Effects of the *rol C* Gene on Hairy Root: Induction Development and Tropane Alkaloid Production by *Atropa belladonna*

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Two series of *Atropa belladonna* hairy root lines were obtained, the first one transformed via *Agrobacterium tumefaciens* harboring *rol C* and *npt II* genes, and the other transformed with *rol ABC* and *npt II* genes. Thirteen hairy root lines were obtained and selected on hormone-free medium. The transformation was confirmed by PCR analysis, and these root lines were first examinated for their growth rate. Then hyoscyamine and scopolamine production was measured after 3 and 4 weeks of culture to evaluate the possible role of *rol C* gene in tropane alkaloid formation. The *rol C* gene alone played a significant role in the hairy root growth rate (17-fold increase). However this effect was much lower than that induced by the *rol ABC* genes together (75-fold increase). In contrast, the *rol C* gene alone was as efficient as the *rol ABC* genes together (mean value of total alkaloids: 0.36% dry weight, i.e., 12-fold times more than in untransformed roots) to stimulate the biosynthesis of tropane alkaloids in *A. belladonna* hairy root cultures.

The parasympatholytic tropane alkaloids, hyoscyamine and scopolamine, accumulated in *Atropa belladonna*, are of great interest for the pharmaceutical industry. They are traditionally obtained by extraction from the leaves of cultured plants. An alternative to extraction from plant tissue could be the use of cell suspension cultures.[1] However, it has been shown that tropane alkaloid biosynthesis was correlated with root differentiation.[2] For this reason, hairy root formation offers an interesting approach to produce these secondary metabolites.[3,4] Hairy root cultures have demonstrated their ability to rapidly produce biomass as well as high contents of tropane alkaloids.[5] In *A. belladonna* hairy roots, high tropane alkaloid production has been obtained after infection with wild strains of *Agrobacterium rhizogenes*.[6−10] The hairy root phenotype was the result of inserting the T-DNA region of the *A. rhizogenes* Ri-plasmid into the plant genome. It has been shown that *rol* genes (*rol A*, *rol B*, and *rol C*) were involved in the induction and development of these transformed roots. The *rol A*, *rol B*, and *rol C* genes together induced root formation and tropane alkaloid biosynthesis in tobacco[11,12] and in *Datura stramonium*.[13] In the case of the *rol C* alone, some investigations have reported the stimulating effects of this gene upon secondary compound production in transformed roots, such as nicotine,[14] indole alkaloids,[15] or ginsenosides.[16] However, to our knowledge, nothing has been reported to date about the capacity of *Atropa* transgenic roots expressing *rol C* or *rol ABC* genes to produce hyoscyamine and scopolamine. In the present study we report on the establishment of *A. belladonna* hairy root cultures transformed by *rol C* alone or by *rol ABC*. We also report the relationship between growth rate, tropane alkaloid production, and the presence in the plant genome of *rol* genes.

Results and Discussion

DNA Analysis. Polymerase chain reaction (PCR) analysis performed on *rol C* clones (Figure 1A) showed that they contained the *rol C* gene (lanes 5−11). Amplification with the *rol C* primers showed a 490 pb band for the seven transformed root lines (lanes 5−11) and for the plasmid GV 3101 (lane 2). No band was observed for the untransformed roots (lanes 4) or for the blank (lane 3). The PCR analysis on *rol ABC* clones (Figure 1B) showed that they contained the *npt II* gene and the *rol A*, *rol B*, and *rol C* genes (lanes 6−9). Amplification with the *npt II* primers showed a 700 pb band for the transformed roots (lanes 6−9) and for the plasmid GV 3101 (lane 2). No band was observed for the untransformed roots (lanes 4, 5) or for the blank (lane 3). When the *rol A* primers were used for

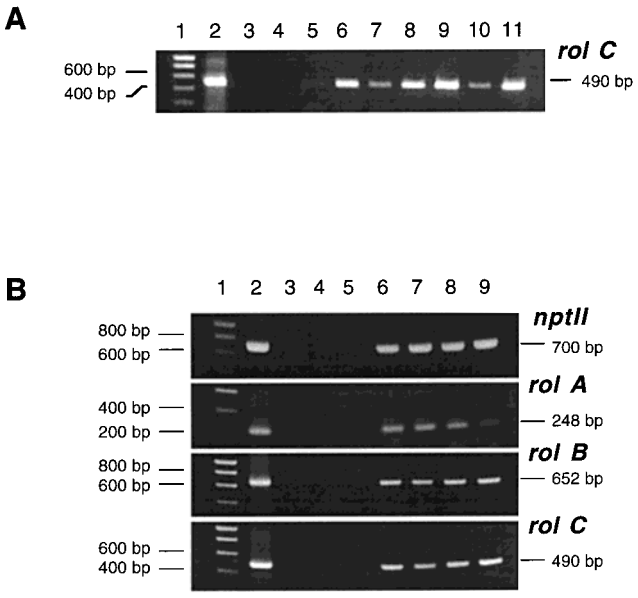

Figure 1. PCR amplified DNA fragments of *npt II*, *rol A*, *rol B*, and *rol C* genes. (A) DNA of *A. belladonna rol C* clones was amplified with *rol C* primers. Lane 1: Smartladder. Lane 2: DNA from the plasmid GV 3101 as positive control. Lane 3: blank (without DNA). Lane 4: DNA from the untransformed roots as negative control. Lanes 5−11: DNA from transgenic *A. belladonna rol C* hairy-roots (C1, C2, C3, C4, C5, C6, and C7). (B) DNA of *A. belladonna rol ABC* clones was amplified with *rol A*, *rol B*, *rol C*, or *npt II* primers. Lane 1: Smartladder. Lane 2: DNA from the plasmid GV 3101 as positive control. Lane 3: blank (without DNA). Lanes 4, 5: DNA from the untransformed roots as negative control. Lanes 6−9: DNA from transgenic *A. belladonna rol ABC* hairy-roots (ABC1, ABC4, ABC5, and ABC6).

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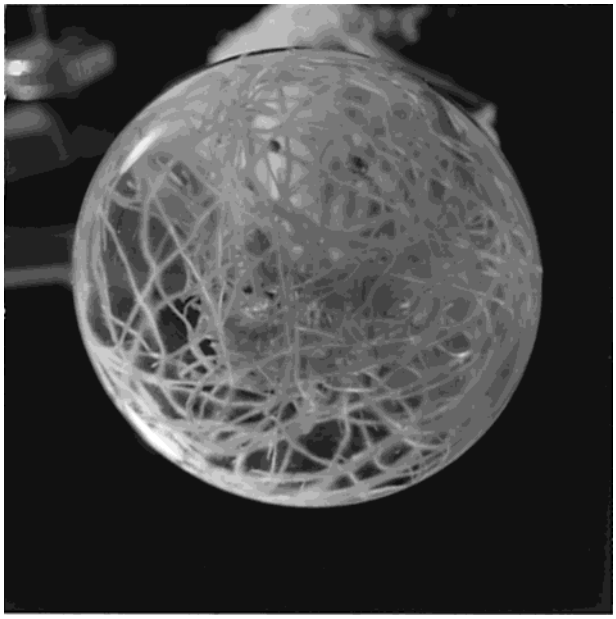

Figure 2. Two-week-old *A. belladonna rol C* transformed root cultures in LS liquid medium.

Table 1. Growth Rate (Fresh Weight of Roots/Fresh Weight of Inoculum) of Seven Hairy Root Lines Obtained from the Infection with (A) *A. tumefaciens rol C* (C1, C2, C3, C4, C5, C6, and C7) and Six Lines from the Infection with (B) *A. tumefaciens rol ABC* (ABC1, ABC2, ABC3, ABC4, ABC5, and ABC6) after 4 Weeks of Culture

	(A) *rol C* root lines						
	C1	C2	C3	C4	C5	C6	C7
growth rate	13	12.5	17	13.5	11	9	13.5
	(B) *rol ABC* root lines						
	ABC1	ABC2	ABC3	ABC4	ABC5	ABC6	
growth rate	58	15	75	31	32	6	

amplification, a 248 pb band was visualized for hairy root clones and for the plasmid GV 3101. With the *rol B* or the *rol C* primers, 652 and 490 pb bands were respectively observed for the four clones (ABC1, ABC3, ABC5, and ABC6) and for the plasmid GV 3101. No band was observed for the untransformed roots or for the blank. For some hairy root lines no data were available due to loss of material following bacterial contamination just prior to molecular biological analysis.

Kinetics of Growth of the Hairy Root Lines. Transgenic root lines were established from *Atropa belladonna* by infecting leaf explants with *Agrobacterium tumefaciens* GV3101 strains containing *rol ABC* genes together or the *rol C* gene alone. Six hairy root lines were obtained with *A. tumefaciens rol ABC* (ABC1, ABC2, ABC3, ABC4, ABC5, ABC6) and seven lines with *A. tumefaciens rol C* (C1, C2, C3, C4, C5, C6, C7). The confirmation of integration of both *rol ABC* or *rol C* and NPTII genes was performed by PCR. The established root lines grew in hormone-free liquid LS medium and showed the characteristic traits of hairy roots (Figure 2) previously described by David et al.[17] They grew quickly and were highly branched with a plagiotropic growth. All the root lines showed a homogeneous morphology called thick morphology (more than 3 mm diameter), as described by Palazon et al.[15] on *Catharanthus roseus* transformed roots. The root line growth was evaluated after 4 weeks of culture (Table 1). This growth rate varied from 9 (C6) to 17 (C3) for *rol C* lines and from 6 (ABC6) to 75 (ABC3) for *rol ABC* lines. It is worth noticing the deep

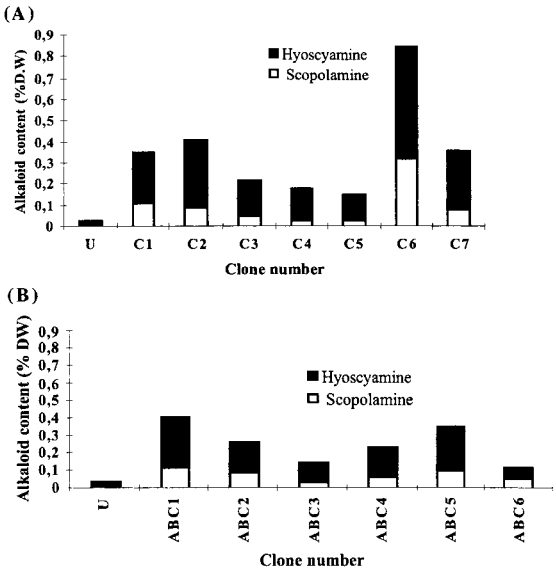

Figure 3. (A) Total tropane alkaloid (hyoscyamine and scopolamine) content of the seven *rol C* hairy root lines and untransformed root line of *A. belladonna* after 3 weeks of culture. (B) Total tropane alkaloid (hyoscyamine and scopolamine) content of the six *rol ABC* hairy root lines and untransformed root line of *A. belladonna* after 3 weeks of culture.

heterogeneity of growth rate values for the six *rol ABC* lines. In contrast to the seven *rol C* root lines, the growth rate was rather homogeneous. The mean growth rate of the *rol ABC* root lines was significantly much higher (36) than that of the *rol C* root lines (13). These results confirmed that the *rol ABC* genes together had a synergistic effect upon the growth of the hairy roots, and the results agreed with those obtained by Spena et al.[11] and Palazon et al.[12] with tobacco. The growth rate was dependent on the gene combination used for transformation. This result was comparable to the result obtained on tobacco by Palazon et al.[14] These results revealed that the presence of the *rol C* gene alone in the *A. belladonna* genome had a dramatic effect on the growth rate of transformed root lines studied. On average these cultures of *rol C* roots showed a growth rate three time less important than *rol ABC* root lines.

Hyoscyamine and Scopolamine Production. The total tropane alkaloid content of the root material was determined by HPLC after 3 and 4 weeks of culture (Figures 3 and 4). The total hyoscyamine and scopolamine was expressed as percent of dry weight. The amount of each alkaloid separately was also determined and the ratio scopolamine/hyoscyamine calculated. As indicated in Figures 3 and 4, all *rol C* and *rol ABC* hairy root lines contained tropane alkaloid concentrations that exceeded 4−28-fold those of control untransformed roots. All these results suggested that *rol* genes have a strong effect on the secondary metabolism in *A. belladonna*. In both series, the alkaloid content greatly varied from one root line to another: 0.15% DW (C5) to 0.85% DW (C6) for *rol C* root lines and 0.11% DW (ABC6) to 0.41% DW (ABC1) for *rol ABC* root lines after 3 weeks of culture. After this time of culture the mean values of the total tropane alkaloids were higher in *rol C* (0.36% DW) than in *rol ABC* (0.26% DW) root lines. Also after three weeks, the C6 root line had the highest capacity to produce tropane alkaloids (0.85% DW). However, after 4 weeks of culture the alkaloid production of this root line decreased to 0.36%. In contrast, after 3 weeks the C3 root line produced a moderate quantity of alkaloids (0.22%). After 4 weeks of culture the production

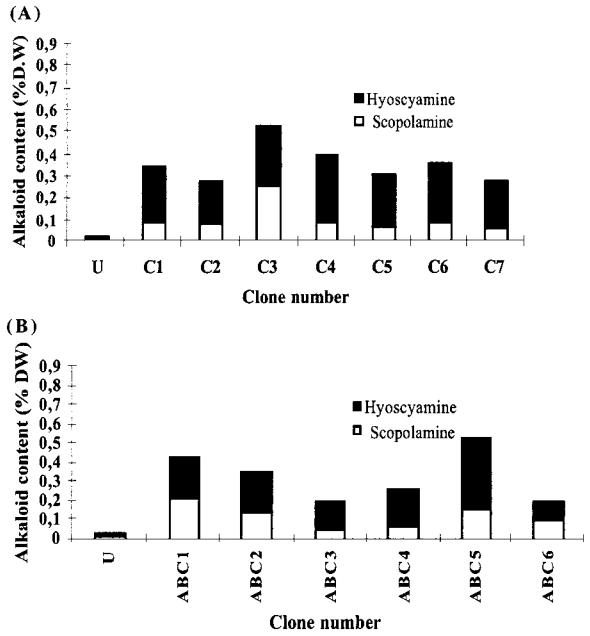

Figure 4. (A) Total tropane alkaloid (hyoscyamine and scopolamine) content of the seven *rol C* hairy root lines and untransformed root line of *A. belladonna* after 4 weeks of culture. (B) Total tropane alkaloid (hyoscyamine and scopolamine) content of the six *rol ABC* hairy root lines and untransformed root line of *A. belladonna* after 4 weeks of culture.

was the highest (0.53%) of the C lines and was quite similar to the highest production of ABC root line (ABC5: 0.52%). After four weeks of culture, the mean values of the total tropane alkaloid yields were similar in *rol C* and *rol ABC* root lines (respectively 0.36% and 0.33% DW). These results indicate a stable alkaloid production (0.36% DW) for *rol C* root lines after 3 and 4 weeks of culture, whereas production varied for *rol ABC* root lines (from 0.26 to 0.33% DW). Hyoscyamine was predominant whatever the combination of genes used, except for the ABC1 and the C3 clones after 4 weeks of culture, in which the contents in hyoscyamine and scopolamine were similar. The mean scopolamine/hyoscyamine ratio increased during the culture in all root lines, i.e., from 0.35% to 0.45% DW for *rol C* lines and from 0.45% to 0.60% DW for *rol ABC* lines. Our results showed that scopolamine production was highest after 4 weeks of culture.

In contrast to the results given by Palazon et al.[12] on tobacco, our work shows that the *rol C* gene alone was deeply involved and sufficient to stimulate the biosynthetic activity of *A. belladonna* hairy root cultures. Our results agree with conclusions of Bulgakov et al.[16] and Palazon et al.,[15] who demonstrated that the *rol C* gene alone may play an important role in stimulating ginsenoside and indole alkaloid production in hairy root cultures.

Experimental Section

General Experimental Procedures. The *Atropa belladonna* hairy roots were freeze-dried, powdered, and extracted twice using 50 mL of chloroform−ammonia (49:1; v/v) during 1 h under reflux. After filtration, each crude extract was evaporated to dryness under reduced pressure. The dried crude alkaloid extract was resuspended in 5 mL of the HPLC mobile phase at 50 °C for 15 min, then filtered through 0.2 μm filters and analyzed by HPLC as previously described by Fliniaux et al.[22] The alkaloid concentrations were calculated according to the external standard method (two-point calibration), with reference to solutions of authentic hyoscyamine and scopolamine. The data were given as the mean of three times the HPLC of the same extract. The variation between several analyses of the same sample did not exceed 5%. The amount of total alkaloids produced in each flask resulted from both the root biomass and the alkaloid content.

Plant Material. *A. belladonna* plantlets obtained from sterilized wild type seeds were micropropagated. They were subcultured every five weeks on a Linsmaier and Skoog (LS) solid medium[18] supplemented with 3% (w/v) sucrose and 0.5 mg/L naphthalenacetic acid (NAA). These cultures were maintained at 22 °C with a daily 16 h photoperiod. Five-week-old plantlets were 5−7 cm high and developed 1 cm long roots and 5 leaves. Untransformed roots issuing from 4-week-old plantlets were used as control roots.

Infection of Plant Explants and Induction of Hairy Root Lines. Two *A. tumefaciens* strains were used to infect *A. belladonna* explants: strain GV 3101 containing *rol C* genes (*A. tumefaciens rol C*) (given by Dr. Spena, Max-Planck Institute, Cologne) in a modified Ti plasmid, in which the pPCV002-C construction was included, as described by Spena et al.,[11] and strain GV 3101 containing *rol ABC* genes (*A. tumefaciens rol ABC*) in a modified Ti plasmid, in which the pPCV002-ABC construction was included. These strains were kanamycin-resistant; they were grown on a solid YEB medium supplemented with 100 mg/L carbenicillin and 25 mg/L kanamycin in the dark at 28 °C. For transformation, leaves of *A. belladonna*, harvested from 5-week-old plantlets, were used. Leaf disks (0.8 cm diameter) were infected by co-culture with these strains according to a slightly modified protocol first described by Horsch et al.[19] The disks were maintained in the dark at 22 °C on a LS solid medium containing 0.5 g/L cefotaxime, to eliminate bacteria, and 0.05 g/L kanamycin as selective agent. Five weeks after infection, hairy roots appeared at the wounded sites. After excision, the hairy roots induced at different sites of the explant were transferred to the same solid LS medium. All the culture media used for the hairy root cultures were hormone-free. In all experiments, about 100 mg (fresh weight) of 4-week-old hairy roots was inoculated into 30 mL of liquid LS medium containing 0.5 g/L cefotaxime, in 100 mL Erlenmeyer flasks, and incubated in the dark at 22 °C on a rotary shaker (130 rpm). For the fresh weight determination, the hairy roots were separated from the medium and then dried on filter paper and weighed. Growth rate was determined after four weeks of culture and was expressed as the ratio between the fresh weight of the root biomass and the fresh weight of the inoculum.

DNA Analysis. DNA was isolated from hairy roots and from the untransformed control roots frozen in liquid nitrogen by the CTAB method as described by Doyle and Doyle[20] and Bousquet et al.[21] Polymerase chain reaction was used to confirm the presence of the *npt II*, *rol A*, *rol B*, and *rol C* genes in roots. PCR experiments were performed by using primers located at the nucleotide position 21-42 (5′-CGTTGTCGGAATGGCCCAGACC-3′) and 268-246 (5′-CGTAGGTCTGAATATTCCGGTCC-3′), designed for the amplification of a 248-bp DNA fragment of the *rol A* gene, and primers at the position 118-138 (5′-ACTATAGCAAACCCCTCCTGC-3′) and 769-749 (5′-TTCAGGTTTACTGCAGCAGGC-3′), for the amplification of a 652-bp *rol B* fragment. Primers at the position 51-70 (5′-TGTGACAAGCAGCGATGAGC-3′) and 550-531 (5′-GATTGCAAACTTGCACTCGC-3′) were used to amplify a 490-bp *rol C* fragment. The primers for the *npt II* gene were from the position 201-221 (5′-GAGGCTATTCGGCTATGACTG-3′) and 900−880 (5′-ATCGGGAGCGGCGATACCGTA-3′), respectively, generating a predicted DNA fragment of 700 bp. Each PCR reaction was carried out in a 50 μL solution containing 200 mM of each dNTP, 2−5 μg of genomic DNA, 1 mM of each primer, 1.25 units of Appligene Taq DNA polymerase, and PCR buffer (containing magnesium chloride). The mixture was amplified in a thermal cycler (Thermojet, Eurogentec). Samples were subjected to 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 60 °C for the amplification of the *npt II* fragment, at 50 °C for the amplification of the *rol A* fragment, and at 55 °C for the amplification of the *rol B* and *rol C* fragments, 1 min extension at 72 °C. Amplified DNA was

detected by ultraviolet light after separation on 1% (W/V) agarose gels containing TAE as electrophoresis buffer and ethidium bromide.

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