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Expression and Conservation of *rol-genes* in Rue, *Ruta graveolens* L., Plants Regeneration from Hairy Roots

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Abstract

In this investigation hairy roots were induced on leave petioles of *Ruta graveolens* L. plants. These roots were white in color and negative geotropism. Paper electrophoresis of hairy roots extract resulted in the separation of black spots of agropine . These transformed roots were cured from *Agrobacterium rhizogenes*, some cultures of these roots were directly produced shoots, and other was orientated to produce callus. Subsequently this callus was capable to produce numerous regenerates. Both groups of shoots were rooted easily and successfully adapted to field conditions. Evidences of molecular biology assessment, including isolation and amplification(PCR) of DNA of each transformed tissues and plants coupled with DNA electrophoresis data proved the transfer of *rol*-genes, except *rol B* , in the regenerated plants.

Conclusion: The genetic modified *R. graveolens* plants possess unique characters due to the conservation of *rol-genes* in their genomes.

Key words: *Ruta graveolens, Agrobacterium rhizogenes R1601, Agropine, PCR, rol-genes.*

Introduction

A recent study reported that *Ruta graveolens* L. plants reacted easily in culture, acceptable to many *in vitro* techniques and possess high regeneration capability[(1)] . Also plants were regenerated from leaf segments derived callus[(2)] and from axillary shoot multiplication[(3)]. It is well-known that this medicinal plant is rich with different industrial plant products, such as alkaloids, coumarine, flavonoids[(4)] and rutin[(5)]. The present study was designed whether *Agrobacterium rhizogenes* R1601, the natural vector, is efficient to create genetically modified tissues and whether genetically engineered plants can produced from them. In such studies experiments involved to demonstrated the transfer of T-DNA, synthesis of agropine, isolation of genomic-DNA of plant, amplification plant DNA by PCR coupled with gel electrophoresis of the amplified DNA [(6)]. The aim of this research is to specify and confirm which *rol*-gene(s) was transferred and expressed.

Materials and Method

Preparation of bacterial suspension .

Agropine type *Agrobacterium rhizogenes* R1601 harboring Kana.^{Rest} and Carb.$^{Res+}$ genes, as genetic markers, on Ri-plasmid was supplied from Prof. E.G. Nester(Washington university, U.S.A.). This strain was grown in agar solidified APM medium [(7)], provided with 100 mg.L^{-1} of each Kanamycin and carbencillin. Agrobacterial suspension was prepared using single colony used to inoculate 50 ml of liquid APM medium supplied with the same above antibiotics. Cultures were kept on rotary shaker 100 rpm for 72 h.. Inoculum was harvested and the precipitated bacterial cells was resuspended in 10 ml liquid APM. Optical density was adjusted to 1.90[(8)].

Direct injection of leaf petioles with *A. rhizogenes* R1601.

Leaves petioles of 3.0 cm length were excised from two years old Rue (*Ruta graveolens* L.) plants, washed with water and surface sterilized through soaking in the sterilizing solution consist of 1:2 (v:v) of sodium hypochlorite NaOCl: water for 15 min.. Explants were washed thoroughly with sterilized water three times 5 min./each time[(1)] . The tip needle tip was immersed into the inoculum and directly inject the top ends of the sterilized petioles in 2-3 sites. Each inoculated petiole was

cultured in vertical position into 25 ml of agar solidified MSO medium contained in 100 ml glass vessel. Hairy roots developed on inoculated samples were excised and transferred to agar solidified MSO medium containing gradual conc. of Cefotaxime to eliminate the habitant agrobacterium, as previously reported[9] .

Detection of agropin in hairy roots by paper electrophresis.

Samples of bacterial-free young hairy roots were cut from their cultures and grinded in eppendrof tubes in the presence of 100 µl 0.1N HCl. The mixture was centrifuged at 6000 rpm for 20 min., volumes of 30 µL of each clarified sample and of standard agropin were spotted on the chromatogrphy paper as described [10].

Plant regeneration from transformed tissues.

Cluster of young hairy roots were transferred to 20 ml of agar solidified MS medium. Friable callus of white-yellow color was developed. Subsequently this callus was differentiated producing number of shoots. In other cultures shoots were produced from the green-tips of hairy roots. Both types of shoots were excised and each shoot was plunged vertically in the rooting medium MS+0.5 mg.L^{-1} IBA [11,12] . All shoots were readily rooted, and adapted using peat-moss medium .

Conservation of *rol-genes* in genomic DNA of transformed plant.

This involved intensive laboratory work including:

Isolation of DNA: The extraction solution CTAB(Cetyltrimethyl Ammonium Bromide)was used for DNA isolation from transformed plant [13]. Purity and concentrations of the isolated DNA were determined at 260 and 280 ηm [14] .

Gel electrophoresis of the amplified isolated DNA: One gram of agarose (Vivantis LE grade Agrose, USA) was dissolved in 100 ml of IX TBE solution. The melted agarose at 40ºC was carefully poured in the tray. Gel layer thickness was 8 mm and of dimension 8.5×10cm. After complete solidification of gel the plastic comb was removed carefully, and the layer was put in the tank (Electrophorasis MS MINI U.K.) in the presence of 250 ml of the extraction solution 1X TBE. The loading samples was each consisted of 8.0µL of DNA mixed with 2.0 µL of loading buffer. One of the terminal well was loaded with 3.0 µL of the ladder λ DNA 50 bp DNA, total size 1350pb. At the end of samples loading electrical current of 50 volt for 90 min. was pass through. Then, remove the layer and transfer to dish containing 500 ml of ethidium bromid stain solution of final conc. 0.5 mg.ml^{-1} for 40min.. Finally examine the layer by UV light of 300 ηm to visualize the separated bands.

Polymerase Chain Reaction test: To detect the putative transfer of *rol*-gene(s), from T-DNA and their integration into plant genomic DNA the specific primers mentioned below (Table 1) used.

Table 1: Types of specific primers, their sequences and molecular weights and Kana.$^{Res+}$ *rol*-genes .

primers	Primers sequences (3' 5')	MW. (bp)	suppliers
Kana.$^{res+}$	F: TAGTCCTGTATCGCAACCGA R: TAGTCTCGTCGGCTAACAGA	593	Geneaid Biotech Ltd.
rol A	F: ATTAAGGTAAGACTTTGTAC R: CCTAATTATGCGTGCACCGA	450	
rol B	F: GTTAAACCCTAGGTAACACT R: TTCGGATTTCGGCTGAACTT	850	
rol C	F: TGTGTCCAGCAGAAGTCGGTA R: ACGTTCAAAGGTTAGCCGATT	650	
rol D	F: AGCGTTTCAACAAACCGGTA R: GCTACCTTGTGCCCGTAATT	1000	

The reaction mixture was prepared in premix tubes containing 5.0 µl of the mixture (Taq DNA polymeras + dNTPS + Tris-HCl + KCl + $MgCl_2$ buffer X10).To this added 1.0 µl of the specific essential primer of conc. 10 Becamol Microliter $^{-1}$. Similar volume and conc. of the reverse primers and of the temple DNA at conc.50 ng μl^{-1}. Complete the final volume to 20 µl by addition 9.0 µl of distilled, deionized sterilized water. Tubes were inserted into the thermal polymer to begin the amplification reaction following the same conditions previously mentioned[15] .

Results

- **Establisment of cured hairy roots cultures.**

The response of petiole explant to direct injection by *A. rhizogenes* R1601 resulted in the formation of adventitious roots on both inoculated and non-inoculated sites (Table 2)

Table 2: Induction of hairy roots on leaf petiole explants of *R. graveolens* to direct injection by *A. rhizogenes* R1601.

Explants	Mean No. of explants inoculated : Responsed	Hairy roots Mean No. : length(cm)
Inoculated	16:9	2.75 : 1.5
Non-inoculated (control)	16 : 0	0 : 0

Hairy roots began to form as tiny bodies (Fig. 1: A), developed to white and fine hairy roots (Fig, 1: B).These hairy roots were excised as cluster or single root. They cultured on agar-solidified MS medium and were negatively geotropism as growing producing mass of these hairy roots. They grew rapidly on the surface of solid MSO medium (Fig. 1: C), whereas samples of excised normal roots failed to continuing their growth. Data indicated that gradual transfer of hairy roots culture three times on100, 200 and 300 $mg.l^{-1}$ of cefotaxine led to the production of bacterial free culture of hairy roots (Fig 1: D). Overall these results considered the first signs of transformation of these roots.

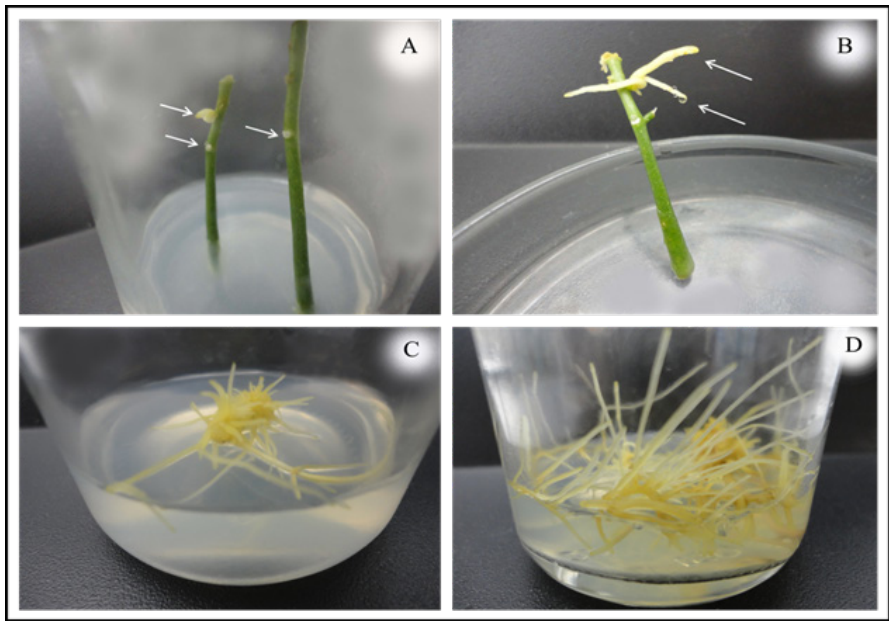

Fig. 1: Production of "agropine- positive" transformed hairy root of *R. graveolens* by *A. rhizogenes* R1601. (A): Formation of small bodies (arrowed) on inoculated petioles. (B): Development of single hairy roots (arrowed) from bodies in (A). (C): Cluster of hairy roots grew on the surface of agar-solidified MSO medium. (D): Development of hairy roots in (C) note their negative geotropism growth.

Detection of the synthesized agropine.

Examination of electrophorctogram proved the separation of agropine that synthesized in these hairy roots. The black spots (approx. 2.0 cm diam.) were corresponded in positions and diam. to spots of standard agropine. This result represent an additional proof that this type of roots and their derived callus are genetically transformed by agropine type *A. rhizogenes* R1601 used in this study.

Regeneration of plant from transformed tissues.

The results revealed that culture of 'agropine positive' hairy roots of *Ruta graveolens* grown on solid hormone-free MS medium express their totipotency and producing number of shoots . Other cultures of hairy root were orientated to the spontaneous formation of callus, Subsequently, this type of callus acquired the green color and numerous shoots were differentiated.

The results indicate that shoots regeneration from bud developed on hairy root culture (Fig. 2. A), also regenerated directly from hairy roots (Fig. 2. B), and from hairy-root derived callus (Fig. 2. C,D). All types of shoots were readily rooted in agar-solidified MS medium supplemented with 0.5 mg L^{-1} IBA(Fig.2.E). Plantlets were adapted and successfully acclimatized to field condition (Fig. 2.E). As they continuing growth clear morphological variations were found comprised the leave size, flower number, shape and flowering period.

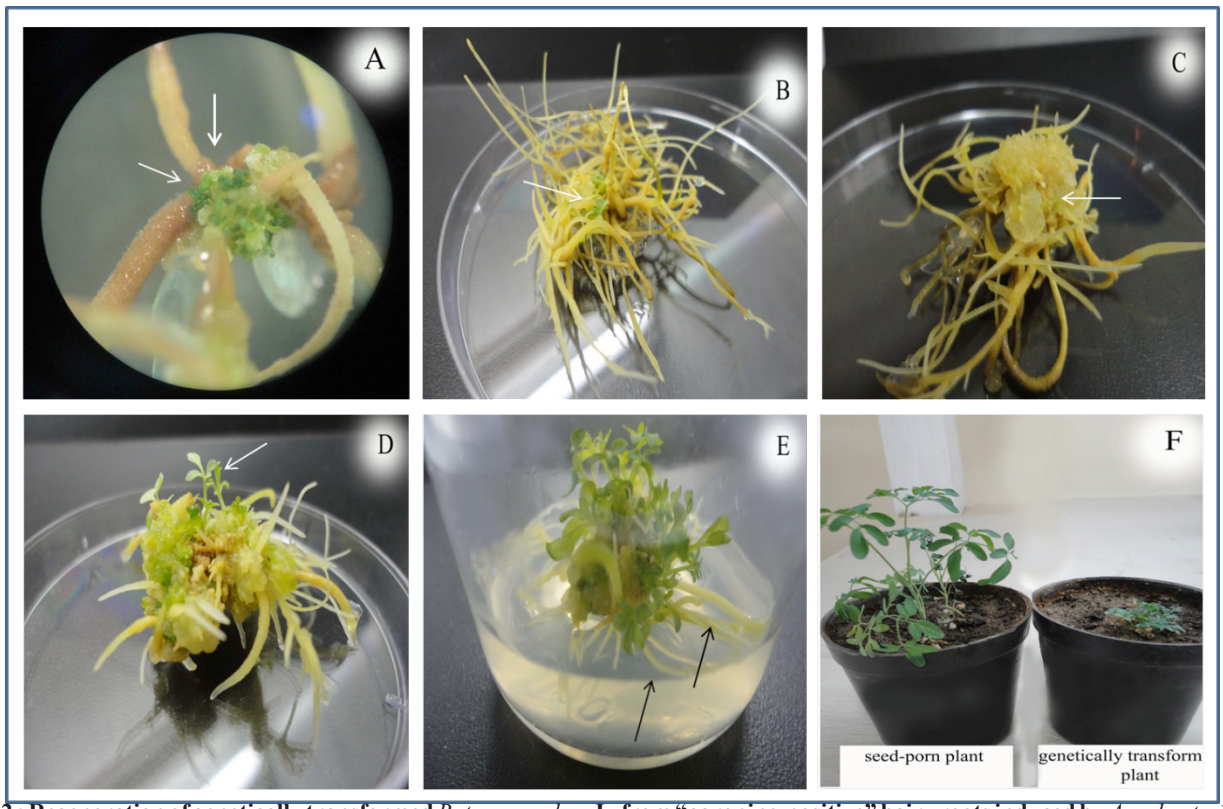

Fig. 2 : Regeneration of genetically transformed *Ruta graveolens* **L. from "agropine-positive" hairy roots induced by** *Agrobacterium rhizogenes* **1601**.

(A): Shoot bud(arrowed) emergence hairy root grown on solid MSO medium start producing shoots (B): Direct shoot regeneration(arrowed) from hairy roots grown on MSO. (C): Spontaneous callus(arrowed) formation from hairy root grown on MSO. (D): Indirect shoot regeneration(arrowed) from callus grown on MSO.(E): Rooting of shoots(arrowed) in (A), in MS medium provided with 0.5 mg.L^{-1} IBA. (F): Transfer and acclimatized plants to field conditions.

Concentrations and purity of DNA.

Data showed that concentrations of DNA of transformed plants regenerated directly from transformed hairy roots and callus was exceed the conc. of DNA isolated from normal callus and plants (Table 3).

Table 3: Concentrations and purity of genomic DNA of *Ruta graveolens* L. plants transformed by *Agrobacterium rhizogenes* 1601.

Source of DNA	Concentration (ng / µl)	purity
Leaves of transformed plant	102.9	1.94
Leaves of normal plant	33.7	1.95
Roots of normal plant	82.3	1.87

Detection of *rol-genes* in genomic DNA of transformed *Ruta graveolens* plant.

Examination of electrophoresis gel layer of PCR products showed the separation of single band of amplified DNA of callus produced from transformed hairy roots. Its M.wt. was equal to the M.wt. of the specific primers for each of Kana.$^{Res+}$ *rol A*, *rol B*, *rol C*, and *rol D* genes as single bands. The separation of these bards confirmed the presence of the responsible genes in cells genome of transformed hairy root, callus and plants regenerated from this type of callus. In the mean time the absence of such bands of amplified DNA isolated from non-transformed callus and plants donate an additional evidence in this aspects(Fig.3).

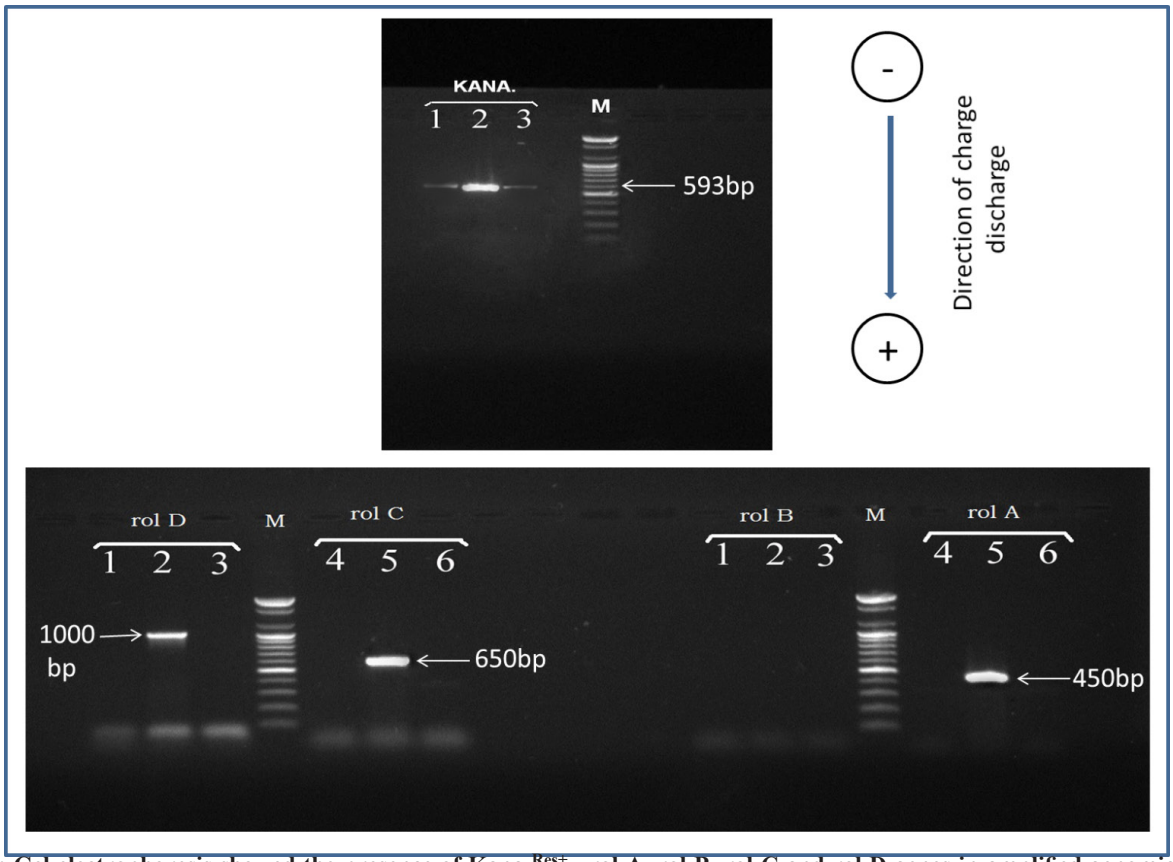

Fig. 3: Gel electrophoresis showed the presence of Kana $^{Res+}$, rol A, rol B, rol C and rol D genes in amplified genomic DNA isolated from leaves of genetically transformed Ruta graveolens L. by Agrobacterium rhizogenes R1601.

Lane (M): Lambda DNA.

Lane (1, 4): amplified DNA isolated from seedling leaves.

Lane (2, 5): amplified DNA isolated from leaves of genetically transform plant.

Lane (3, 6): amplified DNA isolated from seedling roots.

Those results strongly ensured that transformed hairy roots and callus conserved Kana $^{Res+}$ and *rol*-genes group except *rol B* . This indicate their transfer from the bacterial vector to genome of the recipient cells. The

expression of Kana $^{Res+}$ gene enable hairy root derived callus to continue growth on MS medium contained with kanamycin. Whereas expression of *rol*-genes encouraged synthesis of agropine and the development of some variations on plant morphology.

Discussion

The production of genetically modified Rue, *Ruta graveolens* L., plant via *A. rhizogenes* 1601 mediated transformation probably explained the capability of this bacterial vector the rapid transformation of many plant species such as *Vigna radiate*[12] , tomato and potato[16]. In this study data proved the successful interaction between *A. rhizogenes* and petioles leaf, and the incidence of hairy roots pointed out the first sign of biotransformation[17] . During this stage acetosyringone compound release induce the transfer of T-DNA to plant cells[18]. Additionally, this compound stimulate T-DNA genes, of Ri-plasmids, to fuse with cell genome producing a genetically transformed clone[19]. This is supported the spontaneous induction of callus[20] on hormone –free MS medium as occurred with *Solanum nigrum*[21] and *Rubia akane* L.[22] plants. Bacteria was eradicated by the use of cefotaxime antibiotic which recommended by several studies[15]. The expected explanation of agropine synthesis in hairy roots and its callus emphasized that *rol D* gene controlled it's synthesis when *Agrobacterium*-plant functional interaction only take place[23] . In the present work production of genetically- transformed Rue plants from "agropine-positive" tissues is an expected status. At the same time it represented an advanced interested phenomenon. The expectation was not to obtain high transformation frequency (TF)as occured with carrot[15] plants.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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