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Plant Science 168 (2005) 883–888
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Genetic transformation of the medicinal plant *Ruta graveolens* L. by an *Agrobacterium tumefaciens*-mediated method

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Received 13 September 2004; received in revised form 20 October 2004; accepted 3 November 2004
Available online 25 November 2004

Abstract

Ruta graveolens L. is a source of pharmacologically active molecules such as furocoumarins, furoquinolines and acridone alkaloids. A genetic transformation method using *Agrobacterium tumefaciens* was developed for this plant species. The conditions for an efficient regeneration of *R. graveolens* led us to retain hypocotyls among other explants tested. Subsequently, transformation was obtained by co-cultivation of hypocotyls of 2–3 weeks old plants and *A. tumefaciens* strain C58C1Rif^R containing a plasmid harboring neomycin phosphotransferase and β-glucuronidase encoding genes. PCR analyses using β-glucuronidase-primers showed that 78% of the transgenic plants selected for kanamycin resistance were transformed with the gene encoding β-glucuronidase and 67% of them exhibited the corresponding enzymatic activity. Chromosomic integration of β-glucuronidase encoding gene was verified by Southern blotting. Routine transformation efficiency of *R. graveolens* L. was established at 11% and could reach 22% in some experiments. This is the first report of a method to transform genetically *R. graveolens* L.
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Keywords: *Agrobacterium tumefaciens*; *Ruta graveolens*; Hypocotyl; Transgenic plant; *gus*; *npt*II

1. Introduction

Since ancient times, garden rue (*Ruta graveolens* L., Rutaceae) has been among the key plants of the European pharmacopoeia [1]. Its medicinal value is due to the numerous secondary metabolites it contains like furocoumarins, furoquinolines and acridone alkaloids. In plant, furocoumarins exhibit many physiological properties as they protect the tissues against UV light, insects and different pathogens [2]. Amongst furocoumarins, bergapten has been used for decades for the treatment of various skin diseases such as vitiligo and psoriasis [3]. At present, little is known about enzymes and the corresponding genes involved in the synthesis of furocoumarins in plants [4]. These molecules remain one of the least unknown elements of the phenylpropanoid pathway. Genetic transformation is a powerful tool to study gene functions in plants and could help establishing new knowledge on the *R. graveolens* furocoumarin pathway. To date, there is no protocol available to genetically engineer *R. graveolens* L. Two previous reports [5,6] mentioned that *R. graveolens* was susceptible to *Agrobacterium* wild strains. Eilert (1989) and Porter (1991) reported, respectively, the regeneration of possible shooty teratomas and hairy roots from *R. graveolens*. However, these authors neither genetically nor biochemically confirmed the status of these transformants.

Abbreviations: BAP, benzylaminopurin; IAA, indolacetic acid; CEF, cefotaxim; KAN, kanamycin; KAN^R plant, plant resistant to kanamycin; Bp, base pair; w/v, weight/volume; MS, Murashige and Skoog medium [7]; MS(30), MS medium supplemented with 30 g/l sucrose; MS(30)BAP0.1, MS(30) supplemented with 0.1 mg/l BAP; SE (selection efficiency), percentage of selected kanamycin-resistant explants that developed transgenic plants; TE (transformation efficiency), percentage of initial explants that regenerated at least one transgenic plant detected by PCR

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doi:10.1016/j.plantsci.2004.11.001

These two preliminary papers suggested us to choose an *Agrobacterium*-mediated method for the genetic transformation of *R. graveolens* L. This paper describes the experiments that were carried out in order to obtain a fast and reliable method to genetically transform *R. graveolens* L. We successively determined (i) which explant was best adapted to an efficient regeneration of a whole plant and (ii) which conditions to apply to genetically transform explants with an *Agrobacterium*-mediated method. This is the first report of a method to genetically transform *R. graveolens* L.

2. Material and methods

2.1. Culture conditions

Basal medium MS(30) used for the cultures contained MS nutrients [7] and 30 g/l sucrose (pH 5.8). Unless stated otherwise, all MS(30) media described in the experiments were solidified with 0.8% Difco agar. Growth regulators were added to the media before autoclaving (20 min at 120 °C, 10^5 Pa). Cultures were maintained in 15 mm × 57 mm Petri dishes or in glass vessels (70 mm × 55 mm or 100 mm × 55 mm, closed with magenta cap purchased from Sigma) containing 10, 25 or 40 ml of medium, respectively. All cultures were sealed with gas-porous tape and kept in growth chambers at 22 °C and a 16 h photoperiod with 160 μmol m^2 s^{-1} light irradiance.

2.2. Plant material and micropropagation

R. graveolens L. seeds were purchased from Conrad Appel (Samen und Pflanzen GmbH, Darmstadt, Germany). They were scarified 10 min in 95% sulfuric acid, rinsed three times with tap water, then sterilized for 5 min in 7% (w/v) calcium hypochlorite, and finally rinsed three times for 5 min in sterile distilled water before being germinated on MS(30) medium. After 2–3 weeks, apices, hypocotyls, cotyledons, roots and root tips were used as primary explants to determine the best regeneration conditions for *R. graveolens* L. MS(30) medium supplemented with BAP and/or IAA with concentrations ranging from 0 to 2 mg/l was used throughout these experiments.

2.3. Bacterial strains and plasmids

The disarmed *A. tumefaciens* C58C1Rif^R strain carrying the plasmids pGV2260 [8] and the binary vector pBin-*gus* was used as a vector system for genetic transformation. The plasmid pGV2260 carries the *vir* genes necessary to infect the plant plus a gene encoding carbenicillin resistance. The plasmid pBin-*gus* was obtained by replacing the *mGFP5ER* gene by the *gus* gene in the plasmid pBin-*mGFP5ER* obtained from Prof. Jim Haseloff, MRC Laboratory of Molecular Biology, Addenbrookes Hospital, Cambridge, UK. The plasmid pBin-*gus* contains sequences encoding resistance to spectinomycin, streptomycin and ampicillin as well as the T-DNA. This T-DNA is composed of a neomycin phosphotransferase (*npt*II) marker gene driven by the nopaline synthase (*nos*) promoter, allowing kanamycin (KAN) selection, and the β-glucuronidase (*gus*) reporter gene, driven by the cauliflower mosaic virus (CaMV 35S promoter) allowing GUS histochemical assay.

Bacteria were grown for 3 days on Petri dishes with YEB (beef extract, 5 g/l; yeast extract, 1 g/l; peptone, 5 g/l; sucrose, 5 g/l; $MgSO_4$, 0.25 g/l; pH 7.2; difco-agar, 15 g/l) supplemented with 100 μg/l carbenicillin, 100 μg/l rifampicin, 100 μg/l spectinomycin and 300 μg/l streptomycin at 28 °C. For transformation, a colony from the plate was picked up and grown 12–16 h at 28 °C in YEB liquid medium on a shaker. Bacterial cells were collected by centrifugation (3500 rpm for 15 min), washed twice in lambda buffer (10 mM Tris–HCl, 10 mM $MgSO_4$, pH 7.2), and then resuspended in MS(30)BAP0.1 (50 ml for 1 ml of the initial inoculum).

2.4. Transformation and selection

Factors affecting transformation frequency of *R. graveolens* L. were tested including pre-culture of explants (0, 1 or 2 days), addition of acetosyringone to the co-culture medium (0, 50 and 250 μM), and selection methods. Two types of selection methods were tested. The one step selection protocol consisted in selecting explants that resisted to 50 mg/l KAN in the medium. The two step selection protocol used explants cultivated for 3 days on solidified MS(30)BAP0.1 containing 300 mg/l CEF before being transferred onto fresh solidified MS(30)BAP0.1 containing 300 mg/l CEF and 75 mg/l KAN.

For each experiment, 200–250 explants were immersed for 15 min in the *Agrobacterium* suspensions, then blotted with sterile filter paper and incubated for 3 days at 25 °C on co-culture medium.

After co-culture, explants were rinsed once in sterile water for 15 min, blotted on sterile filter paper, rinsed once again for 15 min in MS(30)BAP0.1 containing 500 mg/l CEF and blotted on a new sterile filter paper. Explants were then transferred on selection medium containing CEF and KAN. When pre-culture was tested, explants were preconditioned for 1 or 2 days on agar-solidified MS(30)BAP0.1.

The selection media were composed of 0.8% agar-solidified MS(30)BAP0.1 added with CEF either alone or with KAN. CEF and KAN concentrations used for selection were determined after preliminary experiments revealing lethal and sub-lethal doses (data not shown).

After 4–6 weeks on selection media, developing shoots were excised from the explants and transferred for 3 weeks into 55 mm × 70 mm glass vessels containing MS(30)-BAP0.1 with 300 mg/l CEF and 75 mg/l KAN. A maximum of one shoot was taken from each extremity of the hypocotyl

to ensure that all the transformed shoots were coming from independent transformation events.

The shoots were then transferred for 6–8 weeks in 55 mm × 100 mm glass vessels containing MS(30) for root formation. Plants were acclimated and transferred into a phytotronic chamber.

2.5. GUS assay

For histochemical GUS assay, freshly harvested leaves of entire plants were immersed into a solution of 1 mM 3-bromo-4-chloro-3-indolyl glucuronid (X-gluc) and 100 mM Tris–HCl and 50 mM NaCl, pH 7 and placed at 37 °C for 12–24 h.

2.6. Molecular analysis

Molecular analysis was performed on putative transgenic and control plants by PCR and Southern hybridisation. Qiagen 'DNeasy plant maxi kit' was used to isolate genomic DNA. For PCR analysis, a 552 bp *gus* sequence was amplified using the following primer sets: 5′-ATG GTC CGT CCT GTA GAA ACC CCA ACC CGT GAA ATC-3′ and 5′-CGC AGC GTA ATG CTC TAC ACC ACG CCG AAC ACC TGG GTG GAC GAT A-3′. The PCR conditions were: 95 °C for 5 min followed by 35 cycles at 95 °C for 60 s, 65 °C for 30 s, and 72 °C for 40 s. Amplified DNA was analysed on a 1% (w/v) TAE agarose gel.

Southern blot was performed on eight randomly chosen KAN^R plants and one wild (control) plant. Each Southern blot analysis was carried out on 1 g of DNA extracted from the leaves using Qiagen 'DNeasy plant maxi kit'. DNA (20 μg) from each sample were individually digested for 20 h at 37 °C with a mix of three enzymes (150 units of *Eco*RI, 150 units of *Bam*HI and 70 units of *Bgl*II) added in two times. There is no restriction site for these three enzymes in the *gus* sequence, so the number of insertions of the *gus* gene in the genome of *R. graveolens* L. is equal to the number of fragments obtained. The digested DNA was precipitated with 0.8 vol isopropanol in presence of 0.3 M AcNa pH 5.6. Pellets were resuspended in TAE buffer. Prior to loading on a 0.8% agarose gel, we added loading buffer (TAE, 1×; 50% glycerol; 0.1% Bromophenol Blue; 0.1 SDS) and heated the samples for 10 min at 65 °C. Digested DNA was blotted on a positively charged nylon membrane Hybond N+ (Amersham-Bioscience). The hybridisation reaction was carried out for 16 h at 65 °C using as a probe the *gus* PCR product obtained with the primer set described above and the pBin-*gus* plasmid. This probe was labelled with ^{32}P using Ready-To-Go™ Labelling Beads (-dCTP) according to the manufacturer's recommendation (Amersham-Bioscience).

3. Results

Regeneration of a plant is a prerequisite to further transformation. Therefore, plant tissue and organ culture conditions were established first to efficiently regenerate *R. graveolens* plants. Secondly, transformation conditions were determined using an *Agrobacterium* binary vector method with the objective to get the highest percentage of transformed plant. Finally, histochemical and molecular techniques were used to verify that plants were genetically transformed.

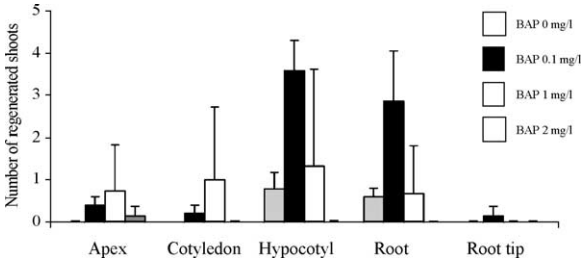

Fig. 1. Effect of BAP on direct shoot regeneration applied to different explants. Apices, cotyledons, hypocotyls, roots and root tips were cultivated on agar-solidified MS(30) containing different concentrations of BAP (0, 0.1, 1 and 2 mg/l). Shoot regeneration was evaluated after 2 weeks for each explant and BAP concentration. The experiment was carried out with 15 replicates and a total of 300 explants.

3.1. Ruta graveolens L. regeneration

Regeneration ability of *R. graveolens* L. was tested by cultivating five types of explants (apices, cotyledons, hypocotyls, roots and root tips) on MS(30) containing different concentrations of BAP (0, 0.1, 1 and 2 mg/l). After 2 weeks, hypocotyls gave the highest number of regenerated shoots (Fig. 1). A maximum of three to four shoots per hypocotyls were regenerated on MS(30) containing 0.1 mg/l BAP (Fig. 1). When reaching a height of 1 cm, these shoots were transferred in glass vessels containing MS(30) with IAA (0, 0.1, 0.5 and 1 mg/l) for rooting. Shoots rooted better when they were grown on MS(30) without IAA than after addition of this compound. After 6 weeks, 55% of the shoots had four roots a least, enough to transfer them into a phytotronic chamber (Table 1).

Hypocotyls appeared to regenerate plants most efficiently. These explants were retained for further genetic transformation.

Table 1
Effect of IAA concentration on shoots rooting

IAA concentration (mg/l)	Rooted shoots (%)
0	56
0.1	24
0.5	18
1	0

Shoots (1 cm-high) were grown on agar-solidified MS(30) medium containing IAA (0, 0.1, 0.5 and 1 mg/l). After 6 weeks, the percentage of shoots having at least four roots was evaluated. The experiments were carried out with a total of 70 shoots.

Table 2
Effects of acetosyringone concentration (0, 50 and 250 μM) in the co-culture medium of *Ruta graveolens* L. and *Agrobacterium tumefaciens*

N[a]	Acetosyringone (μM)	Kanamycin concentration (mg/l)	KAN^R shoots number (%)	KAN^R plants number (%)	SE (PCR + plants, %)[b]	GUS + plants[c] (%)	TE[d] (%)
322	0	75	19 (5.9)	10 (3.1)	ND	ND	ND
164	50	75	30 (18.3)	16 (9.8)	ND	ND	ND
225	250	75	43 (19.1)	32 (14.2)	78	52	11.1
207	250	50	32 (15.5)	19 (9.2)	ND	21	ND

Selection method was done in one step (with 50 mg/l kanamycin) or two step (with 75 mg/l kanamycin). ND: not determined.
[a] Number of *Ruta graveolens* L. hypocotyls infected with *Agrobacterium tumefaciens*.
[b] SE: Percentage of KAN^R plants with the *gus* gene (PCR detection).
[c] Percentage of KAN^R plants with GUS activity detected by histochemical assays.
[d] TE: Transformation efficiency, number of explants that regenerated at least one transformed plant confirmed by PCR on *gus* gene.

3.2. Genetic transformation and regeneration of plants

3.2.1. Effect of acetosyringone

Phenolics like acetosyringone are well-known virulence inducers for *Agrobacterium* [9–11]. The aim of the first transformation experiment was to determine the optimum concentration of acetosyringone in co-culture medium. *Agrobacterium tumefaciens* and hypocotyls were co-cultivated for 3 days on MS(30)BAP0.1 supplemented with 0, 50 or 250 μM acetosyringone. Subsequently, the hypocotyls were treated as previously described for the 'two step' selection method. Explants consisting of 1 cm of developing shoot explants were then transferred to a fresh selection medium containing KAN 75 mg/l for 3 weeks and could reach 2–3 cm at the end of this period. When transferred to a fresh MS(30) medium without antibiotic for 6–8 weeks, they could grow and root. Results shown in Table 2 demonstrate that the transformation of *R. graveolens* L. was improved in the presence of acetosyringone, since 14.2% of the initial explants produced KAN^R plants when 250 μM of acetosyringone was added to the co-culture medium. Selection efficiency (SE) was established at 78% for this acetosyringone treatment.

3.2.2. Effect of pre-culture

Pre-culture of 0, 1 or 3 days was tested in order to improve the transformation efficiency. Hypocotyls were pre-cultured on MS(30)BAP0.1 before being co-cultured with *A. tumefaciens* on MS(30)BAP0.1 with 250 μM acetosyringone. Selection with 75 mg/l KAN was used as previously described. This experiment was duplicated. The transformation efficiency (TE) was about 5%, whatever the time of pre-culture. Consequently, even if the transformation rate of these experiments is lower than those described previously, it demonstrates that pre-culture has no positive effect for genetic transformation of *R. graveolens* L.

3.2.3. Simplification of the protocol

Decreasing the number of steps necessary to obtain transgenic plants would speed up genetic transformation of *R. graveolens* L. Therefore, we tested a more simple protocol by comparing TE obtained with the 'one step' and 'two step' selection method. However, we had previously established that numerous plants cannot support a strong and early selection, therefore, KAN concentration in the 'one step' selection method was diminished from 75 to 50 mg/l. The percentage of KAN^R plants obtained with the two step selection method (14.2%, Table 2) was 1.5 folds the one recorded with the one step selection method (9.2%, Table 2). Consequently, the one step selection method was not used for the following experiments.

3.2.4. Optimised protocol (two step selection method)

Transformation efficiency is above 11.1% for routine experiments and could reach 22.2% in some experiments. Because KAN^R plants obtained in the different transformation experiments are coming from different hypocotyls, all transgenic plants are originating from different transformation events. After selection screening, the shoots that showed good root development were grown *ex vitro* into a phytotronic chamber. All of them exhibited normal development as compared to control plants.

3.3. Molecular analysis of transgenic plants

PCR analysis was carried out to confirm the presence of the *gus* gene in the genome of putative transformants (Fig. 2). This was performed on 21 out of the 27 KAN^R plants. The 550 bp expected *gus* fragment was found in the positive control (pBin-*gus* plasmid) as well as in 78% of the analysed plants. Wild plants (controls) did not show the fragment.

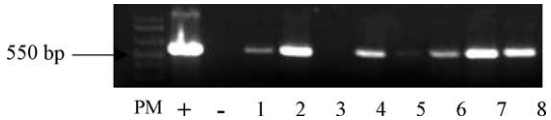

Fig. 2. PCR analysis of putative transformed plants. Genomic DNAs were amplified with primers corresponding to a 550 bp *gus* gene sequence. Lanes: PM, molecular weight ladder; (+) positive control (pBin-*gus*); (−) negative control (wild type plant); lanes 1, 2, 4–8, transformed plants; lane 3, untransformed plant.

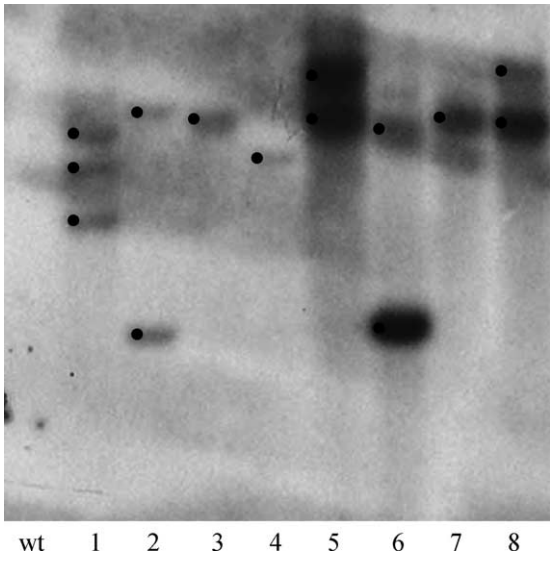

Fig. 3. Southern blot analysis of putative transformed plants. Genomic DNAs were digested with a mix of three enzymes (*Eco*RI, *Bam*HI and *Bgl*II) and probed using a ^{32}P labelled GUS probe, prepared from the plasmid pBin-*gus*. Lane wt: wild type control plant, lanes 1–8: transformed plants. Dots indicate insertion events.

Southern blot analysis was done in order to estimate the number of inserted loci. DNA from eight plants were analysed. Fig. 3 shows the Southern blot hybridisation pattern obtained for the *gus* probe. Three out of eight analysed plants showed a single insert of the *gus* gene (lines 3, 4 and 7), while four lines (2, 5, 6, 8) showed two hybridisation bands for the *gus* gene, and line 1 has three copies. No band was observed in the control plant.

3.4. GUS activity in rooted plants

About 67% of the plants transformed with the *gus* gene expressed β-glucuronidase (Table 2). Staining for GUS expression revealed variable colour intensities in transformed plants. No GUS staining was observed in control plants (Table 2).

4. Discussion

An efficient *Agrobacterium*-mediated transformation protocol for *R. graveolens* L. is presented for the first time. The only related studies are those of Porter [6] and Eilert [5], who infected *R. graveolens* with wild type *Agrobacterium rhizogenes* or *tumefaciens*. However, these authors neither tried to genetically transform *R. graveolens* L. with engineered *Agrobacterium* strains, nor to genetically/biochemically confirm the status of the transformants. Our transformation procedure is based on a 3 days co-culture of seedling hypocotyls with *A. tumefaciens* C58C1Rif^{R} in the presence of 250 μM acetosyringone. Regeneration and selection were obtained in one step on MS(30)BAP0.1 with 300 mg/l cefotaxime, or in two steps on MS(30)BAP0.1 supplemented with 300 mg/l cefotaxime and 75 mg/l kanamycin. Stable transgene integration was confirmed by growth on selection medium for *npt*II, by PCR and Southern blot analyses, and histochemical revelation of GUS activity. Results observed with acetosyringone suggest that this compound is useful to improve genetic transformation of *R. graveolens* L. as for many other species [11]. It has been demonstrated that a two step selection protocol increases the transformation efficiency and minimizes the incidence of escapes even with a higher concentration of selective agent [12]. The remaining escapes could be explained either by *Agrobacterium* endogenous contamination of plant tissues, or by a low transformation frequency versus a high efficiency of shoot formation. In this latter case, the non-transformed cells could be protected by the surrounding transformed cells resulting in chimeric shoots. Pre-culture of hypocotyls, prior to bacterial co-incubation, had no positive effect on transformation efficiency.

Starting from the original seeds, it is possible to obtain transgenic plants growing on soil within 4–5 months. Because the original plant material is composed of seeds, it prevents possible somaclonal variations associated with long lasting *in vitro* culture of callus.

R. graveolens L. produces many different secondary metabolites. The efficiency of this transformation protocol will allow us to easily generate mutants up or down regulated for various key-enzymes involved in secondary pathways. This will help us to understand secondary metabolites regulations, especially in the furocoumarin pathway. A new set of mutants has been recently produced with a modified cinnamate-4-hydroxylase (C4H) expression under the control of the CaMV 35S promoter using this transformation method.

Acknowledgements

We acknowledge the Région Lorraine for financial support of the research program (PRST Bioingénierie) and the French Ministry of Research for Ph.D. grant (Karine Lièvre). We thank Paul Lacoste and Sébastien Doerper for their technical assistance.

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