCT-3'), for the *rol* C (5'-TAC-GTC-GAC-TGC-CCG-ACG-ATG-ATG-3' and 5'-AAA-CTT-GCA-CTC-GCC-ATG-CCT-CAC-3') and for the *rol* D (5'-AGC-TCT-CAA-CGG-CTT-CAT-GTC-GAT-3' and 5'-CTA-TTC-CAA-CAG-GAC-CTT-GCC-AAT-3').

The primers for the *rol* A, B and C were purchased from *Amersham Pharmacia Biotech* (Little Chalfont, England) and for the *rol* D from *Eurogentec* (Seraing, Belgium). The amplification protocol for the *rol* genes was: 5 min melting at 95 °C followed by 36 cycles of 1 min melting at 94 °C, 1 min annealing at 61 °C and 1 min elongation at 72 °C. The respective expected sizes of yielded fragments were 190 bp for the *rol* A, 300 bp for the *rol* B, 350 bp for the *rol* C and 600 bp for the *rol* D.

PCR analysis was also used to check the presence of residual contaminating bacteria in the samples. The pair of primers specific to the *vir* D1 sequence (*Eurogentec*, Seraing, Belgium) of *A. rhizogenes* used were: 5'-ATG-TCG-CAA-GGA-CGT-AAG-CCC-3' and 5'-GGA-GTC-TTT-CAG-CAT-GGA-GCA-3'. The amplification protocol for the *vir* D1 was: 5 min melting at 95 °C followed by 25 cycles of 1 min melting at 95 °C, 1 min annealing at 52 °C and 2 min elongation at 72 °C. The expected size of the yielded fragment was 450 bp. The amplified products were analyzed by 1.5 % agarose gel electrophoresis.

Results and discussion

The hairy root lines establishment: Thin roots emerged from the wounded sites of cotyledons, hypocotyls and radicles (Fig. 1*a*) approximately one month after the inoculation of the androgenic embryos. They were easily distinguishable from the much larger roots of germinating androgenic embryos. Hairy roots, in contrast to untransformed roots, grow autonomously in a PGR-free medium (Tepfer and Casse-Delbart 1987) showing a loss of gravity response (Fig. 1*b*). No adventitious root at all formed from the control embryos. This single trait allowed the isolation of putative transformants. Ten percent of inoculated androgenic embryos developed hairy roots in the presence of acetosyringone during the co-culture period, while the percentage was only 5.3 % in its absence. The adventitious roots were excised and transferred to a MS liquid PGR-free medium. Although 71 hairy root lines were isolated, most of them survived less than a few months, probably due to auxin transgenes silencing. Only 5 lines grew with an equivalent growth rate, over a 4-year period of successive subcultures. The control roots, isolated from uninoculated germinated androgenic embryos, did not grow under these conditions at all. The mass of the *A. hippocastanum* hairy roots increased 6 - 8 fold over the initial mass within a 4-week culture period. Some of the lines stopped growing after a few months and maintained the same mass for almost a year. However, one of these lines then recovered growth and it was still growing after a period of 2 years. We do not know which factor activated the transgene expression. This is consistent with the results obtained by Guivarc'h *et al.* (1999), who reported that only 7 carrot hairy root clones, out of 160, had been maintained for at least 2 years. The rest of them had survived no longer than a few weeks. Tanaka *et al.* (2001) reported similar results for *Trifolium alexandrinum* hairy roots.

The transformation efficiency seems to be quite low (10 %). However, transformation efficiency is significantly lower in woody plants than in herbaceous plants, and it is usually up to 10 %, *e.g.*, in apple rootstock M26 1.8 - 4 % (Maheswaran *et al.* 1992) and 0.92 % (Holefors *et al.* 1998), in apple rootstock M.9/29 6.67 % (Zhu *et al.* 2001), in apple cultivar's Royal Gala 0.4 - 2.8 % (Yao *et al.* 1995), in *Castanea sativa* 1.5 - 1.8 % (Seabra and Pais 1998), in *Populus alba* L. cv. Vilafranca 7 % (Confalonieri *et al.* 2000) and 11 % (Delledonne *et al.* 2001).

The axenic root tips of all lines of *A. hippocastanum* showed a positive reaction with x-gluc (Jefferson *et al.* 1987), in contrast to untransformed root tissue (not shown). All five root lines were submitted to flow cytometry analysis for the ploidy level and were diploids. Guivarc'h *et al.* (1999) reported that carrot rhizogenic calli were mainly diploid with a small portion of polyploid nuclei, while the hairy roots originating from those calli were exclusively diploid.

Molecular confirmation of transformation events: The PCR analysis revealed the presence of the four *rol* genes in all samples. Amplification with an appropriate set of primers resulted in the yield of a 190 bp band for the *rol* A (Fig. 2*a*), a 300 bp band for the *rol* B (Fig. 2*b*), a 350 bp band for the *rol* C (Fig. 2*c*) and a 600 bp band for the *rol* D (Fig. 2*d*). Expectedly, the *uid* A sequence, which is integrated between the *rol* C and the *rol* D, was also present (not shown) in all lines. There were no bands for untransformed control.

Since the DNA delivery method used *Agrobacterium*-mediated gene transfer, we checked the presence of residual contaminating bacteria. PCR analysis was performed using specific primers for the *vir* D1 sequence of *Agrobacterium rhizogenes*. It is obvious from Fig. 2*e*, that no sample was contaminated. Positive signal was obtained for positive control only.

A number of papers have already reported the use of hairy roots for the production of secondary metabolites (Kamada *et al.* 1986, Nabeshima *et al.* 1986, Rhodes

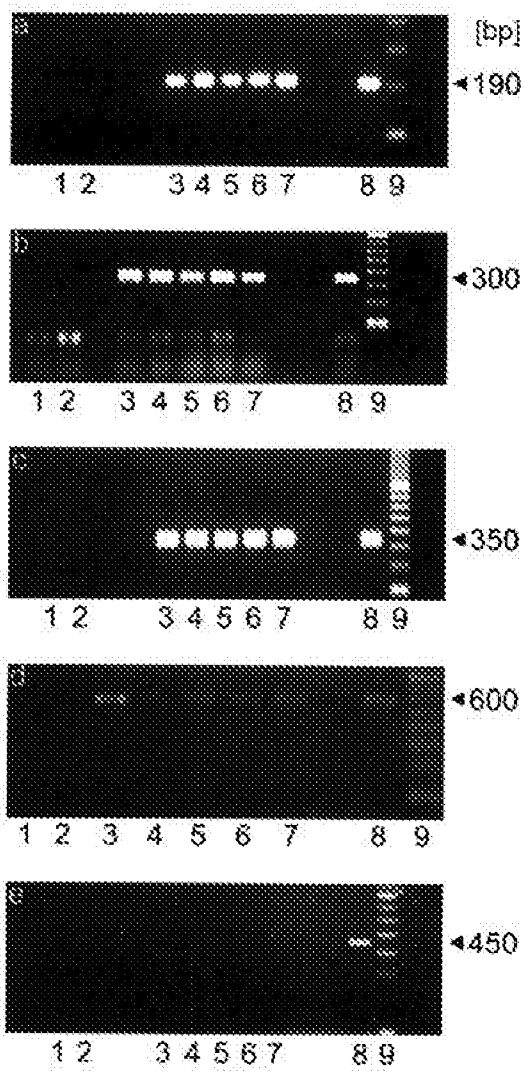

Fig. 2. Gel electrophoresis of a PCR performed to confirm the presence of: *a* - the *rol* A, *b* - the *rol* B, *c* - the *rol* C, *d* - the *rol* D genes in transformed hairy root lines of *A. hippocastanum*, *e* -PCR analysis specific to the *vir* D1 sequence of *A. rhizogenes*, performed to confirm the absence of residual contaminating bacteria in transformed lines of *A. hippocastanum*. *Lane 1*- the blanc control, *lane 2* - negative control (DNA isolated from untransformed androgenic embryos), *lanes 3 - 7* - transformed root lines 31, 36, 39, 43, 47, *lane 8* - positive control (the pLJ1 cosmid DNA of the pRiHRI), *lane 9* - molecular size marker (λHind III DNA).

et al. 1986, Matsumoto and Tanaka 1991, Xie *et al.* 2000). Flores and Curtis (1992) mentioned that more than 30 different plant cell lines allowed the production of plant chemicals at levels much higher than those found in the original plant tissue. According to them, the roots may have an immense biochemical potential. Tissue culture for secondary metabolite production shows advantages over conventional methods: biomass production is more rapid than that of the whole plant, year-round production of pharmaceuticals is possible, the extraction of active principles is less expensive and less laborious.

Conclusions: These results constitute, to the best of our knowledge, the first report on the genetic transformation of the horse chestnut. The data presented here show that *A. rhizogenes* can be used for *A. hippocastanum* genetic transformation. We may conclude that all tested lines are transformed and harbor the *rol* A, B, C and D genes. Several hairy root lines with high growth potential have been established, expecting the presence of secondary metabolites. After their detection, conditions will be optimized for large-scale production of these unique compounds.

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