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BRIEF COMMUNICATION

Hairy roots formation in recalcitrant-to-transform plant *Chenopodium rubrum*

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Abstract

Susceptibility of *C. rubrum* to *Agrobacterium*-mediated transformation was demonstrated by inoculating the petioles of *in vitro* grown plants with *A. rhizogenes* strain A4M70GUS. Hairy roots were produced in 8 % of explants. They were isolated and maintained on plant growth regulator-free solid or liquid half-strength Murashige and Skoog medium for two years. Hairy root fresh mass increased 30 - 90 folds when grown in liquid medium, which was superior to solid medium, where most of the hairy roots produced calli. When these calli were grown on medium supplemented with 0.5 mg dm^{-3} thidiazuron, embryo-like structures were obtained. Transgenic status of long-term callus and hairy root cultures was confirmed by histochemical GUS assay, by PCR specific to the *uidA*, *rolA&B* and *ags* genes and by Southern hybridization.

Additional key words: Agrobacterium rhizogenes, embryo-like structures, GUS activity, thidiazuron.

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Chenopodium rubrum L., a short day plant, has been used as a suitable object for physiological studies on photoperiodic flower initiation for many years (*e.g.* Pavlová and Krekule 1984, Živanović *et al.* 1995, Mitrović *et al.* 2003), as well as for *in vitro* morphogenetic studies (Milivojević *et al.* 2005). *Agrobacterium rhizogenes* induced hairy roots offer the ability to test different genes in plants and are widely used for production of secondary metabolites (Giri and Narasu 2000). Successful *A. rhizogenes*-mediated transformation has already been reported for some species of the family *Chenopodiaceae*, such as *Beta vulgaris* and *Spinacia oleracea* (Kifle *et al.* 1999, Ishizaki *et al.* 2002, Thimmaraju *et al.* 2008). However, the species of the genus *Chenopodium* have been considered as recalcitrant to genetic transformation and regeneration. There are only few reports of their transformation, mostly on *Chenopodium quinoa* (Komari 1990, Jung *et al.* 1992). In *C. rubrum*, after several unsuccessful attempts using standard transformation procedures, only transient GUS expression using sonication-assisted *Agrobacterium*-mediated transformation (SAAT) method was reported by Flores Solís *et al.* (2003). This study was designed to investigate transforming ability of *Chenopodium rubrum* plants with agropine-type strain *Agrobacterium rhizogenes* A4M70GUS using leaf petioles as explants.

Chenopodium rubrum L. (ecotype 184) seeds, collected in 2002, were aseptically germinated under specific regime: 32 °C for 24 h (dark), 10 °C for 24 h (dark), 32 °C for 48 h (light). The seedlings were cultured on medium containing Murashige and Skoog (1962; MS) mineral salts and vitamins, 3 % sucrose and 0.7 % agar. They were grown under non-inductive photoperiodic conditions: (16-h photoperiod, irradiance of 70 μmol m^{-2} s^{-1} and temperature of 25 ± 2 °C). To induce hairy roots, petioles were excised from 4-week-old seedlings and dipped into the suspension of *A. rhizogenes* A4M70GUS (Tepfer and Casse Delbart 1987) for a few

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Abbreviations: BA - benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; GUS - β-glucurodinase; MS medium - Murashige and Skoog medium; NAA - naphthalene acetic acid; PCR - polymerase chain reaction; TDZ - thidiazuron.

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minutes. Explants were dried by filter paper and placed on half-strength MS medium. Seven days after co-cultivation in the dark at 25 °C, the explants were transferred to solid ½ MS medium supplemented with 300 mg dm^{-3} cefotaxime (*Jugoremedia A.D.*, Zrenjanin, Serbia). Roots appeared on the explants after 5 weeks and were excised and cultured on solid or liquid ½ MS medium with 2 % sucrose. *A. rhizogenes* cells were eliminated with 100 mg dm^{-3} cefotaxime in the next three subcultures. In order to induce plant regeneration, calli that spontaneously developed on hairy roots grown on solid ½ MS medium, were transferred to MS medium containing naphthalene acetic acid (NAA) and gibberellic acid (GA_3) or NAA and benzyladenine (BA) at various concentrations as well as 0.2 and 0.5 mg dm^{-3} thidiazuron (TDZ).

Hairy roots (fresh mass 100 mg) were transferred to full- or half-strength MS liquid medium containing 2 % sucrose and cultured for 4 weeks. Five Erlenmeyer flasks were used per clone. Roots excised from intact non-transformed *in vitro* plantlets were used as a negative control. The roots from each sample were blotted dry on filter paper and fresh mass was determined.

Transgenic nature of hairy root clones was confirmed by PCR analysis. PCR amplification of the *uidA* gene and GUS staining were performed under the same conditions as described by Sretenović-Rajičić *et al.* (2006). Oligonucleotide primers used to amplify 1.6 kb fragment of complete coding sequence of the *rolA&B* genes were 5'-ATGGATCCCAAATTGCTATTCC-3' and 5'-ATG GAATTAGCCGGACTAAACG-3'. Another set of primers was used to amplify 1.6 kb segment of agropine synthase (*ags*) gene: 5'-CGGAAATTGTGGCTCG TTGTGGAC-3' and 5'-AATCGTTCAGAGAGCGTCC GAAGTT-3'. PCR conditions for both sets of primers were: a 5-min melting at 95 °C, followed by 35 cycles of 95 °C for 50 s, annealing at 53 °C for 1 min and polymerization at 72 °C for 1 min and final elongation at 72 °C for 10 min. PCR products were separated by electrophoresis on a 1.5 % agarose gel, stained with ethidium bromide and visualized under UV radiation.

Genomic DNA was extracted from hairy roots and callus of the clone CR3 using the method of Murray and Thompson (1980). Non-transformed seedlings were used as a negative control, and horse chestnut hairy root line 36, transformed with the same plasmid (Zdravković-Korać *et al*. 2004), was used as a positive control. The DNA was digested with *Bam*HI (*Fermentas*, Vilnius, Lithuania), separated electrophoretically in a 0.8 % (m/v) agarose gel (16 h, 20 V), and transferred to a positively charged nylon membrane (*Roche*, Mannheim, Germany) by capillary transfer for 24 h. Signal detection was performed using a non-radioactive DNA labelling and detection protocol (*DIG Probe Synthesis Kit*, *Roche*). The membrane was probed with the *rolA&B* (1.6 kb) probe, and then was stripped and re-probed with the *uidA* (366 bp) probe. Both probes were labelled with digoxigenin *(DIG)-dUTP* (*Roche*) by PCR using the pRiA4 DNA as a template. The hybridization was performed in *DIG Easy Hyb* buffer (Roche) at 42 °C for the *rolA&B* and at 44 °C for the *uidA* for 16 h. The membrane probed with the *rolA&B* was washed 2 × 5 min with the following buffers: 2× SSC + 0.1 % SDS at 42 °C, 1× SSC + 0.1 % SDS at 42 °C, 0.5× SSC + 0.1 % SDS at 65 °C and 0.1× SSC + 0.1 % SDS at 65 °C, and the membrane probed with the *uidA* was washed 2 × 5 min with 2× SSC + 0.1 % SDS at 44 °C and 2 × 15 min with 0.1× SSC + 0.1 % SDS at 68 °C. The hybrids were detected with anti-DIG antibody (*Roche*), visualised with chemiluminescent substrate *CDP-Star* (*Roche*) and recorded with X-ray film (*Kodak*, Paris, France).

Initially, 390 petioles excised from 4-weeks-old *in vitro* grown plants were inoculated with agropine-type strain of *A. rhizogenes*, A4M70GUS. Adventitious roots appeared on the infection sites of 31 petioles within 5 weeks. Root formation was never observed in uninfected petioles used as a control, indicating that root formation could not be attributed to physiological stresses. The transformation efficiency, assessed as the number of petioles developing roots per total number of inoculated explants, was 8 %. In comparison to plants transformed with the same strain (Tiwari *et al*. 2008) or some other vectors (Gangopadhyay *et al.* 2008, Zhang *et al.* 2008), the obtained response may be considered as low. Nevertheless, regarding the recalcitrant nature of this species and transformation efficiencies reported for other members of family *Chenopodiaceae* (Kifle *et al.* 1999, Ishizaki *et al*. 2002, Thimmaraju *et al.* 2008), we found efficiency of 8 % to be satisfactory for confirmation of *C. rubrum* susceptibility to *Agrobacterium*-mediated transformation. Adventitious roots were excised and cultured on solid or liquid hormone-free ½ MS media with 2 % sucrose (Fig. 1*A*). Thirty different rapidly growing lines were established. Three of them, retaining high growth capacity for two years, have been chosen for further investigations.

All three lines displayed typical characteristics associated with the hairy root phenotype previously described by Tepfer (1984). They were white or pink to red, highly branched, and developed numerous root hairs with a plagiotropic growth habit. Two phenotypic variations could be distinguished: thin and soft roots (CR1 and CR2 lines), and thick roots (CR3 line). Histological analysis confirmed similarity between these two root types (data not shown). Liquid medium used for cultivation of clone CR3 was coloured pink probably due to the production of betalain pigments. Compared to non-transformed roots, the mass of hairy roots increased about 30 - 90 fold during four weeks of culture in ½ MS liquid medium containing 2 % sucrose, and about 30 - 45 fold on the full-strength MS medium with the same sucrose concentration. Clone CR3 exhibited highest biomass

accumulation (Table 1). Comparing to other species transformed with *A. rhizogenes* A4M70GUS strain (Vinterhalter *et al.* 2006, Tiwari *et al.* 2008), this growth rate was extremely high. An enhanced biomass production offers possibilities for betalain accumulation.

Hairy roots grown on solid hormone-free medium spontaneously formed soft, yellow calli after four weeks of culture. These results indicated that addition of auxins was not necessary for callus induction in Ri-transformed root explants of *C. rubrum*, unlike reported for other species of *Chenopodiaceae* family (Ishizaki *et al.* 2002). The ability of *C. rubrum* hairy roots to grow and form calli without an auxin supply could be related to the presence and activity of auxin biosynthesis *aux* genes, brought by the TR-DNA of the Ri plasmid (Camilleri and Jouanin 1991).

In an attempt to induce somatic embryogenesis from hairy root explants, we followed the procedures established for spinach by Ishizaki *et al.* (2002). The effects of different concentrations of NAA and GA_3, BA and NAA and additionally TDZ at concentration of 0.2 and 0.5 mg dm^{-3} were examined. All explants formed brown mass of hairy roots on media containing NAA and GA_3, while soft or hard yellow and green calli were formed on media containing cytokinins. Numerous starch grains in the callus cells indicated their proembryogenic status (Fig. 1*B*). Embryo-like structures were released from calli grown on 0.5 mg dm^{-3} TDZ medium (Fig. 1*C*), but failed to develop further upon transfer to hormone-free medium. Similar structures were observed in calli obtained from different non-transformed explants of *C. rubrum* cultured on media with 2,4-D (1 - 10 μM) within 3 - 6 weeks (Milivojević *et al.* 2005).

Table 1. Effect of medium strength on the growth [g] of *Chenopodium rubrum* hairy root clones and control, non-transformed roots after four weeks of culture in liquid hormone-free MS medium containing 2 % sucrose. Means ± SE, $n = 5$.

Clones	Full MS	½ MS
Control	0.15 ± 0.02	0.12 ± 0.02
CR1	3.70 ± 0.2	5.81 ± 0.2
CR2	3.21 ± 0.1	4.00 ± 0.6
CR3	6.80 ± 0.2	11.50 ± 1.7

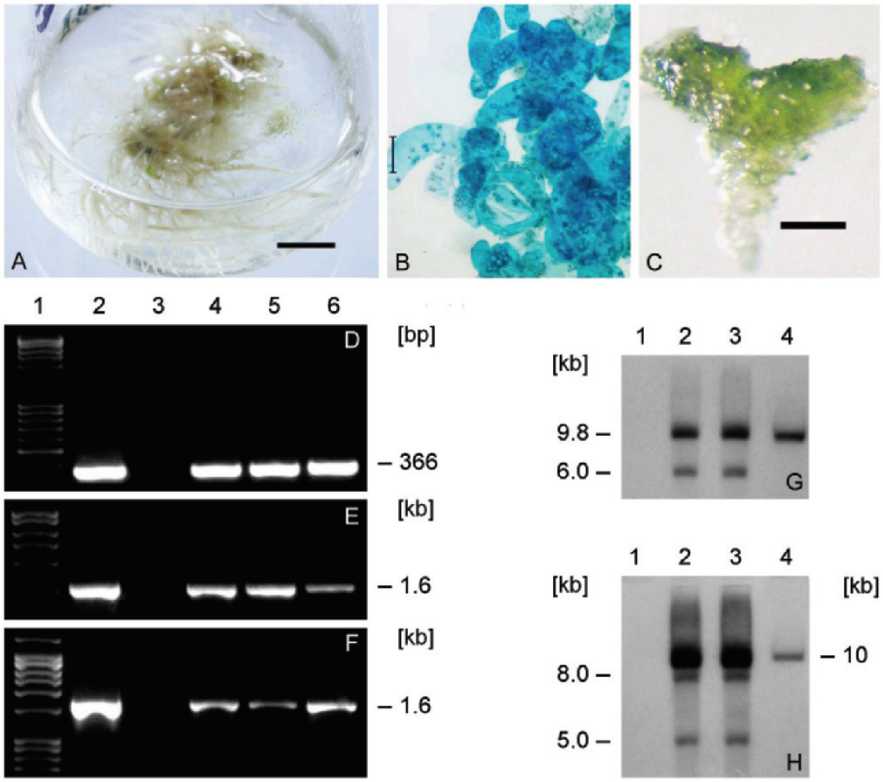

Fig. 1. *A. rhizogenes* A4M70GUS-mediated transformation of *Chenopodium rubrum* L. *A* - Hairy root culture growing on hormone-free ½ MS medium with 2 % sucrose (*bar* = 1 cm). *B* - Hairy-root derived callus after GUS staining (*bar* =100 μm). Numerous starch grains in the cells could be observed. *C* - Embryo-like structure developed from hairy-root derived callus cultured on solid MS medium with 0.5 mg dm^{-3} TDZ (*bar* = 0.5 mm). *D, E, F* - PCR-amplified DNA fragments of the *uidA* (*D*), the *rolA&B (E*) and the *ags* (*F*) genes: *lane* 1 - DNA ladder; *lane* 2 - A4M70GUS plasmid as a positive control; *lane* 3 - untransformed roots as a negative control; *lane* 4 - 6: *C. rubrum* hairy root clones CR1, CR2 and CR3. *G* and *H* - Southern blot analysis of *C. rubrum* genomic DNA digested with *Bam*HI and hybridized to the probe *rolA&B* (*G*) and *uidA* (*H*): *lane* 1 - non-transformed control; *lane* 2 - hairy root clone CR3; *lane* 3 - callus derived from hairy root clone CR3; *lane* 4 - positive control, DNA from horse chestnut hairy root line 36.

High level of the *uidA* expression was observed in both roots and calli of all three clones tested (Fig. 1*B*). Contrary, GUS activity was not detected in non-transformed roots.

PCR analysis was performed with primers designed to amplify the *rolA&B* genes from the TL region of Ri plasmide, the GUS gene inserted into TL region between the *rolC* and the *rolD* genes, and the *ags* gene from the TR region. Amplified PCR products were of the expected size (1.6 kb, 366 bp and 1.6 kb, respectively) and identical to those of the positive control, while they were absent in non-transformed roots (Fig. 1*D-F*). These results indicated the integration of both the TL and the TR-Ri T-DNA in transformed roots.

The integration of the Ri T-DNA in the putatively transformed CR3 clone was further confirmed by Southern blot analysis. Following digestion with *Bam*HI and hybridization with the *rolA&B* probe, *C. rubrum* DNA from both hairy roots and calli of clone CR3, and the positive control produced the expected band of 9.8 kb (Fig. 1*G*), thus indicating stable integration of the TL-DNA. These results demonstrate that *A. rhizogenes* is a potent tool for production of transgenic roots in *C. rubrum*. An unexpected 6-kb band also appeared in *C. rubrum* hairy root CR3 clone, probably indicating an insertion of truncated TL-DNA copy, as was reported in *Convolvulus arvensis* (Jouanin *et al.* 1989) and *Aesculus hippocastanum* (Zdravković-Korać *et al.* 2004). The positive control produced a single 10-kb fragment. The negative control produced no signal in both hybridizations. The copy number of the integrated DNA, estimated by hybridization with the probe *uidA*, was at least three copies for *C. rubrum* (Fig. 1*H*). As the *Bam*HI fragment No. 5 spans the right TL-DNA border, the length of the fragment hybridizing with the *uidA* probe would be variable, depending on the plant flanking sequences. As the minimal expected size of this fragment is 8 - 9 kb, the 5-kb *C. rubrum* fragment indicates an insertion of another truncated TL-DNA copy. In spite of the presence of at least three copies of the TL-DNA, this clone did not lose high growth capacity up to now.

This work demonstrates the establishment of *C. rubrum* hairy root cultures obtained after transformation of petioles with *A. rhizogenes* A4M70GUS. Although the whole plants were not recovered, this system represents a valuable tool for gene functional analyses and for application of a novel tool called the "composite plant system" (reviewed in Veena and Taylor 2007), the alternative strategy for studying the function of genes in roots and entire plant. It also offers the possibility to evaluate genetic engineering potential of the recalcitrant to transform species of the genus *Chenopodium* (Karami *et al.* 2009). The system reported here permits introduction of the *rol* genes and production of hairy root cultures which could contribute to further progress in development of *in vitro* systems for producing betalain pigments (reviewed by Georgiev *et al.* 2008). The experiments, related to regeneration of the whole plants from transformed hairy roots, are in progress, along with pursuing the introduction of transgenes into another model species, the long-day-plant *Chenopodium murale*.

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