EFFICIENT GENETIC TRANSFORMATION OF *IMPATIENS HAWKERII* BULL. (BALSAMIACEAE) USING *AGROBACTERIUM RHIZOGENES*

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Abstract — Transformation of *Impatiens hawkerii* Bull. mediated by *Agrobacterium rhizogenes* strain A4M70GUS was studied. Hairy roots developed 10 days after inoculation were excised from the shoot explants and transferred onto Murashige and Skoog's (MS) basal medium lacking plant growth regulators. More than 20 hairy root clones were established and eight of them were further analyzed. Each clone differed significantly from the others in growth capacity and lateral branching. Clone C2 showed the highest biomass (20.6 g L^{-1}) as well as the highest number of lateral roots (37 ± 2.2). The transgenic nature of the established hairy root clones was confirmed by GUS assay and PCR analysis. In conclusion, hairy roots were developed for the first time in *I. hawkerii* Bull., and transgenic hairy root clones showed a distinct morphological nature and growth patterns.

Key words: *Impatiens hawkerii,* genetic transformation, hairy roots, ornamental plant

INTRODUCTION

New Guinea impatiens (*Impatiens hawkerii* Bull.) belongs to the Balsamiaceae family and is a potted-flower plant, with many cultivars used in gardening. This species is very popular in the floriculture industry because of its appearance, extra-large attractive flowers, and good branching (Morgan, 2007). New Guinea impatiens can only be propagated by vegetative cuttings from stock plants. This explains the great dissemination of pathogens such as viruses, which are transmitted directly or indirectly to the planting material. New Guinea impatiens can suffer from tomato spotted wilt virus (TSWV) and impatiens necrotic spot virus (INSV) carried in the tissues. The development of host resistance to TSWV and INSV is a high priority for New Guinea impatiens breeding in the world. The introduction of foreign genes into plants through genetic transformation is a highly promising alternative to conventional breeding. Different plant tissues from most dicotyledonous and monocotyledonous species have shown the capacity to be infected and genetically transformed by Ri T-DNA of *Agrobacterium*

rhizogenes, which results in formation of hairy roots (Venna and Taylor, 2007). During interaction between the bacterium and plants, partial transfer of the agrobacterial plasmid Ri T-DNA into the chromosome of the plant cell occurs. This plasmid contains *rol*A, *rol*B, *rol*C, and *rol*D genes, which are able to induce neoplastic transformation of plant cells (Christey, 2001). Recently, *A. rhizogenes*-mediated transformation and transformation with *rol* genes from *A. rhizogenes* Ri T-DNA were obtained for several ornamental species in order to improve and modify plant characteristics such as growth morphology, flower shape or color, and resistance to pathogens or abiotic stresses (Casanova et al., 2005). Although micropropagation of New Guinea impatiens has been reported before now (Stephens et al., 1985, 1992), no data are available on genetic transformation by *A. rhizogenes*. The purpose of the present study was to induce hairy roots of *I. hawkerii* Bull. using *A. rhizogenes* strain A4M70GUS. Such hairy roots could then be used for transgenic plant production and further studies on genetic improvement of this important ornamental species.

MATERIAL AND METHODS

Plant material

Plants of New Guinea impatiens (*Impatiens hawkerii* Bull.) cv. 'Tango' were kindly provided by commercial growers in Serbia. Potted plants cultivated in the greenhouse were used in the present study. Stems with leaves (approximately 15-20 mm in length) were dissected from the greenhouse plants and then surface sterilized with commercial bleach solution containing 3% active chlorine for 15 min. Following three rinses with sterilized distilled water, explants were placed on solid MS basal medium (Murashighe and Skoog, 1962) with 30 g L^{-1} sucrose and 7 g L^{-1} agar lacking plant growth regulators.

Bacterial strain

The agropine-type *A. rhizogenes* strain A4M70GUS was used in the present study. The bacterial strain was cultured on solid YEB medium at pH 7.2 (Van Larebeke et al., 1977) supplemented with 100 mg L^{-1} neomycin. The strain was grown for 48 h at 28 ± 2°C and cultures were maintained at 4°C. The bacterial suspension used for transformation was prepared by transferring a single bacterial colony to 10 ml of liquid YEB medium. Bacterial growth was measured by its optical density (OD 600 nm = 0.6) using a spectrophotometer.

Shoot explants with three noduses obtained from *in vitro* grown plants were inoculated with the bacterial strain A4M70GUS. Shoot explants were pricked between the first and second nodus with a sterile needle dipped beforehand in bacterial suspension. Explants pricked with sterile distilled water or YEB medium with 100 mg L^{-1} neomycin served as controls.

Establishment of hairy root cultures

Formed roots were excised from the shoot explants and transferred onto MS basal salt medium containing 30 g L^{-1} sucrose, 7 g L^{-1} agar, and 200 mg L^{-1} cefotaxime (Torical, Jugoremedia, Zrenjanin, Serbia). *Agrobacterium*-free root tips (15 mm in length) of those clones showing fast growth and lateral branching were selected and placed on medium of the same composition lacking the antibiotic. Hairy root cultures were maintained by subculturing root tips every 4 weeks on the same medium.

GUS assays

Hairy root culture GUS assays were performed according to the protocol described by Gallagher (1992). For analysis of hairy roots, plant material was incubated in X-Gluc buffer [2 mM X-Gluc, 100 mM sodium phosphate buffer (pH 8.0), 10 mM EDTA, 1 mM potassium ferricyanide, and 1 mM potassium ferrocyanide] for 24 h at 37°C in the dark. After staining for GUS activity, five representative root samples (cut at 1.5 cm from the root tip) were fixed in FAA containing 5.4 ml of formalin, 65.5 ml of ethanol, 5 ml of glacial acetic acid, and 24 ml of distilled water per 100 ml (Jensen, 1962). Root tips were cut and embedded in Histowax (Sweden). At the beginning of this process, the samples were dehydrated in 2-h steps through a graded series of ethanol (50, 70, 96, and 100%). The samples were then embedded in Histowax for 72 h at 58°C. Slices (15 μm) were cut at room temperature using a rotary microtome (Reichert-Vienna) equipped with type-819 microtome blades (Leica, Germany). Slices were stretched on a bath of distilled water and mounted on slides. Sections were mounted in Canada balsam before microscopic examination (Leica, Leitz DMRB, Germany).

Confirmation of transformed nature of hairy roots by PCR

Genomic DNA was extracted from hairy roots frozen in liquid nitrogen using the CTAB DNA isolation method (Zhou et al., 1994). The DNA from nontransformed *Impatiens hawkerii* roots and plasmid DNA were used as negative and positive controls, respectively. The following primers specific to the *uid*A gene were used for PCR: 5'-TAG CGG GAC TTT GCA AGTG -3'; and 5'-GTT TTT GCA GCA GAA AAG CC- 3'. The amplification protocol for the *uid*A gene was as follows: 5-min melting at 95°C, followed by 35 cycles of 1-min melting at 95°C, 1-min annealing at 62°C, 2-min elongation at 72°C, and final elongation for 10 min at 72°C. The primers used for amplification of the approximately
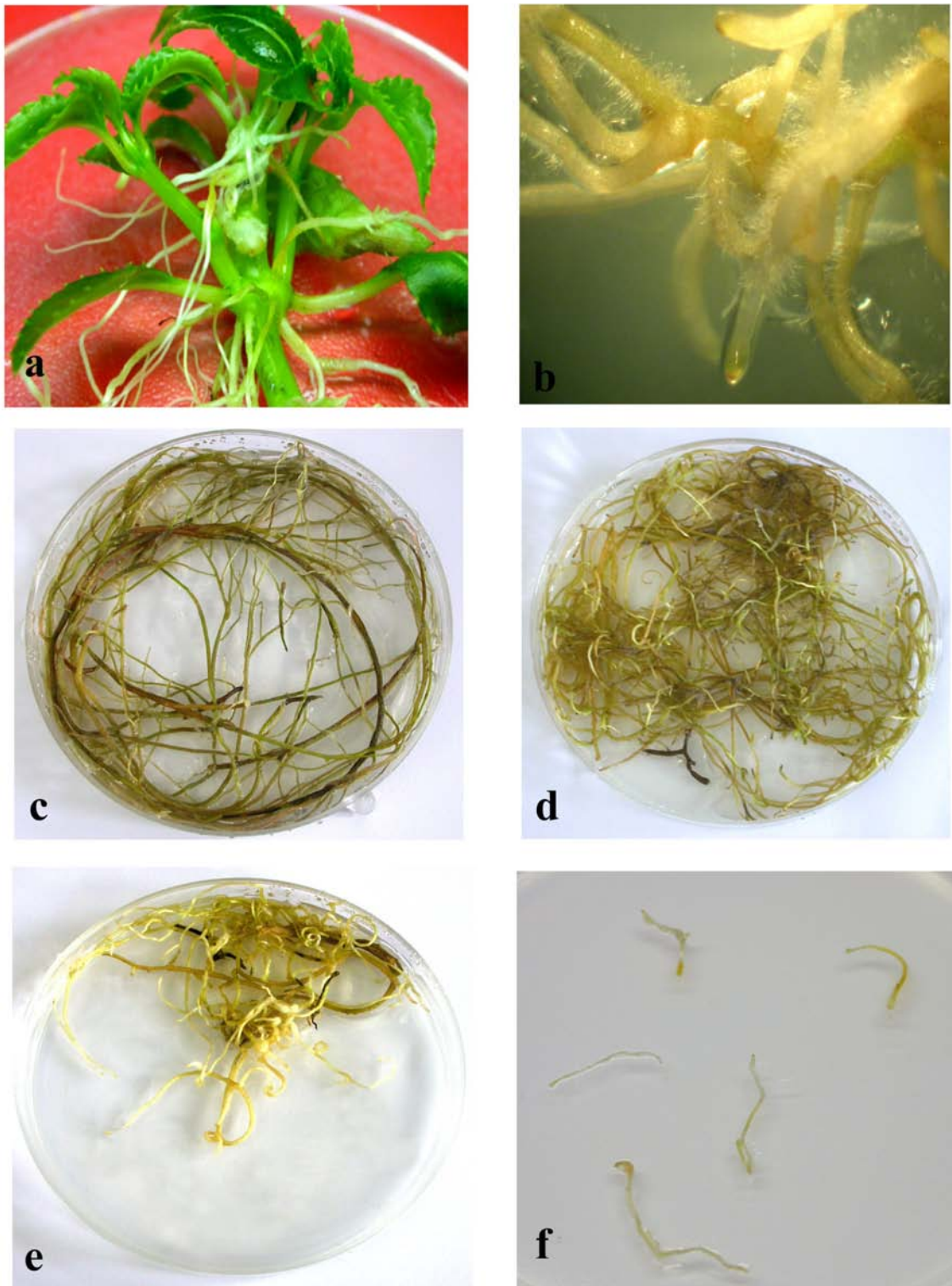
Fig. 1. Induction and establishment of hairy root culture from shoot explants of *I. hawkerii* Bull. in response to *A. rhizogenes* strain A4M70GUS. a) Micropropagated shoot of *I. hawkerii* Bull. with developing hairy roots; b) detail of well-branched hairy roots; c-e) growth of C1 (c), C2 (d), and C19 (e) hairy root clones; f) culture of nontransformed roots.
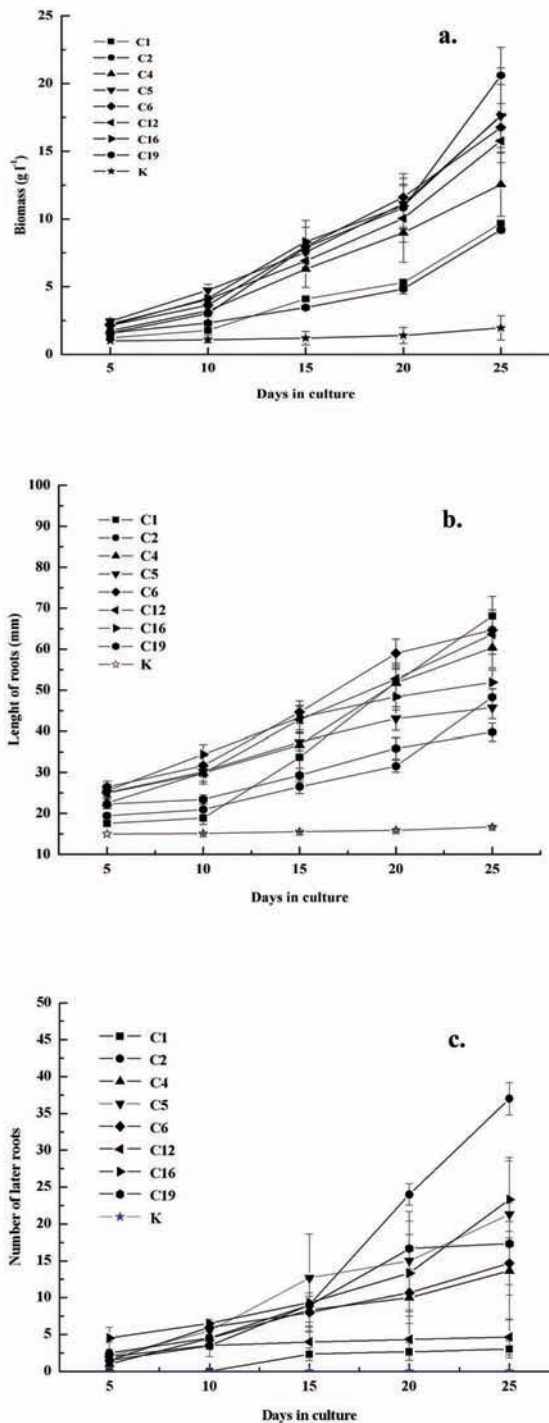
Fig. 2. Growth patterns of hairy root cultures of *I. hawkerii* Bull. on plant growth regulators-free medium after 25 days. a) Biomass increase of hairy root and nontransformed root cultures; b) linear growth of hairy and nontransformed roots; c) number of lateral roots developed from hairy and nontransformed roots during the culture period. The vertical bar is the mean ± SE.

350-bp fragment of the *rol*C gene were: 5'-TAC GTA GAC TGC CCG ACG ATG ATG-3'; and 5'-AAA CTT GCA CTC GCC ATG CCT CAC-3'. The amplification conditions consisted of 5-min melting at 95°C, followed by 35 cycles of 1-min melting at 95°C, 1-min annealing at 55°C, 2-min elongation at 72°C, and final elongation for 10 min at 72°C. The obtained PCR products were analyzed by electrophoretic separation on 1.5% agarose gels (m/v) in 1×TBE buffer and staining with ethidium bromide (Sambrook et al., 1989).

### *Growth patterns of hairy roots*

Once the transformation of hairy root clones was confirmed, they were characterized on the basis of their growth dynamics. For testing biomass accumulation, 50-mg batches of root tips of eight clones and controls were subcultured in 15 ml of MS liquid medium lacking plant growth regulators and grown on a rotary shaker set at 60 rpm in the dark at 25 ± 2°C. Roots from each sample were washed with sterile water and blotted dry after which their fresh weight (FW) was recorded at intervals of 5 days for a total period of 25 days. In addition, the linear growth of hairy roots and number of lateral roots on the primary roots of each sample were estimated at the same intervals. All experiments included at least five replicates, and the data are presented as means ± SE of three independent experiments.

RESULTS AND DISCUSSION

Shoot cultures of *I. hawkerii* Bull. were easily established and maintained for two years under *in vitro* conditions. Shoot explants taken from 4-week-old plants were inoculated with *A. rhizogenes* strain A4M70GUS. As a result of transformation, hairy roots started appearing from wounded regions of the explants 10 days after inoculation (Fig. 1A). They showed typical hairy root characteristics, including lateral branching with many root hairs and the plagiotropic growth habit of the ornamental plant (Fig. 1B). The transformation frequency was 98%. Following transfer to hormone-free MS basal medium, more than 21 clones were established. Among them, eight clones (designated as C1, C2, C4, C5, C6, C12, C16, and C19) were selected for further
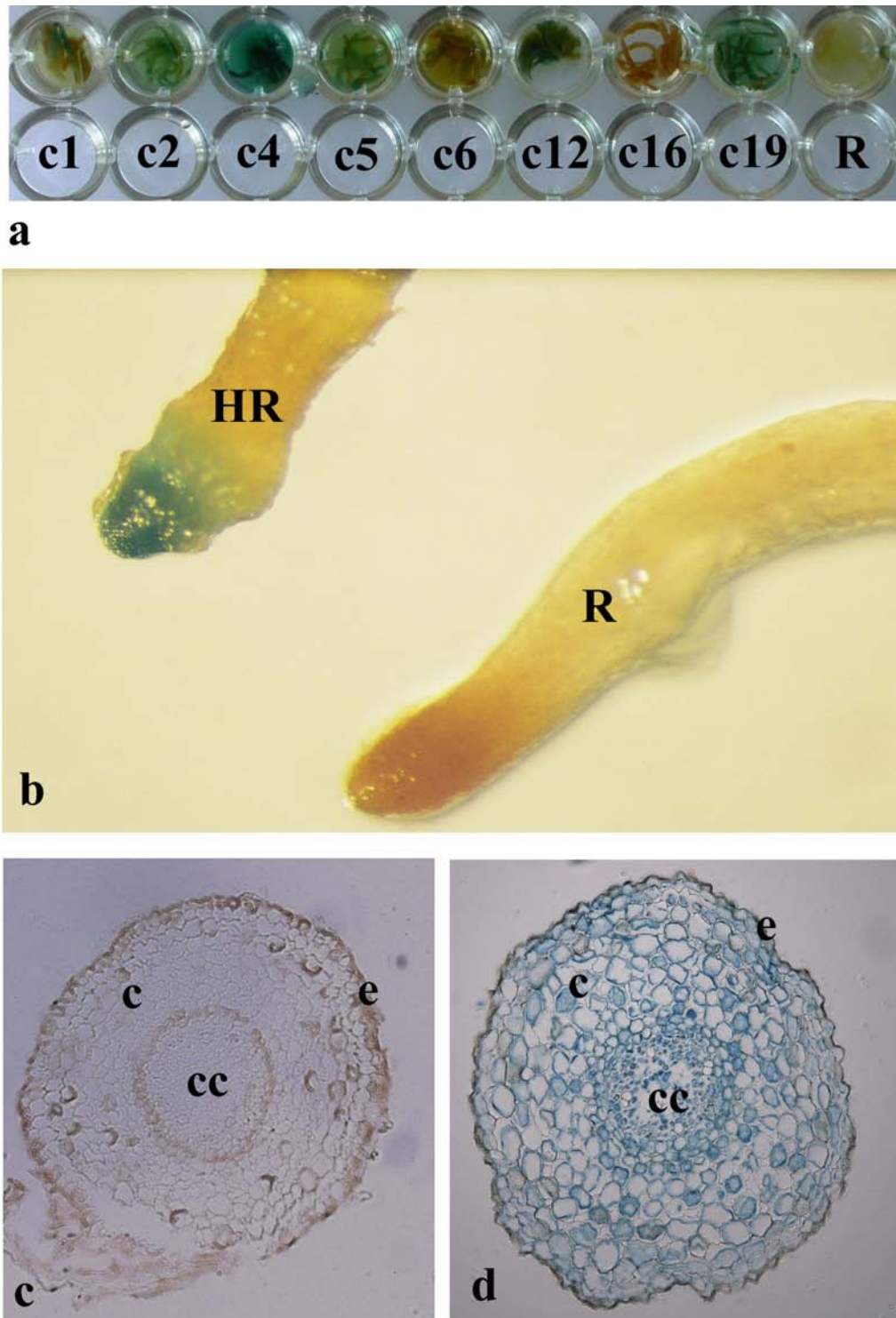
Fig. 3. Histochemical localization of GUS activity in roots of *I. hawkerii* Bull. a) Histochemical staining with X-Gluc (the blue color shows a positive reaction in all clones of hairy roots; b) histochemical staining for GUS activity in hairy (HR) and nontransformed (R) roots; c) histological cross-section of hairy root after staining for GUS activity; d) cross-section section of nontransformed root. e - epidermis; c - cortex; cc - central cylinder.
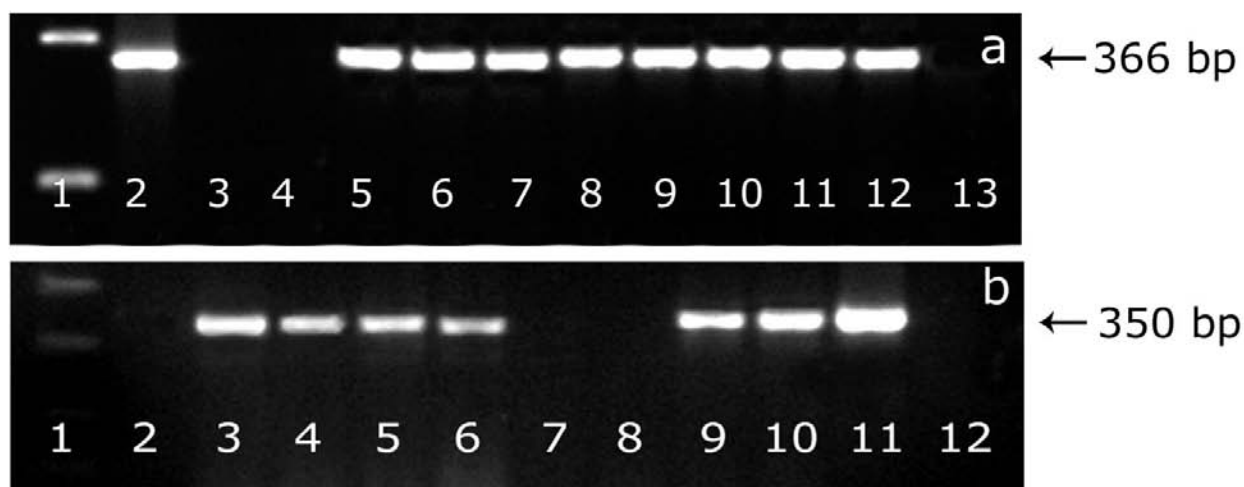
Fig. 4. Use of PCR to detect Ri-plasmids of *A. rhizogenes* A4M70GUS in hairy root clones of *I. hawkerii* Bull. a) uidA: lane 1 - DNA ladder; lane 2 - *A. rhizogenes* A4M70 GUS; lanes 3-4 - negative controls; lane 5 - C1 clone; lane 6 - C2 clone; lane 7 - C4 clone; lane 8 - C5 clone; lane 9 - C6 clone; lane 10 - C12 clone; lane 11 - C16 clone; lane 12 - C19 clone; lane 13 - blank. b) rolC: lane 1 - DNA ladder; lane 2 - negative control; lane 3 - C1 clone; lane 4 - C2 clone; lane 5 - C4 clone; lane 6 - C5 clone; lane 7 - C6 clone; lane 8 - C12 clone; lane 9 - C16 clone; lane 10 - C19 clone; lane 11 - *A. rhizogenes* A4M70 GUS; lane 12 - blank.

investigation. All hairy clones were GUS-positive after being tested with the GUS histochemical assay. No stain could be observed in nontransformed roots (Fig. 3A). Strong GUS staining occurred mainly in the root cap and in the region of cell division (Fig. 3B). Histology of hairy roots grown under the same conditions was compared with nontransformed roots. Nontransformed roots showed a typical root structure consisting of central cylinder, endodermis, and 8-10 cell layers of cortical parenchyma and epidermis (Fig. 3C). In hairy roots, the cells of cortical parenchyma were large-vacuoled, and great intercellular spaces were observed (Fig. 3D).

To confirm transformation, genomic DNAs of all established clones were analyzed by PCR amplification using *uid*A primers. Whereas control plants did not show any positive signal for the 366-bp fragment of the GUS gene, all eight hairy root clones were positive (Fig. 4A), indicating integration of this gene into the host cell genome. The presence of the Ri T-DNA plasmid in hairy root cultures was confirmed employing PCR amplification of the *rol*C gene. A distinct band of ~ 350 bp corresponding to the *rol*C gene was obtained in DNA of the C1, C2, C4, C5, C16, and C19 clones (Fig. 4B). On the basis of morphological traits of hairy root clones, three

different phenotypes can be distinguished. The first type (clone C1) showed very fast linear growth with no lateral roots (Fig. 1C). In the second type (the C4 and C19 clones), the short and thick hairy roots exhibited weak growth but good lateral branching (Fig. 1E). Vigorously growing thin roots were described as a third phenotype (C2, C5, C6, C12, and C16) (Fig. 1D). Roots were green or pale-yellow. Distinct differences in growth capacity were observed among individual hairy root clones (Figs. 2A, 2B, 2C). Among the eight clones extensively studied in terms of biomass production, root length, and lateral branching, the C2 clone grew more rapidly and branched more frequently than all the others (Figs. 2A, 2C). As opposed to C2, growth and branching analyses showed that the C1 clone had the lowest response, but this clone elongated rapidly and starting from the 10th day outgrew all the other clones (Fig. 2B). The highest number of lateral roots was recorded in the C2 and C19 clones (Fig. 2C). All eight clones exhibited moderate production of biomass: the ratios of final fresh weights to their initial fresh weights were 12 to 17 for each subculture. Nontransformed root grew slowly and showed a low growth rate (1.90). The obtained growth rates were comparable to those of hairy roots of *Linum flavum* (Lin et al., 2003) and *Atropa belladonna* (Bonhomme

et al., 2000). Different characteristics among hairy root clones might be related to variation of the T-DNA insertion, i.e., of the copy number and location of *Ri* genes integrated into the plant genome (Doran, 2002). The variation in morphological and growth characteristics can also be attributed to the effect of *rol* genes. The different levels of their expression among hairy root clones could alter the endogenous auxins/cytokinins ratio or sensitivity to the previous plant growth regulator balance of each genotype (Shen et al., 1988). Endogenous auxins had specific effects on hairy root growth, increasing branching and stopping linear growth. Only a few studies have been devoted to analyses of hairy root growth (Bonhomme et al., 2001; Tanaka et al., 2001) and lateral branch development (Morgan and Shanks, 2000; Balvanyos et al., 2001). In the present study, it was evident that the obtained hairy root clones of *I. hawkerii* Bull. have distinct morphological alterations, and the precise causes of those differences will be further studied. Hairy root cultures following *A. rhizogenes* A4M70GUS-mediated transformation have been established in many plant species, e.g., *Gentiana* spp. (Momčilović et al., 1997), *Lotus corniculatus* (Nikolić et al., 2003-2004), and *Hypericum perforatum* L. (Vinterhalter et al., 2006). Using the same strain, we now report successful transformation of an important ornamental plant, *I. hawkerii* Bull. The efficient transformation protocol achieved here offers possibilities of generating *I. hawkerii* Bull. transgenic plants with improved virus resistance or certain important ornamental traits.

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ЕФИКАСНА ГЕНЕТИЧКА ТРАНСФОРМАЦИЈА *IMPATIENS HAWKERII* BULL. (BALSAMIACEAE) ПОМОЋУ *AGROBACTERIUM RHIZOGENES*

СНЕЖАНА МИЛОШЕВИЋ, АНГЕЛИНА СУБОТИЋ, А. ЦИНГЕЛ, СЛАЂАНА ЈЕВРЕМОВИЋ и СЛАВИЦА НИНКОВИЋ

Одељење за физиологију биљака, Институт за биолошка истраживања „Синиша Станковић“, Универзитет у Београду, 11060 Београд, Србија

Проучавана је генетичка трансформација *Impatiens hawkerii* Bull. посредством *Agrobacterium rhizogenes* соја A4M70GUS. Десет дана после инокулације формирали су се трансгени коренови на експлантатима изданака, а затим гајени на Murashige and Skoog's (MS) основној хранљивој подлози без биљних регулатора растења. Успостављене су културе више од 20 клонова, а 8 је даље анализирано. Клонови су се међусобно значајно разликовали у односу на капацитет растења и бочног гранања. Клон C2 је имао највећу биомасу (20.6 g L^{-1}), као и највећи број бочних коренова (37 ± 2.2). Присуство страних гена у клоновима трансгених коренова је потврђено GUS есеја и PCR анализе. Трансгени коренови су добијени први пут код *Impatiens hawkerii* Bull. и показују значајне разлике у морфологији и параметрима растења.