Journal of Plant Biology, June 2005, 48(2) : 258-262 **SHORT COMMUNICATION**

Genetic Transformation of *Ajuga multiflora* Bunge with *Agrobacterium rhizogenes* and 20-Hydroxyecdysone Production in Hairy Roots

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An efficient transformation system for *Ajuga multiflora* Bunge was established by using *Agrobacterium rhizogenes* strain A4. After inoculation with the bacteria, we obtained a number of hairy-root clones from micro-calli of the explant petioles. One fast-growing line showed the highest production of 20-hydroxyecdysone (20-HE). PCR amplification of rooting locus (*rol*) genes revealed that the left hand-transferred DNA of the root-inducing plasmid was inserted into the genome of our transformed *Ajuga* hairy roots. This integration was further confirmed by DNA-DNA hybridization. The 20-HE content in hairy roots was 10 times higher than that measured in the wild type.

Keywords: Ajuga multiflora, hairy roots, 20-hydroxyecdysone, Ri-plasmid, *rol* genes

Agrobacterium rhizogenes, a causal bacterium of hairy root disease in dicotyledonous plants, harbors a large, root-inducing (Ri) plasmid (White and Nester, 1980). Two sets of pRi genes -- *aux* genes in the TR region and *rol* (root loci) genes in the TL region -- are involved in this induction process (Jouanin, 1984). In addition, *ags* genes responsible for opine biosynthesis in transformed hairy roots are located in the TR region (Binns and Tomashow, 1988). Various culture systems, such as cell-suspension, adventitious-root, and whole-plant, have been employed for mass production of useful compounds (Lian et al., 2002; Kim et al., 2004a, b). In particular, the use of hairy roots, as induced by pRi genes, is a valuable tool for studying secondary metabolites because those tissues grow faster than other types (Shimomura et al., 1991; Maldonado-Mendoza et al., 1992; Bhadra et al., 1993).

20-Hydroxyecdysone (20-HE) is the physiological inducer of molting and metamorphosis in arthropoda (Camps and Coll, 1993). This compound is biosynthesized in both insects and plants, especially in *Ajuga* species. Several studies have focused on the high rate of 20-HE production by that genus; some researchers have now established methods for culturing *Ajuga reptans* hairy-root lines (Matsumoto and Tanaka, 1991; Tanaka and Masumoto, 1993a; Uozumi et al., 1995). Although several types of phytoecdysteroids, including 20-HE, have been found in *A. reptans* roots, they have not yet been reported from *A. multiflora*, which is a horticultural groundcover plant used in Korea. Therefore, our aim in this investigation was to transform explants of *in vitro*-grown plants of *A. multiflora* with *A.rhizogenes* in order to obtain hairy-root cultures. In addition, we compared 20-HE contents between hairy-root transformants and the wild type.

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MATERIALS AND METHODS

Plant Materials

Plants of *A. multiflora* Bunge were collected from the BaikYang Mountain, Chonnam Province, Korea. Shoots were surface-sterilized for 7 to 9 min in 3% sodium chlorite (v/v) containing 0.1% Tween 20, then washed three times with sterile water. They were then placed under lights at 25°C in plastic bottles containing 80 mL of an MS medium (Murashige and Skoog, 1962) supplemented with 0.8% agar. After 1 d, their leaves and petioles were excised and used to induce

hairy roots.

Agrobacterium Culture

We selected the A4 strain, a single clone of *A. rhizogenes* that harbors the agropine-type plasmid pRiAb. The bacterium was cultured overnight in 50 mL of potato dextrose broth (Difco, USA) in the dark at 28°C on a rotary shaker (160 rpm). Afterward, the bacterial solution was collected and centrifuged, then re-suspended in a growth regulator-free liquid MS medium for inoculation.

Induction and Culture of *A. multiflora* Hairy Roots

To induce hairy root formation, we tested both leaf and petiole explants and found that transformation rates were higher for the latter type (data not shown). Therefore, we excised 10-mm sections of those petioles and inoculated their cut ends. These tissues were then cultured at 25°C in the dark on a half-strength MS medium supplemented with 3% sucrose and 0.8% agar. After 1 d of co-culture the bacteria were washed away with sterile distilled water and the explants were transferred to the same half-strength MS medium, now supplemented with 500 mg L^{-1} cefotaxime (Sigma, USA). Hairy roots were excised from the parent tissues and transferred to fresh half-strength MS media (plus 500 mg L^{-1} cefotaxime) until all residual bacteria had been killed.

Liquid cultures were established from 10-mm-long tips of 21-d-old hairy roots. Five root tips were inoculated in a 100-mL Erlenmeyer flask containing 30 mL of a half-strength antibiotic-free MS medium. These roots were then grown at 25°C on a rotary shaker (100 rpm) in the dark.

Identification of Transformed *A. multiflora* Hairy Roots by PCR

Genomic DNAs from both non-transformed leaves and hairy roots were isolated by the cetyldimethylethylammonium bromide method (Doyle and Doyle, 1987). DNA was dissolved in TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. After RNase treatment, the DNA was stored at –20°C. PCR was performed to detect the *rolA* and *rolB* genes in the T-DNA of the pRiAb plasmid and the genomic DNAs of the non-transformed leaves and hairy roots. Plasmid DNA of *A. rhizogenes* strain A4 was used as a positive control. PCR primers were designed as follows, according to DNA sequences: for *rolA* gene amplification of a 454-bp fragment, the primer pair was 5′-GAGTGTGGTTGTAGGTTCAATTAT-3′ and 5′-TTAA-CAGAACATATTCGATATCATCTCC-3′; for *rolB* gene amplification (858-bp fragment), the primers were 5′-CTTATGACAAACTCATAGATAAAGGTTG-3′ and 5′-TCGTAACTATCCAACTCACATCAC-3′. The PCR mixture comprised approximately 200 ng of plant genomic DNA, 0.4 mM of each primer, 0.2 mM of each dNTP, 1.5 U Taq DNA polymerase (5 U μl^{-1}; Takara, Japan), and 10 µL 10× buffer, in a final volume of 100 µL. PCR was run under the following conditions: initial denaturation at 94°C for 5 min; then 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and elongation at 72°C for 1 min; followed by a final extension at 72°C for 10 min. The amplified products were examined by electrophoresis on a 1.5% (w/v) agarose gel.

DNA-DNA Hybridization

After PCR amplification, the electrophoresed DNAs were transferred to a blotting membrane (Bio-Rad, USA), then hybridized with probe DNA that had been radio-labelled with α-[^{32}P]-dCTP by a random primer kit (Roche, USA). The probe DNA fragments, amplified with plasmid DNA serving as template, were used as probes for the *rolA* and *rolB* genes. The blot was first washed twice, for 60 min each, at 65°C with 5% SDS that contained 1 mM EDTA and 40 mM Na_2HPO_4, then twice (60 min each) at 65°C with 1% SDS containing 1 mM EDTA and 40 mM Na_2HPO_4.

Analysis of 20-HE

20-HE was isolated from the hairy roots of *A. multiflora* as described by Matsumoto and Tanaka (1991) for analysis by TLC (Thin-layer chromatography) on a silica gel 60 F_{254} plate (Merck, Germany). Both a standard 20-HE methanol solution and the solution for the extracted sample were dropped on a plate. A 72:25:3 (v:v:v) mixture of chloroform:methanol:water was used as the mobile phase. The plate was sprayed with 20% H_2SO_4, then heated at 100°C for 5 min. Quantitative determinations were accomplished via HPLC (monitored at 242 nm) by separating the 20-HE on a Waters C_{18} µ-Bondapak column (3.9 mm × 300 mm) that used an 18:2:80 CH_3CN: methanol: H2O (v/v/v) mixture mobile phase at a flow rate of 1.2 mL min^{-1}.

RESULTS AND DISCUSSION

Transformation and Establishment of Hairy Root Cultures

We initially tested two explant types (leaves and petioles) from *A. multiflora* to induce hairy roots by transformation. After 3 weeks of inoculation, a few hairy-root lines were obtained from both types. Although hairy roots were induced from the micro-calli of petioles in a half-strength MS medium, none was directly obtained from their cut edges. At that time, transformation rates were 1.52-times higher from petioles than from leaves (data not shown). Consequently, further infection experiments were performed only with the petiole explants, from which 43% formed putative transformants. Shi and Kintzios (2003) have also reported high induction frequencies with *Pueraria phaseoloides* hairy roots from petiole explants. Although morphological patterns and hairy root-induction rates from infected tissues vary (Bercetche et al., 1987; Ottani et al., 1990), those differences may depend on the species, organs used, or infection sites.

To propagate our transformed hairy roots, we excised their apical tips (about 10 mm long) and transferred them to half-strength MS solid or liquid media. Of the 18 selections made, 4 hairy-root cultures that manifested different growth patterns were established 12 weeks after their induction (Fig. 1). One line in particular (AM5) showed active elongation with numerous lateral roots on a hormone-free medium. That line also had the best growth rate after 35 d of culture (data not shown).

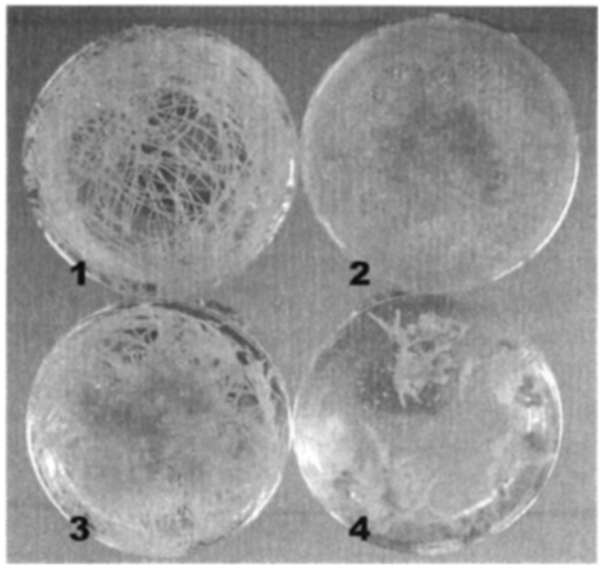

Figure 1. Four clones of *A. multiflora* hairy roots transformed with *A. rhizogenes* A4 strain. 1, AM1; 2, AM5; 3, AM7; 4, AM11.

Analysis of Transgenic Cultures

To confirm that *A. rhizogenes* plasmid T-DNA had been integrated into the *A. multiflora* genome, we

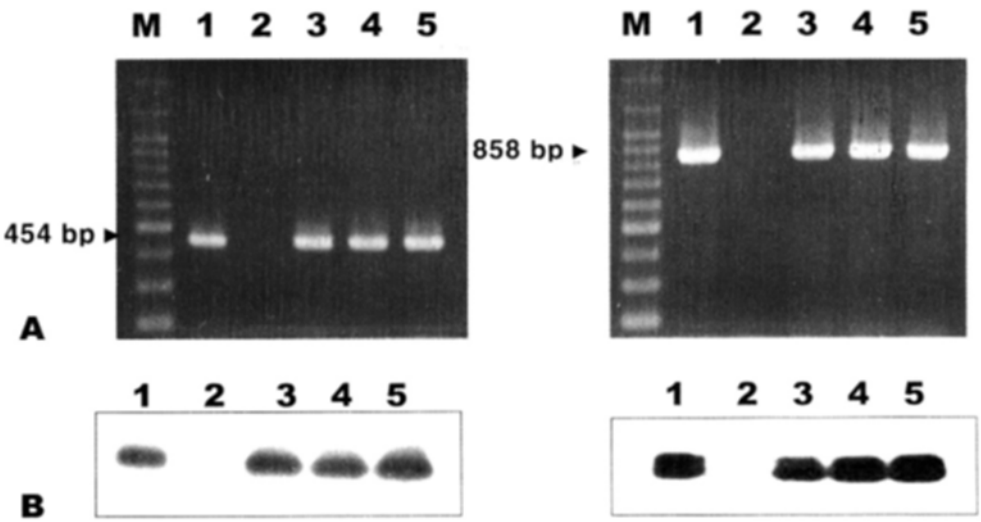

Figure 2. PCR analysis and DNA-DNA hybridization with *rolA* (left) and *rolB* (right). **(A)** Identification of *rol* genes in hairy roots by PCR amplification. Lane M, Molecular-weight marker; lane 1, plasmid DNA isolated from *A. rhizogenes* A4 strain; lane 2, DNA isolated from non-transformed leaves of *A. multiflora*; lanes 3-5, DNA isolated from hairy roots of transformed lines AM1, AM5, and AM 7, respectively. **(B)** Confirmation of amplified DNA by blotting to nylon membrane followed by DNA-DNA hybridization with radio-labelled *rolA* or *rolB* gene probe.

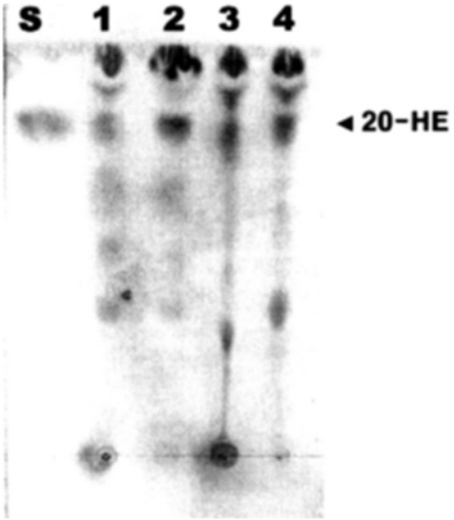

Figure 3. TLC analysis of 20-HE in *A. multiflora* hairy roots. Lane S, authentic (standard) 20-HE; lane 1, AM1; lane 2, AM5; lane 3, AM7; lane 4, AM11.

conducted PCR analysis with primers designed according to *rolA* and *rolB* gene sequences. Using DNAs from three hairy-root lines (AM1, 5, and 7) and one with non-transformed leaves (Lane 2) as templates, we were able to detect 454-bp and 858-bp fragments from the hairy-root lines that corresponded to *rolA* and *rolB*, respectively, whereas no fragments were detected from the non-transformed plants (Fig. 2A). DNA-DNA hybridization was conducted to further confirm the introduction of *rolA* and *rolB* into the *A. multiflora* genome (Fig. 2B). The expected bands for *rolA* and *rolB* were observed in the amplified DNA from all three hairy-root lines (lanes 3 to 5), while no hybridization band was produced for the non-transformed plant (lane 2). These results demonstrate that *rolA* and *rolB* from the *Agro-*

Table 1. Contents of 20-HE in *A. multiflora* root tissues, as detected by HPLC.

Root type	20-HE content (mg g^{-1} dry wt.)
Untransformed roots (wild-type)	0.6±0.03
Hairy roots induced by pRiA4b	
AM1	1.5±0.12
AM5	6.4±0.25
AM7	0.3±0.05
AM11	4.8±0.22

Results are mean ± standard error (n = 5).

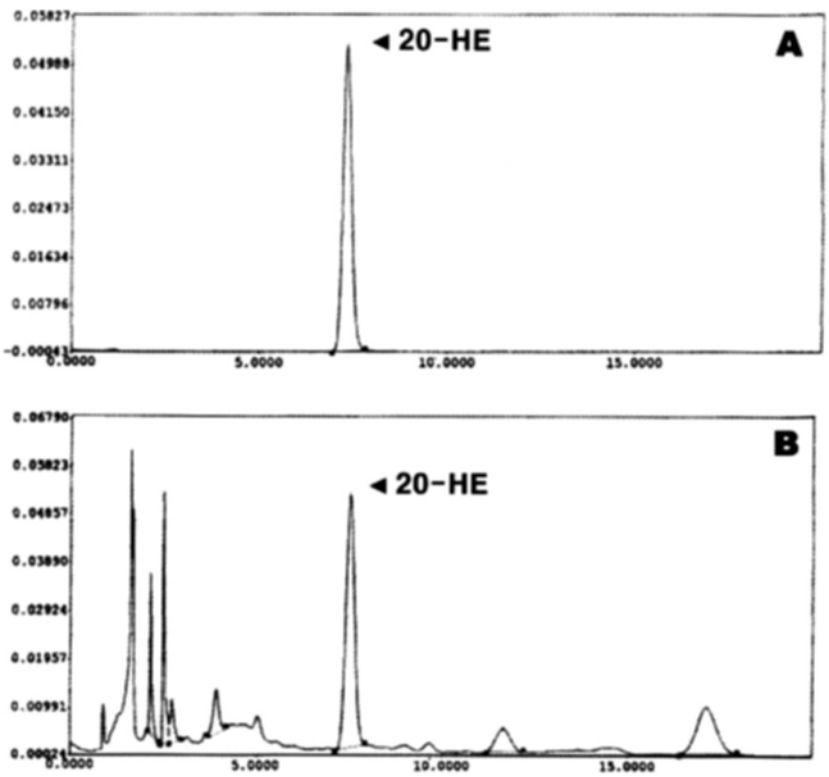

Figure 4. HPLC analysis of 20-HE. **A,** standard 20-HE; **B,** 20-HE isolated from hairy roots.

bacterium Ri plasmid were indeed integrated into the hairy-root *A. multiflora* genome.

Analysis of 20-HE Production in Hairy Roots

Hairy roots were cultured on a half-strength MS medium without any supplemental growth regulators. After 35 d, they were harvested to determine their 20-HE contents via TLC. 20-HE was detected in the *Agrobacterium*-transformed lines (Fig. 3). Our HPLC spectrum also showed that isolated 20-HE had a single, strong single peak at 7.01 min, the same as that for the 20-HE standard (Fig. 4). Among the four transgenic lines, AM5 had the highest level of 20-HE. We also compared the amount of 20-HE in Line AM5 with that measured in the wild-type roots (Table 1). For *A. multiflora* hairy roots cultured for 35 d on a half-strength MS medium, the 20-HE content was 6.4 mg g^{-1} dry weight, a level 10 times greater than that recovered from the roots of untransformed plants. Moreover, this hairy-root concentration was about six times higher than that reported from *A. reptans* hairy roots by Tanaka and Matsumoto (1993b). Based on these results, we believe that our hairy-root transformation system for *A. multiflora* is highly efficient, with the proven ability to produce more 20-HE than from wild-type roots.

Here, we have described the establishment of an efficient transformation system for 20-HE production from *A. multiflora*, using the *A. rhizogenes* A4 strain. We conducted PCR analysis and DNA-DNA hybridization to confirm the integration of T-DNA into the *Ajuga* genome. One transformed line in particular, AM5, showed both the fastest growth of hairy roots and the highest production and content of 20-HE when compared with the performance of wild-type roots. Therefore, the objective in future studies will be to optimize culturing conditions, such as media components and elicitors, in order to scale-up 20-HE production in that species.

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