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Regeneration of *Ruta graveolens* Transgenic Plants

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ABSTRACT

Here we report the construction of transgenic *Ruta graveolens* L. plants. The plants were obtained *via* direct shoot regeneration from leaves and petioles on Murashige and Skoog (MS) solidified medium supplemented with 6-benzyl-aminopurine (1.0 mg L^{-1}) and α-naphthylacetic acid (0,05 mg L^{-1}) in case of *A. tumefaciens*-mediated transformation as well as *via* direct shoot regeneration from rue "hairy" root culture on hormone free half-strength MS medium in case of *A. rhizogenes*-mediated transformation. Frequency of "hairy" root formation was up to 30%. All transgenic root lines were able to form shoots. Using of *A. tumefaciens* allowed to obtain transgenic rue plants with high frequency up to 100%. Indolil butyric acid addition to the medium in concentration 0.5 mg L^{-1} enabled transgenic plant rooting. The shoots regenerated from rue "hairy" root culture were more sensitive to the growth regulator addition and rooted in 2 weeks. Rooting of the shoots obtained after *A. tumefaciens*-mediated transformation was observed only in 6 weeks under these cultivation conditions.

Keywords: *Ruta graveolens* L., *A. tumefaciens, A. rhizogenes*, genetic transformation, plant regeneration, "hairy" root culture.

Introduction

Ruta graveolens L. is a medical plant of Rutaceae family. Numerous compounds of medical properties are known to be synthesized in rue plants. Rutin, alkaloids, essential oils and other chemicals were found in its aerial parts and roots (Orlita *et al*., 2008; Ekiert *et al*., 2005; França Orlanda *et al*., 2015). Rue extracts are characterized by antimicrobial (Ivanova *et al*., 2005; França Orlanda *et al*., 2015), antifungal (Meepagala *et al*., 2005), analgesic (Cunha *et al*., 2015) activity. The antioxidant and anti-inflammatory properties of *Ruta graveolens* L. were analysed earlier (Raghav *et al*., 2006). The study of its anti-tumor activity demonstrated that rue extracts were cytotoxic for lymphoma and carcinoma cells (Preethi *et al*., 2006). Ethanolic rue extracts were not toxic but caused the death of skin melanoma cells (Ghosh *et al*., 2015). So, *R. graveolens* plants could be used as a natural source for medical compounds production and may be used in pharmaceutical industry.

Biotechnological methods of plant improvement include construction of the transgenic plants of numerous species characterized by high level accumulation of valuable natural compounds (Sharafi *et al*., 2013; Zhang *et al*., 2014; Thiruvengadam *et al*., 2014; Majumdar *et al*., 2012). At the same time transgenic plants are able to synthesize the recombinant proteins of medical properties. So the transgenic plants are the promising source both of natural and recombinant biological active substances.

Transgenic plants construction was started at the end of 20th century. *Agrobacterium tumefaciens* and *A. rhizogenes* bacterial strains were used for this purpose. Plant transformation using *A. tumefaciens* strain resulted in direct (from plantlets) or indirect (from primarily formed callus tissue) shoot formation. Transgenic plants can also be obtained *via* shoot regeneration from "hairy" root culture initiated after *A. rhizogenes* – mediated transformation of plants. So, the shoot regeneration methods must be developed

both in case of *A. rhizogenes* or *A. tumefaciens* – mediated transformation. Different types of explants (leaves, roots, hypocotyls, petioles, stems) can be used for shoot regeneration. Methods based on using of growth regulators allow to obtain the regenerated shoots (Diwan *et al*., 2008; Ahmad *et al*., 2010).

A. tumefaciens-mediated transformation method was optimized for *R. graveolens* transgenic plants obtained *via* direct plant regeneration from hypocotyls (Lièvre *et al*., 2009). Transformation efficiency reached up to 22% in case of using for the transformation pTDE4 plasmid harboring genes encoding neomycin phosphotransferase and beta-glucuronidase. Hypocotyls, callus and shoots of *R. graveolens* were used for genetic transformation experiments but "hairy" root culture establishment appeared successful only after inoculation of hypocotyls with *A. rhizogenes* wild *strain (Sidwa-Gorycka et al., 2009).*

The aim of the present study was to obtain transgenic *R. graveolens* plants. We used *A. tumefaciens* GV3101 strain carrying plasmid *pCB124 (*Luchakivskaya *et al*., 2011*)* harboring target *ifn-α2b* gene coding human interferon-α2b synthesis as well the selective neomycin phosphotransferase II gene. We also used *A. rhizogenes* A4 strain for establishment of "hairy" root culture and obtaining of the regenerated transgenic rue plants.

Material and Methods

Plant material

Leaves and petioles of aseptic *Ruta graveolens* plants cultivated on half-strength Murashige and Skoog medium (1/2MS) (Murashige and Skoog 1962) were used for agrobacterial infection.

Determination of selective concentration of antibiotic kanamycin

To find out the optimal concentration of kanamycin for transformant selection we placed the shoots of *in vitro* rue plants on solidified ½MS medium with different concentrations of kanamycin (0–200 mg/l) ("Kievmedpreparat", Ukraine) and cultivated them in Petri dishes for 4 weeks. The medium with minimal concentration of kanamycin which inhibited growth of green plants was chosen as selective one.

Bacterial strains and plasmid vectors

A. tumefaciens GV3101 nopaline strain with the binary pCB124 vector was used for genetic transformation. The T-DNA of the vector contained the neomycin phosphotransferase selective *nptII* gene under the control of nopaline synthase promoter. The used vector carried the target *ifn-α2b* human interferon gene (pCB124 vector) driven by cauliflower mosaic virus *35S* promoter. We also used *A. rhizogenes* agropine A4 strain for rue genetic transformation and "hairy" root culture initiation.

The bacteria were cultivated overnight at 28°C in liquid LB medium (10 g/l of casein hydrolyzate, 5 g/l yeast extract, 10 g/l NaCl, pH 7.2, 100 mg/l carbenicillin and 50 mg/L rifampicin) on rotary shaker (200 rpm). After overnight cultivation the bacterial cultures were resuspended in liquid MS medium (OD_{600} 0.5–0.6).

Genetic transformation, selection and regeneration of transformants

The petiole and leaf segments were inoculated by the bacterial suspension for 30 min and then transferred onto solidified MS medium supplemented with 1.0 mg/L 6-benzylaminopurine (BA), 0.05 mg/L α-naphthylacetic acid (NAA), 30 g/L sucrose, 0.7 % agar for direct shoot regeneration in case of *A. tumefaciens*-mediated transformation. The infected plantlets were transferred on the mentioned medium for 1-4 days period and then were cultivated on the same medium with 600 mg/L cefotaxime (Darnitsa, Ukraine) for bacteria elimination. The plantlets were put on MS medium supplemented with 1.0 mg/L BA, 0.05 mg/L NAA, 600 mg/L cefotaxime and 25 mg/L kanamycin after *A. tumefaciens*-mediated transformation for selection of transgenic plants. Regenerated shoots of 1.5–2 cm long were transferred onto 1/2MS medium supplemented with 25 mg/L kanamycin and 600 mg/L cefotaxime. The obtained shoots were cultivated at 24°C under 16-hour photoperiod.

In case of *A. rhizogenes*-mediated transformation the explants were cultivated on 1/2MS solidified medium for "hairy" root induction. The roots formed on the plantlets were transferred to hormone-free 1/2MS medium with 600 mg/L cefotaxime in 3-8 days. The same medium with the antibiotic in mentioned concentration was used for stimulation of transgenic shoot regeneration on "hairy" root culture.

The rooted plants were transferred into soil and grown in the greenhouse.

Molecular analysis of transgenic plants

Total plant DNA was extracted from leaves of the selected transformed and wild-type plants and also from the "hairy" root culture samples by CTAB-method (hexadecyl trimethyl ammonium bromide). The total agrobacterial DNA was extracted according to Draper *et al*., (1988). We used primers 5'-cctgaatgaactccaggacgaggca-3' and

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5'-gctctagatccagagtcccgctcagaag-3' for amplification of 622 bp *nptII* gene fragment; primers 5′-ttgatgctcctggcacag-3′ and 5′-ttctgctctgacaacctc-3′ for amplification of 396 bp *ifn-α2b* gene fragment; 5'-atggatcccaaattgctattccttccacga-3' and 5'-ttaggcttctttcttcaggtttactgcagc-3' for amplification of 780 bp (*rolB*) using PCR method.

The amplification of the gene fragments was carried on under following conditions: *nptII* gene fragment amplification - 3 min at 94 °C - 33 cycles (30 s at 94 °C; 30 s at 65 °C; 30 s at 72 °C) - 3 min at 72 °C; *ifn-α2b* - 3 min at 94 °C - 30 cycles (30 s at 94 °C; 30 s at 60 °C; 30 s at 72 °C) - 3 min at 72 °C; *rolB* - 3 min at 94 °C - 33 cycles (30 s at 94 °C; 30 s at 56 °C; 30 s at 72 °C) - 3 min at 72 °C. All samples were fractionated in 1% agarose gel in TBE buffer.

Results

Determination of kanamycin selective concentration

Green shoots were formed if the medium contained less than 25 mg/l kanamycin. Whitening of shoots was observed in case of higher concentrations of antibiotic. Basing these data, we consider 25 mg/l kanamycin to be the best for selection of rue transformants.

Transgenic shoot regeneration after A. tumefaciens-mediated transformation

Earlier we demonstrated the possibility of high efficient shoot regeneration from leaves, roots, petioles and stems of *in vitro* cultivated *R. graveolens* plants (Matvieieva *et al*., 2015). For transgenic shoot regeneration after *A. tumefaciens*-mediated transformation we used the medium optimized for effective plant regeneration. This medium contained 1.0 mg L^{-1} BA+0.05 mg L^{-1} NAA growth regulators. The regeneration frequency of *R. graveolens* plants was found to be affected not only by media composition but also by type of explants (Matvieieva *et al*., 2015). Cultivation of rue petioles and leaves on the medium mentioned above resulted in shoot regeneration of 100% frequency. We consider petioles to be more preferable explants because of their greater length comparing to the length of internodes of *in vitro* cultivated rue plants (Fig. 1 a). So the petiole and leaf explants were chosen for *A. tumefaciens* inoculation because of their high regenerative capability.

We reported the possibility of direct shoot regeneration after plantlet cocultivation with *A. tumefaciens* bacterial culture. Period of plantlet cultivation on the medium without cefotaxime addition was shown to be an important factor for successful shoot regeneration. At the same time the prolongation of the cultivation period on the medium without antibiotic adding from one to four days resulted in increase of frequency of shoot formation from 61.1+1.9 to 100%. The direct shoot regeneration was observed in 10-14 days after transformation (Fig. 1 b). The presence of *nptII* gene in the constructs enabled the selection of transformants so that green plants were formed on the medium containing 25 mg/l kanamycin. The frequency of transformation (number of explants with regenerated shoots) depended on the type of explants (leaves or petioles respectively).

The shoots 10-15 mm long were transferred onto 1/2MS medium for further growth and rooting. Root formation was observed only after 8 weeks of shoot cultivation on hormone free medium (Fig. 1 c). The rooted plants were further cultivated under greenhouse conditions (Fig.1 d).

"Hairy" root culture induction and shoot regeneration

We analyzed the dependence of root formation frequency on the time of plantlets cultivation on the medium without cefotaxime addition (3-8 days) because rue extract was found to inhibit *A. rhizogenes* growth. No root formation was observed in case of cefotaxime addition to 1/2MS medium in 3-5 days after transformation. Increasing of duration of plantlet cultivation on the medium without antibiotic up to 8 days enabled the agrobacterial gene transfer and "hairy" root culture initiation. Root formation on the plantlets started in 14-16 days after their cocultivation with bacterial suspension (Fig. 2 a, b). The frequency of root formation was up to 30%. The induced root culture was characterized by typical "hairy" root phenotype which is associated with the transfer of T_L fragment with *rol* genes from *A. rhizogenes* T-DNA of the agropine type Ri plasmid (Tepfer 1990) and did not require any external auxines addition to the medium for their growth.

The obtained roots were subcultured every two weeks on the medium supplemented with cefotaxime (Fig. 2 c). Cultivation of *R. graveolens* "hairy" roots on hormone-free 1/2MS medium resulted in direct shoot regeneration in 6 months after the transformation (Fig. 2 d). The shoots were taken from the root culture and then grown on the same medium for rooting. Roots formation was observed in 3 weeks of their cultivation on hormone-free medium. The period required for plant rooting was considerably smaller than the one necessary for root formation in case of *A. tumefaciens*-mediated transformation. We consider this fact as an effect of *A. rhizogenes* genes transfer to the plants.

The PCR analysis proved the presence of *nptII* and *ifn-α2b* genes for 100% of studied plants regenerated after *A. tumefaciens*–mediated transformation and also the presence of *rolB* gene in case of study of DNA of the plants regenerated on the "hairy" roots (Fig. 3).

As we observed the time differences in rooting of the transgenic plants regenerated after *A. rhizogenes*- and *A. tumefaciens*–mediated transformation we studied the effect of Indolilbutiric acid (IBA) growth regulator in order to find out the way to make the period of root formation shorter. Using of 0.5 mg L^{-1} IBA resulted in initiation of root formation in 6 or 2-week period on the shoots regenerated after *A. tumefaciens*-mediated transformation and the ones regenerated on the "hairy" roots respectively. So this regulator can be used to advance the period of *R. graveolens* rooting.

There is very limited information about *R. graveolens* genetic transformation though these plants are known to be susceptible to agrobacterial infection (Porter 1991). *R. graveolens* "hairy" root cultures were established after inoculation of hypocotyls with wild *A. rhizogenes* strain LBA 9402 (Sidwa-Gorycka *et al.,* 2009) in order to study the level of pinnarin, rutacultin, bergapten and other compounds accumulation. It should be noted that transgenic plants were not obtained from the "hairy" root in these experiments.

A. tumefaciens bacteria were used earlier for *R. graveolens* transformation by Lièvre *et al.,* (2009). Authors reported obtaining of transgenic plants after hypocotyl co-cultivatation with *A. tumefaciens* strain C58C1Rif containing plasmid pTDE4 harbouring neomycin phosphotransferase and beta-glucuronidase encoding genes using acetosyringone as virulence inducer. Routine transformation efficiency of *R. graveolens* was 11% in these experiments.

Here we firstly demonstrated the possibility of *R. graveolens* plants initiation after *A. rhizogenes*-mediated transformation. The obtained shoots were characterized with more fast rooting compared with the shoots regenerated after *A. tumefaciens*-mediated transformation.

We also demonstrated an opportunity for direct regeneration of transgenic rue plants carrying human interferon-α2b gene. Optimization of transformational conditions in particular the prolongation of the cultivation period on the medium without antibiotic cefotaxime adding from one to four days resulted in increase of frequency of shoot formation up to 100%. We would like to note that acetosyringone as *Agrobacterium* virulence inducer were not used in out experiments. Lièvre *et al.,* (2009) noted a significant increase in the number of kanamycin-resistant plants by using acetosyringone. However, our research has shown also the possibility of obtaining of transgenic *R. graveolens* plants with high frequency without use of this compound.

Conclusions

Thus the transgenic *R. graveolens* plants can be obtained *via* direct shoot regeneration from leaves and petioles in case of *A. tumefaciens*-mediated transformation as well as after shoot regeneration from rue "hairy" roots in case of using *A. rhizogenes* for transformation. Using of *A. tumefaciens* allows the obtaining of transgenic rue plants with higher frequency up to 100%. Frequency of "hairy" root formation was up to 30% because of antibacterial activity of rue plants against *A. rhizogenes*. Time of explant co-cultivation on the medium without selective antibiotic had an effect on the successful *A. rhizogenes*-mediated transformation and "hairy" root formation on the plantlets. The ability to root of the shoots of rue "hairy" root origin exceeded the same ability of the plants obtained *via A. tumefaciens*-mediated transformation. IBA addition to 1/2MS medium in concentration of 0.5 mg/L resulted in root growth stimulation.

The described transgenic technique could be applied to improve plant characteristics and transfer of foreign genes to rue plants. Moreover, such approach enables the obtaining of transgenic *R. graveolens* plants carrying gene coding synthesis of recombinant medicinal compounds.

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Figure 1. Regeneration of rue shoots after *A. tumefaciens*-mediated transformation: *in vitro* cultivated plants used for transformation (a); green shoot regeneration on the selective medium (b); transgenic plants under *in vitro* conditions (c) and in greenhouse (d)

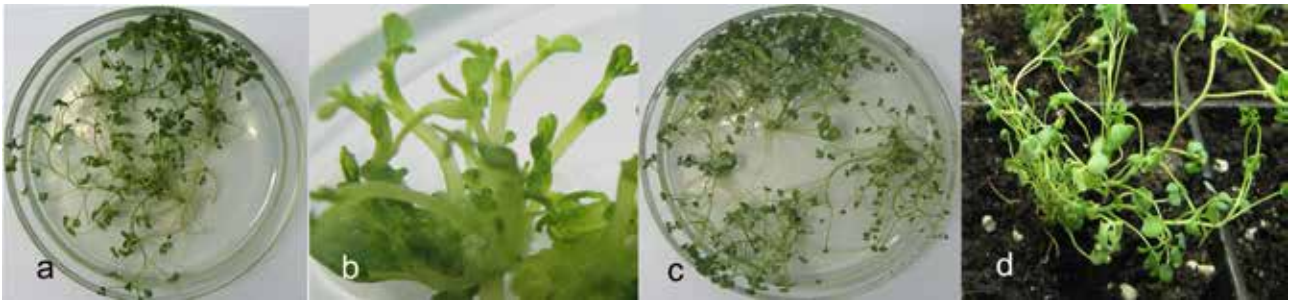
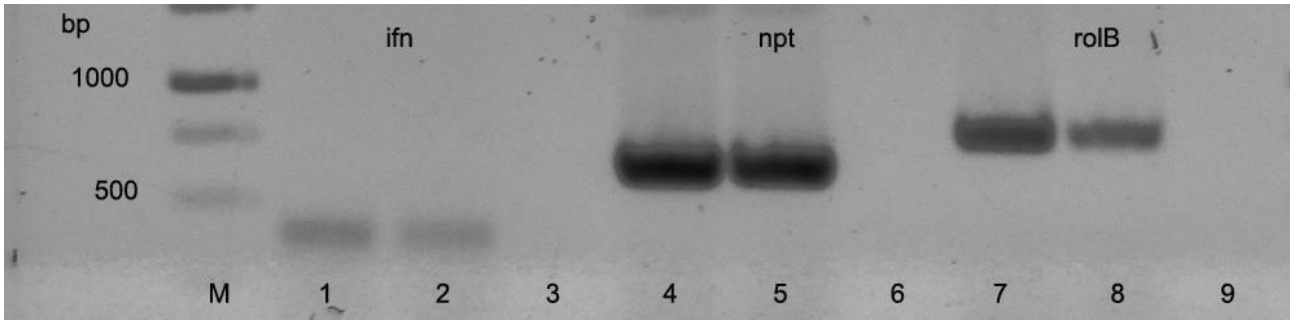


Figure 2. Initiation of *Ruta graveolens* "hairy" root culture and shoot regeneration after *A. rhizogenes*-mediated transformation: root formation on rue petiole (a) and leaf explants (b); "hairy" root culture (c); direct shoot regeneration on hormone free 1/2MS medium (d)

Figure 3. PCR analysis of the rue plants: 1, 2, 4, 5 - DNA of the plants regenerated after *A. tumefaciens*–mediated transformation; 7, 8 - DNA of the plants regenerated on the "hairy" root clones; 3, 6, 9 -negative control (untransformed plant DNA); npt, ifn, rolB - *nptII* (622 bp), *ifn-α*2b (396 bp), and *rolB* (780 bp) genes fragments; M - DNA marker ladder, Fermentas.



References

Ahmad N, Faisal M, Anis M, Aref IM (2010) *In vitro* callus induction and plant regeneration from leaf explants of *Ruta graveolens* L. South African J of Bot 76:597-600

Cunha MR, Melo TS, Magri FMM, Delorenzi JC (2015) Analgesic activity of *Ruta graveolens* L. (Rue) extracts. African J. of Pharmacy and Pharmacol 9:1-5. DOI: 10.5897/AJPP2014.4152

Diwan R, Malpathak N (2008) Novel technique for scaling up of micropropagated *Ruta graveolens* shoots using liquid culture systems: A step towards commercialization. N Biotechnol 5:85-91. DOI: 10.1016/j.nbt.2008.02.002

Draper J, Scott R, Armitage P, Walden R (1988) Plant Genetic Transformation and Gene Expression. A Laboratory Manual. Oxford; Boston: Blackwell Scientific Publications.

Ekiert H, Abou-Mandour AA, Czygan FC (2005) Accumulation of biologically active furanocoumarins in *Ruta graveolens* ssp. divaricata (Tenore) gams *in vitro* culture. Pharmazie 60:66-68

França Orlanda JF, Nascimento AR (2015) Chemical composition and antibacterial activity of *Ruta graveolens* L. (Rutaceae) volatile oils, from São Luís, Maranhão, Brazil South African J of Bot 99:103-106. DOI:10.1016/j.sajb.2015.03.198

Ghosh S, Sikdar S, Mukherjee A, Khuda-Bukhsh AR (2015) Evaluation of chemopreventive potentials of ethanolic extract of *Ruta graveolens* against A375 skin melanoma cells *in vitro* and induced skin cancer in mice *in vivo*. J Integr Med 13:34-44. DOI: 10.1016/S2095-4964(15)60156-X

Ivanova A, Mikhova B, Najdenski H, Tsvetkova I, Kostova I (2005) Antimicrobial and cytotoxic activity of *Ruta graveolens*. Fitoterapia 76:344-347. DOI:10.1016/j.fitote.2005.02.008

Lièvre K, Tran TL, Doerper S, Hehn A, Lacoste P, Thomasset B, Bourgaud F, Gontier E (2009) *Agrobacterium*-mediated transformation of Ruta *graveolens L.* Methods Mol Biol 547:235-248. DOI: 10.1007/978-1-60327-287-2

Luchakivskaya Yu, Kishchenko O, Gerasymenko I, Olevinskaya Z, Simonenko Yu, Spivak M, Kuchuk M (2011) High-level expression of human interferon alpha-2b in transgenic carrot (*Daucus carota* L.) plants. Plant Cell Rep 30:407-415. DOI:10.1007/s00299-010-0942-5

Majumdar S, Garai S, Jha S (2012) Use of the cryptogein gene to stimulate the accumulation of Bacopa saponins in transgenic *Bacopa monnieri* plants. Plant Cell Rep 31:1899-1909. DOI:10.1007/s00299-012-1303-3

Matvieieva NA, Drobot, KO, Pasichnyk L.A. N.V. Zhytkevych An efficient regeneration system and antimicrobial activity of *Ruta graveolens* L. plants/ Ecological Engineering and Environment Protection.-2015, № 1.-P. 33-39

Meepagala KM, Schrader KK, Wedge DE, Duke SO (2005). Algicidal and antifungal compounds from the roots of *Ruta graveolens* and synthesis of their analogs. Phytochem 66:2689-2695. DOI:10.1016/j.phytochem.2005.09.019

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plantarum 15: 473-497

Orlita A, Sidwa-Gorycka M, Kumirska J, Malinski E, Siedlecka EM, Gajdus J, Lojkowska E, Stepnowski P (2008) Identification of *Ruta graveolens* L. metabolites accumulated in the presence of abiotic elicitors. Biotechnol Prog 24:128-133. DOI: 10.1021/bp070261d

Porter J.R. (1991) Host range and implications of plant infection by *Agrobacterium rhizogenes*. Crit Rev Plant Sci 10:387-421. DOI:10.1080/07352689109382318

Preethi KC, Kuttan G, Kuttan R (2006) Anti-tumour activity of *Ruta graveolens* extract. Asian Pac J Cancer Prev 7:439-443

Raghav SK, Gupta B, Agrawal C, Goswami K, Das H (2006) Anti-inflammatory effect of *Ruta graveolens* L. in murine macrophage cells. J. Ethnopharmacol 104:234-239

Sharafi A, Hashemi Sohi H, Mousavi A, Azadi P, Dehsara B, Hosseini Khalifani B (2013) Enhanced morphinan alkaloid production in hairy root cultures of *Papaver bracteatum* by over-expression of salutaridinol 7-o-acetyltransferase gene via *Agrobacterium rhizogenes* mediated transformation. World J Microbiol Biotechnol 29:2125 -2131

Sidwa-Gorycka M, Krolicka A, Orlita A, Malinski E, Golebiowski M, Kumirska J, Chromik A, Biskup E, Stepnowski P, Lojkowska E (2009) Genetic transformation of *Ruta graveolens* L. by *Agrobacterium rhizogenes*: hairy root cultures a promising approach for production of coumarins and furanocoumarins. Plant Cell Tiss Org Cult 97:59-69. DOI: 10.1007/s11240-009-9498-x

Tepfer D (1990) Genetic transformation using *Agrobacterium rhizogenes* Physiol. Plant 79:140-146

Thiruvengadam M, Praveen N, Kim EH, Kim SH, Chung IM (2014) Production of anthraquinones, phenolic compounds and biological activities from hairy root cultures of Polygonum multiflorum Thunb. Protoplasma 251:555-66. DOI:10.1007/s00709-013-0554-3

Zhang Y, Yan YP, Wu YC, Hua WP, Chen C, Ge Q, Wang ZZ (2014) Pathway engineering for phenolic acid accumulations in *Salvia miltiorrhiza* by combinational genetic manipulation. Metab Eng 21:71-80. DOI:10.1016/j.ymben.2013.10.009

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