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Expression of the *rol*C gene and nicotine production in transgenic roots and their regenerated plants

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Abstract Transformation of *Nicotiana tabacum* cv. Xanthi leaf sections with the pPCV002-ABC (*rol* genes A, B and C together under the control of their own promoter) or pPCV002-CaMVC (*rol* gene C alone under the control of the CaMV 35S promoter) construction present in trans-acting *Agrobacterium tumefaciens* vectors yielded several transgenic root lines. The two types (*rol*ABC and *rol*C) of transgenic root lines were examined for their nicotine productivity in relation to growth rate and the amount of *rol*C gene product measured with specific antibodies. In all cases, the changes in the amount of this polypeptide were positively correlated with the capacity of the transgenic roots to grow and produce nicotine. Both capacities were greatly increased when the *rol*A, *rol*B and *rol*C genes were present together, which demonstrates that the activity of the three *rol*-gene-encoded functions is synergistic. Consistent observations were also made in the corresponding regenerated plants.

Key words Nicotine · *rol* genes · Transgenic roots · Transgenic plants · *Nicotiana tabacum*

Introduction

The *rol*A, *rol*B and *rol*C genes of *Agrobacterium rhizogenes* A4 are located on the TL-DNA of the Ri plasmid and correspond to open reading frames 10, 11 and 12, respectively, of this T-DNA section (Slightom et al. 1986). Recently, it was shown that *rol*A, *rol*B and *rol*C genes can induce the full hairy root syndrome in tobacco (Cardarelli et al. 1987; Spena et al. 1987; Jouanin et al. 1987; Altabella et al. 1995; Palazón et al. 1997). Moreover, each of these genes is able on its own to promote root formation in tobacco, but they differ in their efficiency (*rol*B is more efficient than *rol*A or *rol*C). Transgenic roots can regenerate into transgenic plants (Jouanin et al. 1987; Spena et al. 1987). In this case too, the full hairy root syndrome is established only in transgenic plants expressing all three *rol* genes, whereas plants transgenic for single *rol* genes do not show all the phenotypic traits of this syndrome. The characteristic symptoms of the hairy root syndrome are adventitious root formation, high growth rate of roots in culture, reduced apical dominance both in stems and roots, altered leaf morphology and plagiotropic root growth (Tepfer 1984).

It has been shown in tobacco leaf protoplast that the expression of *rol* genes A, B and C enhances the auxin sensitivity of stimulation of the transmembrane electrical potential difference (Maurel et al. 1991). Besides this functional indication, almost nothing was known until very recently concerning the nature of *rol*-gene-encoded proteins. In the case of *rol*C, direct biochemical data are available showing that the polypeptide is a β-glucosidase able to hydrolyse cytokinin N- and O-glucosides, therefore releasing active hormone from the inactive conjugates (Estruch et al. 1991a). However, other investigations showed that *rol*C activity in transgenic plants reduces the rate of synthesis of cytokinins and gibberellins (Nilsson et al. 1993). On the other hand, although high-level nicotine production by tobacco roots transformed by a wild strain of *A. rhizogenes* has previously been reported (Hamill et al. 1986; Parr and Hamill 1987), to our knowledge nothing has been reported about the capacity of *rol* gene transgenic tobacco roots and regenerated plants to produce nicotine, the major alkaloid present in tobacco plants. This alkaloid is synthesized in the roots and then transported to the leaves where it accumulates (Waller and Novacki 1978). In the present study, in addition to obtaining antibodies against the polypeptide encoded by the *rol*C gene, we report the establishment of tobacco roots transgenic for three *rol* genes (A, B and C) and the *rol*C gene alone, and regener-

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ation of their corresponding transgenic plants. We also report that changes in nicotine production of both transgenic roots and regenerated plants are correlated with changes in the phenotype induced by the presence of *rol* genes and with the amount of the polypeptide encoded by the *rol*C gene.

Materials and methods

Bacterial strains and plasmids

The following constructions were obtained from A. Spena (Max-Planck Institut, Cologne): pPCV002-ABC (*rol* genes A, B and C together under the control of their own promoter) and pPCV002-CaMVC (*rol*C gene under the control of the CaMV 35S promoter). These constructions were obtained from the subcloning of different fragments from the Ri TL-DNA of *A. rhizogenes* strain A4 into the binary vector pPCV002 (Koncz and Schell 1986). The binary plasmid also contained, in its T-DNA region, the neomycin phosphotransferase gene driven by the nopaline synthase promoter. All constructions were maintained in *Escherichia coli* HB101 and then mobilised to the disarmed *A. tumefaciens* GV3101 strain according to Höfgen and Willmitzer (1988).

Plant tissue culture and transformation

Root induction on leaf discs of sterile plant culture of *Nicotiana tabacum* cv. Xanthi were carried out as described earlier (Altabella et al. 1995). Roots began to appear after 4 weeks and single roots were picked off individually after 6 weeks and split into two parts to establish the cultures of transgenic root lines and regenerated plants. To obtain the latter, one of the two explants was exposed to kanamycin selection (50 mg/l) during callus induction on Murashige and Skoog (1962) (MS) medium supplemented with 30 g/l saccharose, 0.6 mg/l naphthaleneacetic acid and 0.2 mg/l kinetin. Transgenic plants were raised from these root-derived transgenic calli on MS medium containing 30 g/l saccharose, 1 mg/l benzylaminopurine and 50 mg/l kanamycin. Shoots were rooted on hormone-free MS medium with 30 g/l saccharose under kanamycin selection. The transgenic plants thus obtained and the untransformed plants obtained from sterile seeds were grown on solid MS medium in a controlled chamber (25°C, 25 W/m^2, 16 h photoperiod). Untransformed roots were excised from sterile untransformed plants. Transgenic and untransformed roots were grown in 40 ml of hormone-free MS liquid medium supplemented with 30 g/l saccharose in 100-ml flasks on an orbital shaker at 100 rpm. All root cultures were initiated from inocula of 0.1 g fresh weight and maintained in the dark at 25°C and subcultured every 6 weeks. Growth was measured as dry weight increase per 100 ml flask in six replicates per sample.

Antibody generation

The polyclonal antibodies directed towards the 20.1-kDa polypeptide encoded by the *rol*C gene (Slightom et al. 1985) were made by expressing in *E. coli* the *rol*C open reading frame subcloned as a fragment of 986 bp (from nucleotide 12459 to 13445; Slightom et al. 1986) into the expression vector pDS56-RBSII-2 (Stüber et al. 1990). The construction pDS56-RBSII-2-*rol*C was a gift from A. Spena (Max-Planck Institut, Cologne). *E. coli* M15 containing the repressor plasmid and the plasmid bearing the *rol*C gene were induced at 37°C with 1 mM isopropyl-β-D-thiogalactoside (for details see Stüber et al. 1990). After 1 h on ice, the suspension was sonicated until it was clear. After spinning at 6000 *g* for 30 min, the supernatant was aliquoted and frozen. The protein concentration was determined by the method of Bradford (1976) using the Bio-Rad dye reagent concentrate and bovine globulin as standard. Purification of the fusion protein from the inclusion bodies of the bacteria and rabbit immunisation were performed as described in the FEXFIT manual (Genofit, Geneva, Switzerland). Before the primary immunisation, the rabbits were bled to collect preimmune serum and were subsequently injected with 5 mg of purified fusion protein emulsified with complete Freund adjuvant (Sigma). The animals received booster injections three times, at 3-week intervals, with the same antigen preparation mixed with incomplete Freund adjuvant. The antibodies specific for the *rol*C polypeptide were affinity-purified as described by Schneider et al. (1982).

Protein extraction and immunoblot analysis

Proteins were extracted from root cultures and from plants (roots and leaves separately) by grinding tissue in liquid nitrogen and boiling for 5 min in a denaturation buffer containing 20 mM Tris-HCl pH 8.6, 1% SDS and 2% 2-mercaptoethanol. The supernatant obtained after centrifugation at 15000 g for 5 min was used for SDS-PAGE and immunoblot analysis as described previously by Piñol et al. (1996). Immune serum was utilised at 1/500 dilution and the second antibody coupled to alkaline phosphatase (Bio-Rad) was utilised at 1/5000 dilution. The deposits of the alkaline phosphatase reaction were photographed with a Polaroid MP-4 machine. Films were scanned with an Artiscan 1200C Scanner and then quantified using a PHORETIX 1D Gel Analysis (REXEL Clearview Binder) program.

Nicotine determination

The nicotine content was determined according to the method described by Saunders and Blume (1981), with some modifications. Cultured roots and plants were harvested after 6 weeks of growth and separated into roots and medium (cultured roots) or roots and leaves (cultured plants). The dry plant material was ground and the powder (500 mg) extracted with 10 ml of 25 mM sodium phosphate buffer (pH 7.8) at room temperature for 24 h under constant agitation. The different aqueous extracts were centrifuged (to remove the cell debris) and filtered through a 0.45-µm Millipore filter and the different samples were stored in sealed vials at –20°C. On the other hand, aliquots of root medium were made alkaline (ca. pH 11) with NH_4OH, and extracted with an equal volume of $CHCl_3$. The $CHCl_3$ phase was re-extracted with 1 vol diluted acid (HOAc, pH 3) and the aqueous layer was centrifuged and filtered as before. The different samples were also stored in sealed vials at –20°C. The HPLC system used consisted of an LKB 2158 Uvicord SD detector equipped with a 254-nm filter, 2150 HPLC pump, and Spectra-Physics SP 4270 integrator. Nicotine was quantitively separated using a Waters µ-Bondapak C18 reverse-phase column (30×0.4 cm), eluted with an isocratic mobile phase of 40% (vol/vol) methanol containing 2% (vol/vol) phosphoric acid buffered to pH 7.25 with triethylamine, at a flow rate of 0.8 ml/min. The sample peak areas corresponding to nicotine (20-µl injection volume), with the same retention time as authentic standard (Sigma), were integrated by comparison with an external standard calibration curve. The data shown are the average value of six replicates per sample.

Results and discussion

Characterisation and nicotine production of cultured root lines and regenerated plants

Transgenic root lines were established from *N. tabacum* cv. Xanthi by infecting leaf sections with the *A. tumefaciens* GV3101 strain containing the *rol* genes A, B and C (under the control of their own promoter) or the *rol* gene C (under the control of the CaMV 35S promoter) cloned

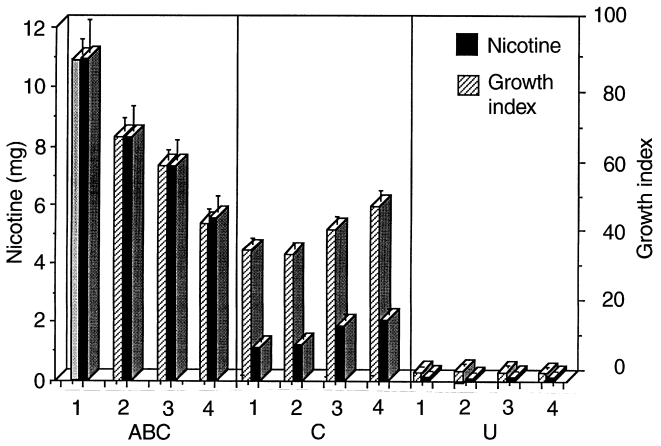

Fig. 1 Growth and nicotine production by the *rol*ABC and *rol*C transgenic root lines and untransformed root lines of *N. tabacum*. An inoculum of 0.1 g fresh weight as 10 mg dry weight was added to each flask with 40 ml of MS liquid medium without phytohormones. Dry weight and nicotine production were determined after 6 weeks of culture. The *growth index* represents harvest dry weight per inoculum dry weight. Measurements are based on six replicates ±SD (*U* untransformed roots)

in the binary vector pPCV002, which has a neomycin phosphotransferase gene in its T-DNA region. We have used pPCV002-CaMVC because, as was previously observed by Spena et al. (1987), the *rol*C gene under the control of its own promoter was not able to induce root formation on tobacco leaves. The individual roots produced at the site of the infection were tested for kanamycin resistance in order to assess their transgenic nature and then used to establish the respective cultures of root lines and regenerated plants as described in Materials and methods. Unlike untransformed roots which grew only slowly without branching of lateral roots, the transgenic roots subcultured every 6 weeks in MS liquid medium without phytohormones grew fast throughout the whole culture period and presented the typically branched hairy root appearance which, as described by us previously (Palazón et al. 1997), was more pronounced in roots harbouring the three *rol* genes. These characteristics remained stable during subsequent transfers.

At the end of the culture period, marked differences were found in the growth and nicotine alkaloid production between the two types (*rol*ABC and *rol*C) of transgenic root lines considered (Fig. 1). As may be deduced from the growth index values (harvest dry weight per inoculum dry weight), in comparison with the mean of the untransformed root lines, the growth mean of the lines harbouring the *rol*A, *rol*B and *rol*C genes together was 22-fold greater, while that of the lines harbouring the *rol*C gene alone was 14-fold greater ($P<0.001$, *t*-test). At the same time, as can be seen in Fig. 1, total nicotine production (nicotine in the roots and the medium) in *rol*ABC transgenic roots lines was always notably higher than in *rol*C transgenic root lines and dramatically higher than in untransformed root lines. Calculating the mean nicotine content for each, the result is 86 mg for the *rol*ABC root lines, 17 mg for the *rol*C root lines and only 0.8 mg for the untransformed root lines.

Nicotine synthesis is known to be critically dependent on a variety of factors including the stimulus of meristematic activity, while the arrest of growth paralyses alkaloid synthesis (Mothes et al. 1957). The marked differences in nicotine production observed in the root lines harbouring the same construction (*rol*ABC or *rol*C, Fig. 1) could have arisen from the secondary effects of the transformation on root line growth. As T-DNA can be integrated at different sites in the plant genome (Ambros et al. 1986), the occurrence of genetic changes as a consequence of transformation might provide an explanation in some cases. In *Nicotiana,* the variation in the number of T-DNA integrations does not seem to be associated with the capacity of the different root lines to produce nicotine (Parr and Hamill 1987).

With respect to the corresponding regenerated plants (Fig. 2), those carrying the *rol*A, *rol*B and *rol*C genes together displayed phenotypic alterations typical of the hairy root syndrome. When compared with untransformed

Fig. 2 General view of *N. tabacum* plants after 6 weeks culture on MS solid medium: plants transgenic for *rol*ABC genes (**A**), plants transgenic for the *rol*C gene (**B**), and untransformed plants (**C**). The capacity of *rol*C transgenic plants for developing roots and forming root biomass was higher than that of untransformed plants but not as high as that of *rol*ABC transgenic plants

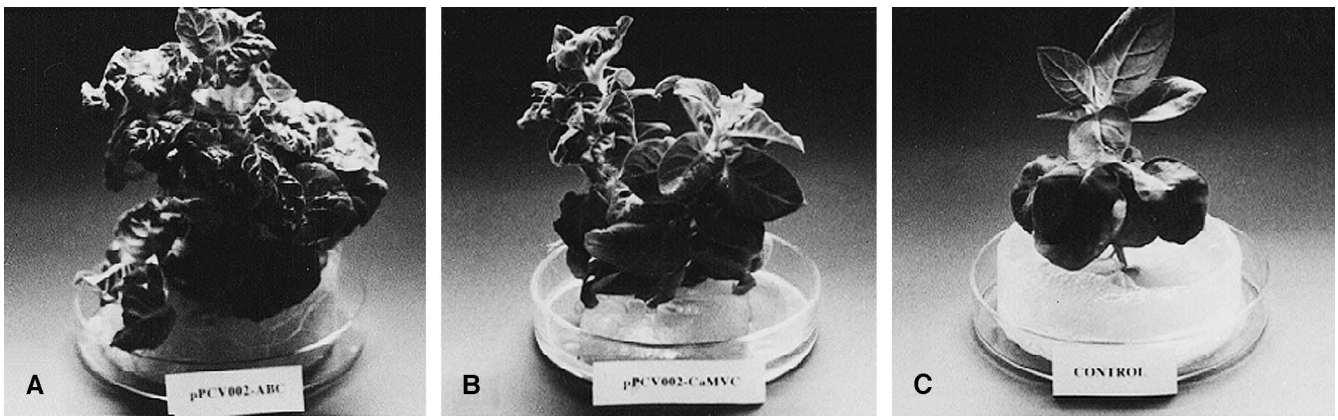

Table 1 Biomass production and nicotine content by *Nicotiana tabacum* transgenic plants. To obtain complete transgenic plants from each root line containing the three *rol* genes (A, B and C) or the *rol*C gene alone, the corresponding shoots (about 5 mm long) raised from these root-line-derived transgenic calli were rooted on hormone-free MS medium with 30 g/l saccharose under kanamycin selection and cultured on the same medium in a controlled chamber. Determinations from roots and leaves were made separately after 6 weeks of culture. Measurements correspond to the average values of five plants of each type (untransformed, *rol*ABC and *rol*C) ± SE (*DW* dry weight, *U* untransformed)

Whole plant organs	Biomass (g DW)			Nicotine (mg)		
	rolABC	rolC	U	rolABC	rolC	U
Roots	0.334±0.021	0.183±0.012	0.061±0.009	0.412±0.038	0.142±0.015	0.070±0.010
Leaves	0.402±0.031	0.225±0.028	0.084±0.010	1.539±0.102	0.456±0.038	0.139±0.009

plants, in addition to their higher rooting rate and dramatically greater capacity to develop root biomass, these transgenic plants showed a shorter internodal distance, altered leaf morphology and reduced apical dominance both in stems and roots. Similarly, transgenic plants carrying the *rol*C gene alone showed a high capacity to form roots and develop root biomass but this capacity was significantly lower than that of the plants transgenic for three *rol* genes. With regard to phenotypic alterations affecting stems, *rol*C transgenic plants were more branched than untransformed plants and also presented a rather altered leaf morphology as was previously described by Schmülling et al. (1988) for *rol*C transgenic plants where this *rol* gene was also under the control of the 35S promoter.

To determine the growth and nicotine production values of these plants, five different plants regenerated from each transgenic root line were tested. Similar growth and nicotine production were observed among the different plants harbouring the same construction and also among the untransformed plants. Hence, only the average values for each type of plant (untransformed, *rol*ABC and *rol*C) are presented in Table 1. However, because nicotine is synthesised in the roots of the tobacco plant and then transported to the leaves where it accumulates, the growth and nicotine content of the roots and leaves are shown separately. The determinations were made at 6 weeks when plants were still in the vegetative state. As shown in Table 1, the plants transgenic for the three *rol* genes (A, B and C), whose root biomass (measured as grams dry weight at 6 weeks) was more than five times higher than that of untransformed plants, accumulated more nicotine (6-fold greater in the roots and 11-fold greater in the leaves). With respect to plants transgenic for the *rol*C gene alone, the root biomass was 3 times higher than that of untransformed plants, and the nicotine content in roots and leaves about 2 and 3 times higher, respectively, compared to untransformed plants. These results reveal a close positive correlation between root biomass and the capacity of these organs to produce nicotine in the three types (untransformed, *rol*ABC and *rol*C) of tobacco plants.

Immunoblot analysis

We have generated specific polyclonal antibodies to the Ri TL-DNA *rol*C gene 20.1-kDa product (Slightom et al. 1985), as described in Materials and methods, to investigate whether changes in the amount of this polypeptide could be correlated with changes in nicotine alkaloid production in the *rol*ABC and *rol*C transgenic tobacco root lines and their corresponding transgenic plants. Figure 3 demonstrates that the antibodies against the *rol*C gene product specifically recognized a band of about 20.1 kDa in the total protein fraction of *E. coli* containing the expression vector pDS56-RBSII-2-*rol*C. The fact that this band was not observed when the total protein fraction of the bacterium was immunoassayed with preimmune serum confirmed the indicated specificity of the generated antibodies.

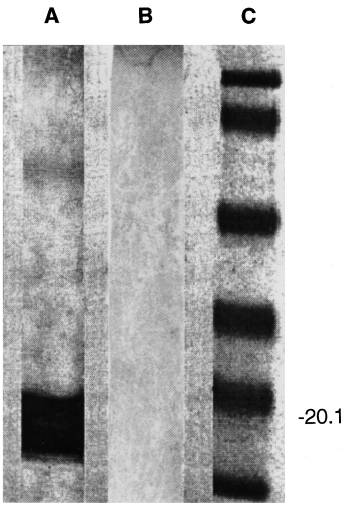

Fig. 3 Specificity of the reaction of antibodies with total protein fraction of *Escherichia coli* containing the plasmid bearing the *rol*C gene. *Lanes A and B*: 100 µg of total protein fraction of *E. coli* containing this plasmid; *lane C*: molecular weight standards of 80, 49.5, 32.5, 27.5, and 18.5 kDa from top to bottom. *Lanes A and B* were blotted to nitrocellulose and immunodetected with either immune serum (*A*) or preimmune serum against the 20.1-kDa polypeptide encoded by the *rol*C gene (*B*); *lane C* was stained for protein with Coomassie R-250

The immunoassays using the specific antibodies directed towards the polypeptide encoded by the *rol*C gene were made at 6 weeks of culture for both the cultured transgenic root lines and their regenerated plants. Each lane of the gel was charged with the same amount of total protein fraction, and therefore the reactivity intensity provided a

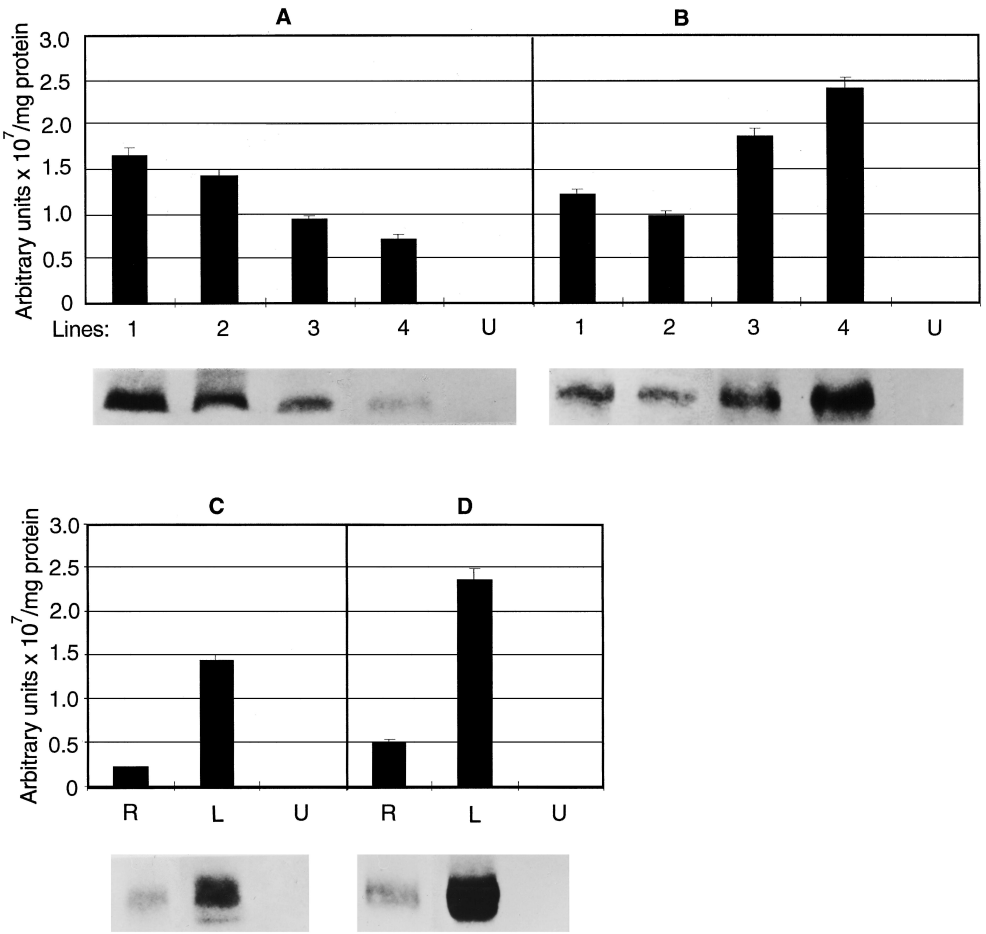

Fig. 4 Immunoblot analysis of *N. tabacum* root lines transgenic for *rol*ABC genes (**A**) and the *rol*C gene (**B**) and their corresponding regenerated transgenic plants (*rol*ABC plants, **C**; *rol*C plants, **D**) after 6 - weeks of culture. A total of 100 µg per lane of total protein fraction from each root line considered and from the respective roots (**R**) and leaves (**L**) of the regenerated plants was analysed by PAGE-SDS, blotted to nitrocellulose and immunodetected with antiserum against the *rol*C protein product. The values for the amount of *rol*C gene product are the average of four determinations. Protein size (20.1 kDa) was determined by comparison with a standard marker (*U* untransformed)

relative measurement of the *rol*C gene product. As shown in Fig. 4A, B, the immunoblot analysis results revealed that a clear reaction band of 20.1 kDa could be seen when the antibodies were used with the total protein fraction of each *rol*ABC or *rol*C transgenic root line considered. The amount of the polypeptide encoded by the *rol*C gene quantified as described in Materials and methods was 1.6×10^7, 1.4×10^7, 9.3×10^6 and 7.2×10^6 arbitrary units/mg protein for *rol*ABC lines 1, 2, 3 and 4, respectively, and 1.2×10^7, 9.7×10^6, 1.8×10^7 and 2.4×10^7 arbitrary units/mg protein for *rol*C lines 1, 2, 3 and 4, respectively. At the same time, it could be observed that, among the root lines harbouring the same construction (i.e. *rol*ABC or *rol*C), the differences in the intensity of this reaction band and consequently in the level of the *rol*C gene product, were closely correlated with the biomass of the roots and their capacity to form nicotine (see the growth index and nicotine production values in Fig. 1). Thus, among the different *rol*ABC root lines, lines 1 and 4 presented the highest and lowest amount, respectively, of *rol*C gene product, growth rate and alkaloid production, and among the different *rol*C root lines the same phenomenon was observed with the highest values in line 4 and the lowest in line 2. We suggest that this stimulatory effect on nicotine production in both types of transgenic roots was mainly due to an increase in root biomass production (relative to untransformed roots in culture) in direct proportion to the amount of *rol*C gene product accumulated in the cells of the respective transgenic root lines.

With regard to the regenerated plants transgenic for the *rol* genes A, B and C or the *rol*C gene alone, the immunoblot analysis results revealed that when the antibodies directed toward the polypeptide encoded by the *rol*C gene were used with the total protein fraction of roots and leaves of both types of transgenic plants, a 20.1-kDa polypeptide also reacted with these antibodies (Fig. 4C, D). In this case, the amount of *rol*C gene product was similar for the different plants harbouring the same construct (*rol*ABC or *rol*C), possibly as a consequence of growth correlation at the whole-plant level, and at the same time significantly higher in the roots than the leaves of all considered plants (up to 6.5- and 4.6-fold for *rol*ABC and *rol*C plants, respectively, $P<0.001$). Not only do these results reveal that the different capacity to accumulate the *rol*C gene product in the transgenic root lines carrying the same construction (see above) was not manifested in the corresponding regenerated plants but they also show that *rol*C gene expression was organ specific in both types of transgenic plant. In several studies of the expression of Ri T-DNA, different transcription patterns have been observed depending on the transformed tissues or plant parts analysed (Taylor et al. 1985; Ooms et al. 1986).

The fact that when compared with root lines and plants transgenic for the three *rol* genes (A, B and C), those transgenic for the *rol*C gene alone had up to 1.3- and 1.6-fold more *rol*C gene product, respectively ($P<0.001$), may be attributed to the control of the *rol*C gene by its own promoter in the first case and by the 35S promoter in the second and for this reason overexpressed. The 5′-flanking sequences of cauliflower mosaic virus 35S transcript (35S promoter) constitute a strong unregulated promoter. However, it is clear that under the conditions of our experiment, the overexpression of the *rol*C gene was not sufficient to replace the stimulant effect of the presence of *rol*A and *rol*B genes on the growth rate of roots in culture or attached to the plant, and their ability to synthesise nicotine. The available data show that the transgenic root lines and plants expressing the *rol*C gene under the control of the 35S promoter (i.e. transgenic for the *rol*C gene alone) exhibited the highest levels of *rol*C gene product and, at the same time, the lowest levels of root biomass and nicotine production when compared with transgenic root lines and plants expressing the *rol*C gene under the control of its own promoter (i.e. transgenic for *rol*ABC genes).

On the other hand, the fact that the *rol*C gene product can by itself stimulate root formation in tobacco leaves indicates that the product of this *rol* locus must interact with plant factors controlling organ differentiation. If these factors are similar or identical to those responsible for the effects of growth hormones in plants, it is understandable that they could be different in different host species, since the *rol*C gene product can by itself stimulate root formation in tobacco but not in kalanchoe (Spena et al. 1987). In relation to *rol*C gene function, an important observation made by Estruch et al. (1991b) has been that in subcellular fractions of homogenates from 35S-*rol*C transgenic leaves, the *rol*C polypeptide was present exclusively in the cytosolic fraction. Evidently, this observation would be compatible with the idea that the *rol*C gene product affects plant growth and morphogenesis by modifying either directly or indirectly the activity of endogenous plant hormones.

Although the precise functions of the *rol* gene products remain unknown, we conclude that the genetic changes involved in *rol*ABC- or *rol*C-mediated transformation have apparently not only modified cell differentiation in favour of root formation, but also resulted in normal stimulation of root-specific secondary metabolism (nicotine is synthesised in the roots of tobacco plants) in root lines and their corresponding regenerated plants. In both cases, this stimulation was positively correlated with the level of the polypeptide encoded by the *rol*C gene and, at the same time, more pronounced when the *rol*A, *rol*B and *rol*C genes were introduced together, which clearly demonstrates a synergistic action of the three *rol* genes in determining root growth rate and nicotine synthesis capacity in our transgenic tobacco roots and plants.

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References

Altabella T, Angel E, Biondi S, Palazón J, Bagni N, Piñol MT (1995) Effect of the *rol* genes from *Agrobacterium rhizogenes* on polyamine metabolism in tobacco roots. Physiol Plant 95:479–485

Ambros PF, Matzke AJM, Matzke MA (1986) Localization of *Agrobacterium rhizogenes* T-DNA inplant chromosomes by *in situ* hybridization. EMBO J 5:2073–2077

Bradford MM (1976) A rapid sensitive method for the cuantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Cardarelli M, Spano L, Mariotti D, Mauro ML, Van Sluys MA, Constantino P (1987) *Agrobacterium rhizogenes* T-DNA genes capable of inducing hairy root phenotype. Mol Gen Genet 209: 475–480

Estruch JJ, Chriqui D, Grossmam K, Schell J, Spena A (1991a) The plant oncogene *rol*C is responsible for the release of cytokinins from glucoside conjugates. EMBO J 10:2889–2895

Estruch JJ, Parets-Soler A, Schmülling T, Spena A (1991b) Cytosolic localization in transgenic plants of the *rol*C peptide from *Agrobacterium rhizogenes.* Plant Mol Biol 17:547–550

Hamill JD, Parr AJ, Robins RJ, Rhodes MJC (1986) Secondary product formation by cultures of *Beta vulgaris* and *Nicotiana rustica* transformed with *Agrobacterium rhizogenes.* Plant Cell Rep 5:111–114

Höfgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res 16:1877

Jouanin L, Vilaine F, Tourneur J, Tourneur C, Pautot V, Muller JF, Caboche M (1987) Transfer of a 4.3-kb fragment of the TL-DNA of *Agrobacterium rhizogenes* strain A4 confers the pRi transformed phenotype of regenerated tobacco plants. Plant Sci 53:53–63

Koncz C, Schell J (1986) The promoter of TL-DNA gene controls the tissue-specific expression of chimeric genes carried by a novell type of *Agrobacterium* binary vector. Mol Gen Genet 204:383–396

Maurel C, Banbier-Brygoo H, Spena A, Tempé J, Guern J (1991) Single *rol* genes from the *Agrobacterium rhizogenes* TL-DNA alter some of the cellular responses to auxin in *Nicotiana tabacum.* Plant Physiol 97:212–216

Mothes K, Engelbrecht L, Tschoepe KH, Trefftz-Huts-Chereuter G (1957) Root activity and nicotine formation. Flora 144:518–523

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

Nilsson O, Moritz T, Imbault N, Sandberg G, Olsson O (1993) Hormonal characterization of transgenic tobacco plants expressing the *rol*C gene of *Agrobacterium rhizogenes* TL-DNA. Plant Physiol 102:363–371

Ooms G, Twell D, Bossen ME, Hoge JHC, Burrell MM (1986) Developmental regulation of Ri TL-DNA gene expression in roots, shoots and tubers of transformed potato (*Solanum tuberosum* cv desire). Plant Mol Biol 6:321–330

Palazón J, Cusidó R, Roig C, Piñol MT (1997) Effect of *rol* genes from *Agrobacterium rhizogenes* TL-DNA on nicotine production in tobacco root cultures. Plant Physiol Biochem 35: 155–162

Parr AJ, Hamill JD (1987) Relationship between *Agrobacterium rhizogenes* transformed hairy roots and intact, uninfected *Nicotiana* plants. Phytochemistry 26:3241–3245

Piñol MT, Palazón J, Cusido RM, Serrano M (1996) Effects of Ri T-DNA from *Agrobacterium* on growth and hyoscyamine production in *Datura stramonium* root cultures. Bot Acta 109: 133–138

Saunders JA, Blume DE (1981) Quantitation of major tobacco alkaloids by high-performance liquid chromatography. J Chromatogr 205:147–154

Schmülling T, Schell J, Spena A (1988) Single genes from *Agrobacterium rhizogenes* influence plant development. EMBO J 7: 2621–2629

Schneider C, Newman RA, Sutherland R, Asser U, Greaves MF (1982) A one-step purification of membrane proteins using a high efficiency immunomatrix. J Biol Chem 257:10766–10769

Slightom JL, Jouanin L, Leach F, Drong R, Tepfer R (1985) Isolation and identification of TL-DNA plant junctions in *Convolvulus arvensis* transformed by *Agrobacterium rhizogenes* strain A4. EMBO J 4:3069–3077

Slightom JL, Duran-Tardif M, Jouanin L, Tepfer D (1986) Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. J Biol Chem 261:108–121

Spena A, Schmülling T, Koncz C, Schell JS (1987) Independent and synergistic activity of *rol*A, B and C *loci* in stimulating abnormal growth in plants. EMBO J 6:3891–3899

Stüber D, Matile H, Garotta G (1990) System for high level productions in *Escherichia coli* and rapid purification of recombinant proteins: application to epitope mapping, preparation and antibodies and structure function analysis. In: Lefkovits I, Pernis, B (eds) Inmmunological methods. Academic Press, London, pp 121–152

Taylor BH, White FF, Nester EW, Gordon MP (1985) Transcription of *Agrobacterium rhizogenes* A4 T-DNA. Mol Gen Genet 201:546–553

Tepfer D (1984) Transformation of several species of higher plants by *Agrobacterium rhizogenes:* sexual transmission of the transformed phenotype and genotype. Cell 47:959–967

Waller GR, Novacki EK (1978) Sites of alkaloid formation. In: Alkaloid biology and metabolism in plants. Plenum, New York, pp 121–141