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Independent and synergistic activity of *rol A, B* and *C* loci in stimulating abnormal growth in plants

A.Spena, T.Schmülling, C.Koncz and J.S.Schell

Max-Planck-Institut für Züchtungsforschung, 5000 Köln 30, FRG

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The Ri plasmid A4 of *Agrobacterium rhizogenes* contains within its T-DNA genetic information able to trigger root formation in infected plants. Tobacco plants regenerated from transformed roots display the hairy root (hr) syndrome. We show that DNA fragments containing the *rol B* locus alone are able to induce root formation both in tobacco and kalanchoe tissues. The *rol A* and the *rol C* loci by themselves are also able to induce root formation in tobacco but not in kalanchoe. This capacity to induce root formation in either host is greatly increased when the *rol A* and/or *C* loci are combined with the *rol B* locus. Root induction is shown to be correlated with the expression of the *rol* loci. Transgenic plants exhibit all the characteristics of the hairy root syndrome only when all three loci are present and expressed. Although the activity of the *rol* encoded functions is synergistic, each of them appears to independently influence host functions involved in the determination of root differentiation.

Key words: hairy root/Ri plasmid/*rol* loci/transgenic plants/hairy root syndrome

Introduction

Infection of a plant by *Agrobacterium rhizogenes* usually results in root formation at the site of infection (Riker *et al.*, 1930; Hildebrand, 1934; Bevan and Chilton, 1982). This morphogenic event is due to the transfer of genetic information carried by Ri plasmids from bacteria to the plant cells (Moore *et al.*, 1979; Chilton *et al.*, 1982; Spano *et al.*, 1982; White *et al.*, 1982). In this regard *A.rhizogenes* behaves similarly to *A.tumefaciens*. The integration and expression of the transferred DNA (T-DNA) of Ti plasmids causes metabolic changes mainly determined by the *iaaM* and *iaaH* genes which code for enzymes involved in indoleacetic acid synthesis and the *ipt* gene whose product catalyses the first step in cytokinin biosynthesis (Akiyoshi *et al.*, 1984; Schröder *et al.*, 1984; Inze *et al.*, 1984; Barry *et al.*, 1984; Tomashow *et al.*, 1984; Buchmann *et al.*, 1985; Yamada *et al.*, 1985).

Similarly infection of a wounded tissue by *A.rhizogenes* strains, harbouring Ri plasmids, results in transfer of T-DNA to the plant genome. The T-DNA of the *A.rhizogenes* strain A4 consists of two non contiguous stretches of DNA, the T_L- and the T_R-DNA (Huffmann *et al.*, 1984; Jouanin, 1984; De Paolis *et al.*, 1985). The T_R-DNA contains two genes homologous to the *iaaM* and *iaaH* genes of Ti plasmids but only plays a conditional and non-essential role in the hairy root formation (Boulanger *et al.*, 1986; Cardarelli *et al.*, 1987).

Many features differentiate the morphogenic action of Ri plasmids from that of Ti plasmids (Cardarelli *et al.*, 1987). Ri-transformed roots can be cultivated *in vitro* on hormone-free medium and, in some species, regenerated into plants (Ackermann, 1977; Tepfer, 1984). These transgenic plants show an altered phenotype, called hr syndrome, which, despite some variable traits, has characteristic features in several plant species (Tepfer, 1984; Ooms *et al.*, 1985). The invariable and characteristic traits of the hr syndrome in tobacco are: (i) high growth rate of roots in culture; (ii) reduced apical dominance in roots and stems; (iii) wrinkled leaves with increased width to length ratio; and (iv) plagiotropic roots (i.e. with altered geotropism). These phenotypic traits are due to the presence of several loci in the T_L-DNA delivered to the plant genome (Tepfer, 1984; Durand-Tardif *et al.*, 1985).

Four loci involved in the hairy root formation have been identified through insertional mutagenesis in the T_L-DNA. They do not show any homology with the T-DNA of Ti plasmids (Huffmann *et al.*, 1984; Jouanin, 1984) and were called root loci (*rol*) *A, B, C* and *D* (White *et al.*, 1985). The genetic analysis showed that insertions in the *rol B* locus were completely avirulent on kalanchoe leaves, whilst insertions in the *rol A, C* and *D* loci gave an attenuated response and consequently pointed to a pivotal role of the *rol B* locus in root induction on kalanchoe leaves (White *et al.*, 1985). The *rol A, B, C* and *D* loci correspond, most likely, to open reading frames (ORFs) 10, 11, 12 and 15 of the T_L-DNA (Slightom *et al.*, 1986). Tobacco plants, transformed only with a portion of the T_L-DNA lacking ORFs 1–7 and lacking the T_R-DNA altogether displayed all the characteristic traits of the hr syndrome (Tepfer, 1984; Durand-Tardif *et al.*, 1985).

In this work we analyse the functions of the *rol A, B* and *C* loci in two different systems, kalanchoe and tobacco, by producing plant cell clones as calli or as transgenic plants which contain different combinations of the *rol A, B* and *C* loci.

Results

EcoRI fragment 15 of the Ri plasmid A4 controls root formation in transformed calli

Tobacco protoplasts were transformed by co-cultivation with *A.tumefaciens* strains harbouring the constructions pPCV002-ABC1 and -ABC2 shown in Figure 1. These vectors contain part of the T_L-DNA of *A.rhizogenes* strain A4 covering the *rol* loci *A, B* and *C* (Slightom *et al.*, 1986). Half of the kanamycin resistant calli (22 out of 45) resulting from transformation with pPCV002-ABC1 or pPCV002-ABC2 developed roots (Figure 2). Both constructions were equally able to induce root formation indicating that root induction is independent of the orientation of the *Eco*RI fragment 15 in the pPCV002 vector. The transformed tissues showed root formation even in the absence of exogenous auxin (MS3 medium). Kanamycin resistant calli transformed with the binary vector pPCV002 by itself did not form roots. This result shows that *Eco*RI fragment 15, consisting of 4374 bp of the pRiA4 T_L-DNA, contains genetic information able to induce root formation in tobacco calli.

Transcriptional analysis of the rol A, B and C loci

The transcription pattern of the *rol A, B* and *C* loci was analysed in a transformed tobacco callus, clone ABC2-N (Figure 3a). The

results of a Northern blot analysis (Figure 3b) performed on poly(A)$^+$ RNA extracted from callus material grown on medium containing 0.5 mg/l of benzylaminopurine (BAP), can be summarized as follows: (i) *Eco*RI fragment 15, used as a probe, detected five transcripts of 650, 850, 1050, 2100 and 2300 nucleotides (Figure 3b, lane 1). Three of these transcripts were also evident on a shorter exposure (lane 9). (ii) A 986 bp *Hpa*I−*Eco*RI probe (Figure 1), spanning the *rol C* locus, hybridizes to transcripts of 850 and 2300 nucleotides (Figure 3b, lane 2). (iii) The internal *Hin*dIII fragment of 1773 bp (Figure 1) hybridizes to transcripts of 650, 1050 and 2100 bases (Figure 3b, lane 3). However, single strand specific probes, derived from the same fragment and therefore spanning the *rol B* and part of the *rol A* locus, hybridize either to the 650 and 2100 nucleotide-long transcripts (lane 4) or to the 1050 nucleotide transcript (lane 5). (iv) A probe containing most of the *rol B/rol C* intergenic region (derived from the 899 bp *Dra*I fragment; Figure 1) hybridizes to the transcript of approximately 2100 bases (lane 6). (v) An 873 bp *Hpa*I−*Hin*dIII probe obtained from the *rol B/rol C* intergenic region of fragment *Eco*RI 15 (Figure 1) does not hybridize to any transcript (data not shown). (vi) A 1410 bp *Eco*RI−*Pst*I probe fragment corresponding to the *rol A* locus hybridizes to the transcripts of 650 and 2100 nucleotides (lane 7). The same hybridization pattern is obtained with a probe consisting of the *Nco*I−*Pst*I fragment of 353 bp (lane 8).

The possibility that any of the transcripts, and in particular transcripts A′ and C′, result from initiations outside of *Eco*RI fragment 15 is excluded by the fact that in transgenic tobacco plants the vector DNA, flanking *Eco*RI fragment 15 to the right, gave a single defined mRNA of ~ 1.25 kb derived from the neomycin phosphotransferase II gene (Koncz and Schell, 1986), and no mRNA at all derived from the leftwards flanking sequences (data not shown).

Therefore these hybridization data suggest that the *rol C* locus generates two transcripts: an abundant one of 850 nucleotides (C) and another less abundant one of 2300 nucleotides (C′) which is probably a readthrough product of the smaller transcript. The *rol B* locus gives rise to a transcript of 1050 nucleotides (B). The *rol A* locus codes for the 650 nucleotide long transcript (A) and presumably for a longer readthrough of 2100 nucleotide (A′).

The *rol B* transcript is therefore transcribed from the opposite strand of transcript A′. The possibility of transcriptional interference and the presence of complementary sequences on these transcripts could somehow curtail gene expression (Coleman *et al.*, 1984). Previous studies in *Nicotiana* species (Taylor *et al.*, 1985; Durand-Tardif *et al.*, 1985) have detected 650, 850 and 1050 nucleotides long and other transcripts in the region spanning the *rol A, B* and *C* loci.

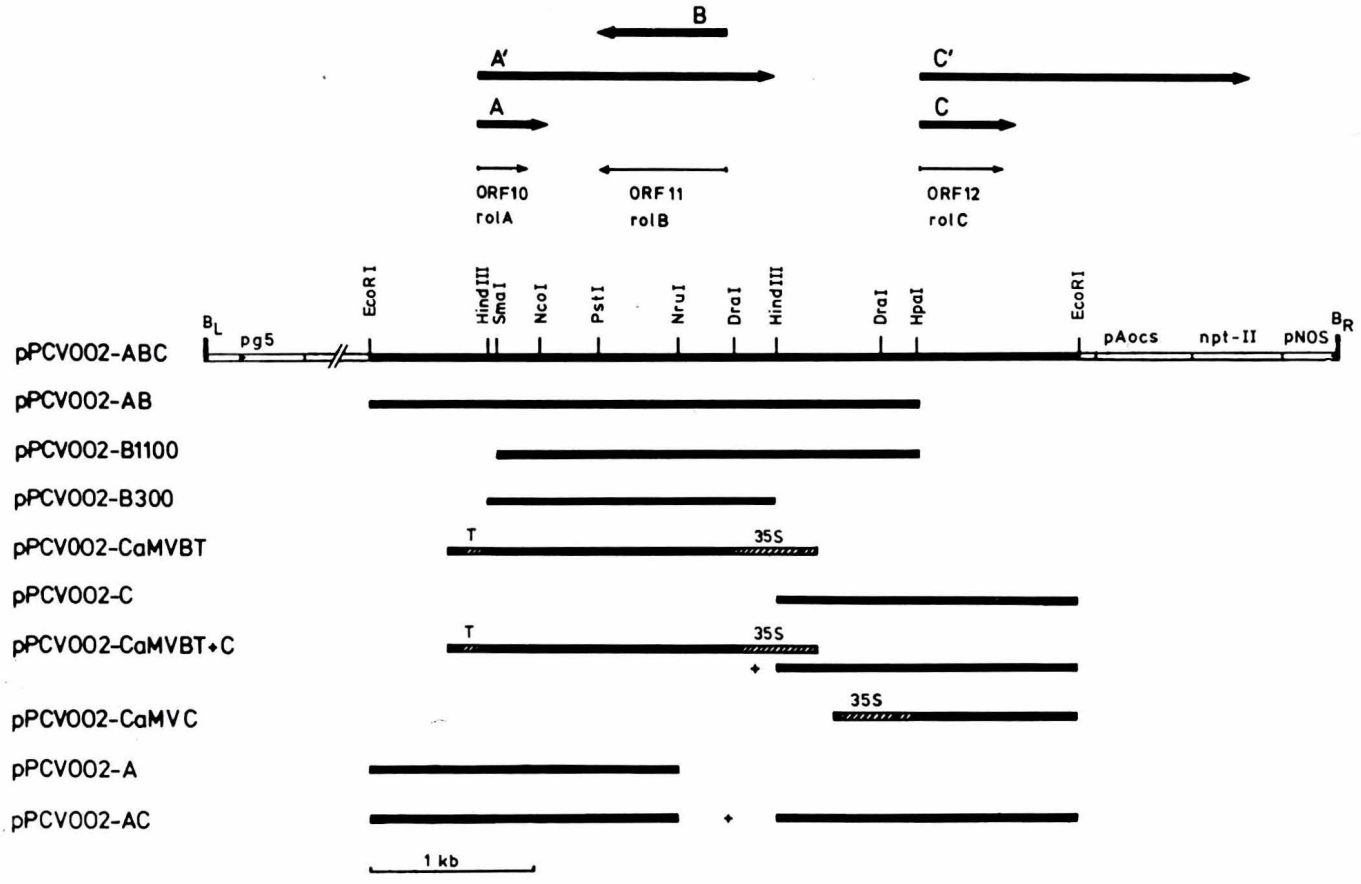


Fig. 1. Schematic drawing of the constructions. pPCV002-ABC contains the *Eco*RI fragment 15 of the Ri plasmid A4 in both orientations, called pPCV002-ABC1 and pPCV002-ABC2. The location of the kanamycin resistant gene and of the gene 5 promoter of the pPCV002 vector (Koncz and Schell, 1986) are also indicated. Above the map with relevant restriction sites, are shown the positions of orfs 10, 11 and 12 (Slightom *et al.*, 1986) corresponding to the *rol A*, *rol B* and *rol C* loci respectively. The approximate location and size of the *rol* transcripts as determined in this paper (see Figure 3b) is also indicated. Below the map are shown the different fragments and chimeric constructions delivered to the plant genome. Abbreviations: 35S, cauliflower mosaic virus 35S promoter; T, cauliflower mosaic virus 35S terminator; kb, kilobase; B_L and B_R, left- and right border sequences of vector T-DNAs; pg5, truncated promoter of T_L-DNA gene 5; pNOS, promoter of nopaline synthase gene; pAocs, polyadenylation sequence of octopine synthase gene; NPT-II, neomycin phosphotransferase gene of transposon Tn5.

Root formation correlates with the expression of the rol loci

Despite the identical growth conditions, only 22 out of 45 kanamycin resistant calli were root-forming. Since transformed calli and transgenic plants show variation in the level of expression of newly introduced genes (Horsch *et al.*, 1984; Czernilofsky *et al.*, 1986; Fluhr *et al.*, 1986; Spena and Schell, 1987), we have anlaysed root-forming and non root-forming calli to test whether the morphogenic response correlates with the expression of the *rol A, B* and *C* loci. Figure 4 shows a northern blot with poly(A)$^+$ RNA extracted from root-forming (lanes 1, 2 and 4) and non-root-forming calli (lans 3 and 5) transformed with the pPCV002-ABC1 construction and grown on MS3 medium. It is striking that, whereas all root-forming calli contained different *rol* transcripts, no transcripts at all were detectable in non-root-forming calli. The relative amounts of different *rol* transcripts in root-forming calli varied considerably and especially for *rol B*. The lack of detectable transcripts in non-root-forming calli is not due to gross DNA rearrangements, as indicated by Southern blot analysis (data not shown). In some cases *rol* transcripts were detected in transformed calli some weeks before root appearance.

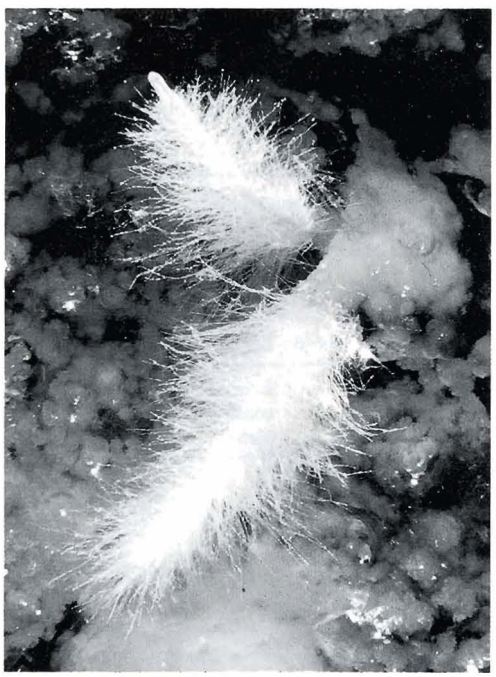
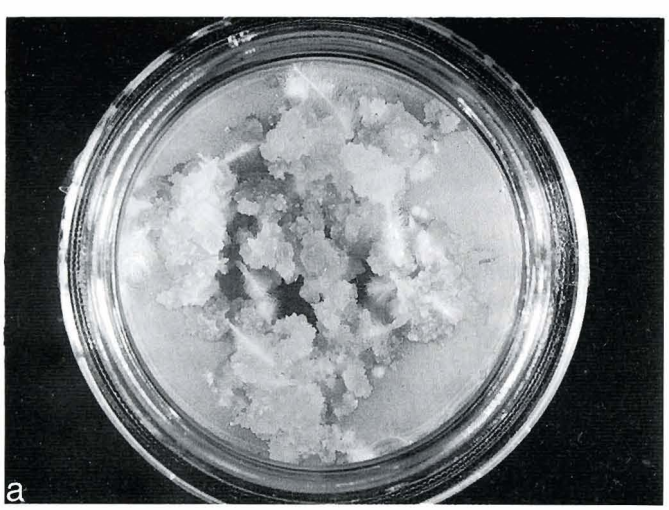


Fig. 2. Selection of transformed calli. *N.tabacum* protoplasts were co-cultivated with *A.tumefaciens* GV3101 harbouring a Ti plasmid defective for its T-DNA and the pPCV002-ABC1 construction. Small calli resistant to 50 mg/l of kanamycin sulfate were visible after 5–6 weeks. Half of them (22 out of 45) were forming roots and in some cases root formation took place before subculturing to solid medium.

The rol A, B and C loci are independently able to induce root formation in tobacco

We have further dissected *Eco*RI fragment 15 in order to test whether each of the *rol A, B* and *C* loci alone would be sufficient to induce root formation in transgenic tobacco calli.

Tobacco protoplasts were transformed by co-cultivation with the construction pPCV002-B300 shown in Figure 1. Within 14–17 weeks of subculture, 5 out of 22 individual calli transformed with pPCV002-B300 had formed roots. However, root formation in this case was less efficient than with pPCV002-ABC transformed calli of which 22 out of 45 had already shown root formation during the first 8 weeks of culture. Transgenic calli (pPCV002-B300) were forming roots on media containing either low (MS2) or high auxin concentrations (MS1). Figure 5 shows a Northern blot performed with poly(A)$^+$ RNA extracted from root-forming (lanes 1 and 7) and non-root-forming calli transformed with pPCV002-B300 (lanes 3, 4, 5 and 6). A detectable level of the 1050 nucleotide long transcript is observed with mRNA obtained from the root-forming calli, whereas non-root-forming calli did not usually express the *rol B* transcript. In one case (rolB300-10) *rol B* transcripts were detected in callus which



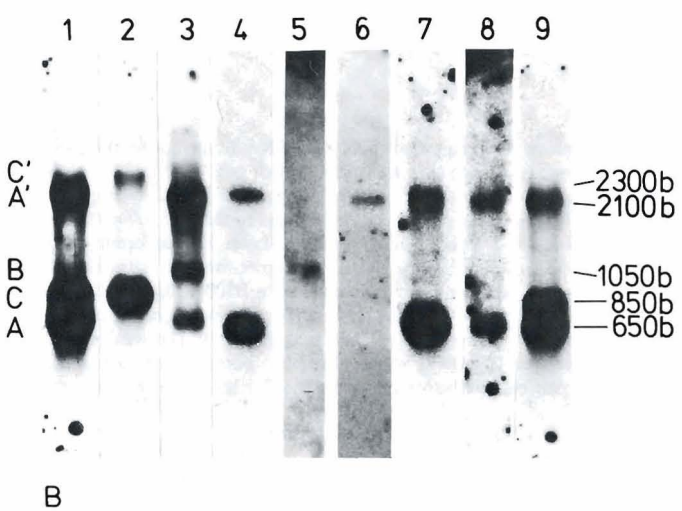


Fig. 3. (**a**) Callus clone ABC2-N was forming roots when subcultured on MS3 medium containing 0.5 mg/l of BAP, an hormonal treatment which usually induces shoot formation. (**b**) Transcriptional analysis of the *rol A, B* and *C* loci. ^{32}P-labelled probes were hybridized to northern blots of poly(A)$^+$ RNA extracted from callus clone ABC2-N grown on MS3 medium. Approximately 1.7 μg of poly(A)$^+$ RNA were loaded in each slot. The following probes were used (restriction sites correspond to those shown in Figure 1): **Lane 1**, *Eco*RI fragment 15; **lane 2**, 990 bp *Hpa*I–*Eco*RI fragment; **lane 3**, the internal 1773 bp *Hin*dIII fragment; **lane 4**,, single strand specific probe for *rol A* (orf 10); **lane 5**, single strand specific probe for *rol B* (orf 11); **lane 6**, the internal 899 bp *Dra*I fragment; **lane 7**, the 1410 bp *Eco*RI–*Pst*I fragment; **lane 8**, the 353 bp *Nco*I–*Pst*I fragment; **lane 9**, *Eco*RI fragment 15, a shorter exposure of the same blot shown in **lane 1**. Transcript C′ (2300b) and B (1050b) cannot be seen after this short exposure confirming the observation in **lane 2**, that the C′ transcript has a much lower abundance than the C transcript. Transcript size was determined by comparison to a standard marker (BRL RNA ladder).

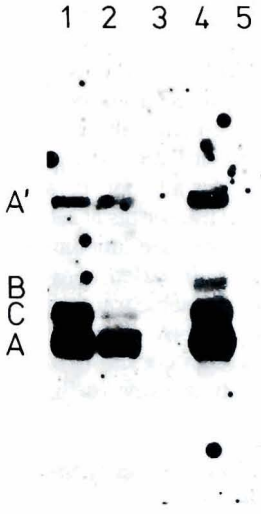


Fig. 4. Transcriptional analysis of the *rol* loci in root-forming and non-root-forming calli grown on MS3 medium. Poly(A)$^+$ RNA (2 μg) was fractionated in a 1.5% agarose formaldehyde gel, transferred to nitrocellulose filters and hybridized to the *Eco*RI fragment 15 of 4374 bp spanning the *rol A, B* and *C* loci. **Lane 1**, root-forming callus clone ABC2-e; **lane 2**, root-forming callus clone ABC2-X; **lane 3**, non root-forming callus clone ABC1-IX; **lane 4**, root forming callus, clone ABC1-II; **lane 5**, non root-forming callus clone ABC1-I.

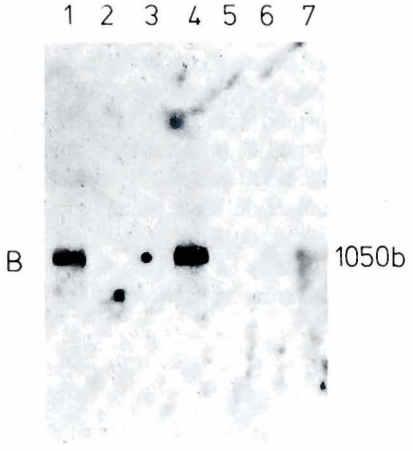


Fig. 5. Northern blot analysis of poly(A)$^+$ RNA from root-forming and non-root-forming tobacco calli transformed with pPCV002-B300. Poly(A)$^+$ RNA (1.5 μg) was fractionated in a 1.5% agarose formaldehyde gel, transferred to nylon membranes and hybridized to the *Pst*I–*Hin*dIII fragment of 1.1 kb spanning the *rol B* locus. **Lane 1**, root-forming callus clone B300–22; **lane 2**, empty; **lane 3**, non-root-forming callus clone B300–1; **lane 4**, non-root-forming callus clone B300–10; **lane 5**, non-root-forming callus clone B300–25; **lane 6**, non-root-forming callus clone B300–26; **lane 7**, root-forming callus clone B300–9. The weak signal of the transcript seen in **lane 7**, was confirmed to correspond to a real band by several independent northern blots of poly(A)$^+$ RNA extracted from the same callus.

did not show root formation, not even after prolonged cultivation (up to 9 months) (lane 4). It is intriguing that in this exception the *rol B* transcript was expressed quite vigorously.

Our constructions (Figure 1) were then tested in a tobacco leaf disc system (see Materials and methods for details). Table I summarizes the results obtained 4 weeks after inoculation. All three *rol* loci were able to trigger root formation in this system although at quite different frequencies. The construction pPCV002-B1100 elicited the strongest biological response. More intense and earlier

Table I. Root induction in tobacco leaf discs

Construction	Root formation
pPCV002	–
pPCV002-ABC1	+++++
pPCV002-AB	+++++
pPCV002-B1100	++++
pPCV002-B300	+
pPCV002-CaMVBT	+
pPCV002-CaMVBT+C	+++
pPCV002-C	–
pPCV002-CaMVC	+
pPCV002-A	++
pPCV002-AC	+++

Tobacco leaf discs were inoculated with *A.tumefaciens* strain GV3101 harboring the constructions shown in Figure 1. The tobacco leaf discs were kept on hormone free MS medium and analysed up to 4 weeks after inoculation.

Table II. Hairy root induction on *Kalanchoe diagremontiana* leaves

Construction	Infection with construction alone	Coinfection with *A.tumefaciens* strain pGV3297
pPCV002	–	–
pPCV002-ABC1	–	++++
pPCV002-AB	–	+++
pPCV002-B1100	–	++
pPCV002-B300	–	–
pPCV002-CaMVBT	–	+
pPCV002-CaMVBT+C	–	++
pPCV002-C	–	–
pPCV002-CaMVC	–	–
pPCV002-A	–	–
pPCV002-AC	–	–

Kalanchoe leaves were inoculated with *A.tumefaciens* strain GV3101 harboring the constructions shown in Figure 1. Root formation only took place on leaves coinoculated with *A.tumefaciens* strain pGV3297 harboring a Ti plasmid deficient for the *ipt* gene but which still has the auxin producing genes (Joos *et al.*, 1983). Inoculations with *A.tumefaciens* strain pGV3297 alone were avirulent. Plants were analysed up to 12 weeks after inoculation.

root formation was repeatedly observed with this construct than with pPCV002-A, -CaMVC or -B300. The pPCV002-B300 (containing only 300 bp of *rol B* 5′ flanking sequences) and -CaMVC constructions also induced root formation but at a lower frequency than pPCV002-A. Combinations of different *rol* loci evoked biological responses considerably stronger than those elicited by the single constructions. It is interesting to note that the CaMVBT chimeric gene, where ORF 11 (*rol B*) is under the control of a strong promoter, is eliciting a response weaker than -B1100, where the ORF 11 is under the control of its own weaker 5′ flanking sequences. On the other hand, ORF 12 (*rol C*) was not able to induce root formation when driven by its own 5′ flanking sequences, but induced root formation when controlled by the cauliflower mosaic virus 35S promoter.

The rol B locus by itself is able to induce root formation on kalanchoe leaves

Since the *rol* loci were originally defined by inoculation of agrobacteria insertional mutants of the T-DNA of Ri plasmids on kalanchoe leaves (White *et al.*, 1985), we also tested our constructions (Figure 1) in this experimental system. Table II summarizes the results. None of the constructions were able *per se*

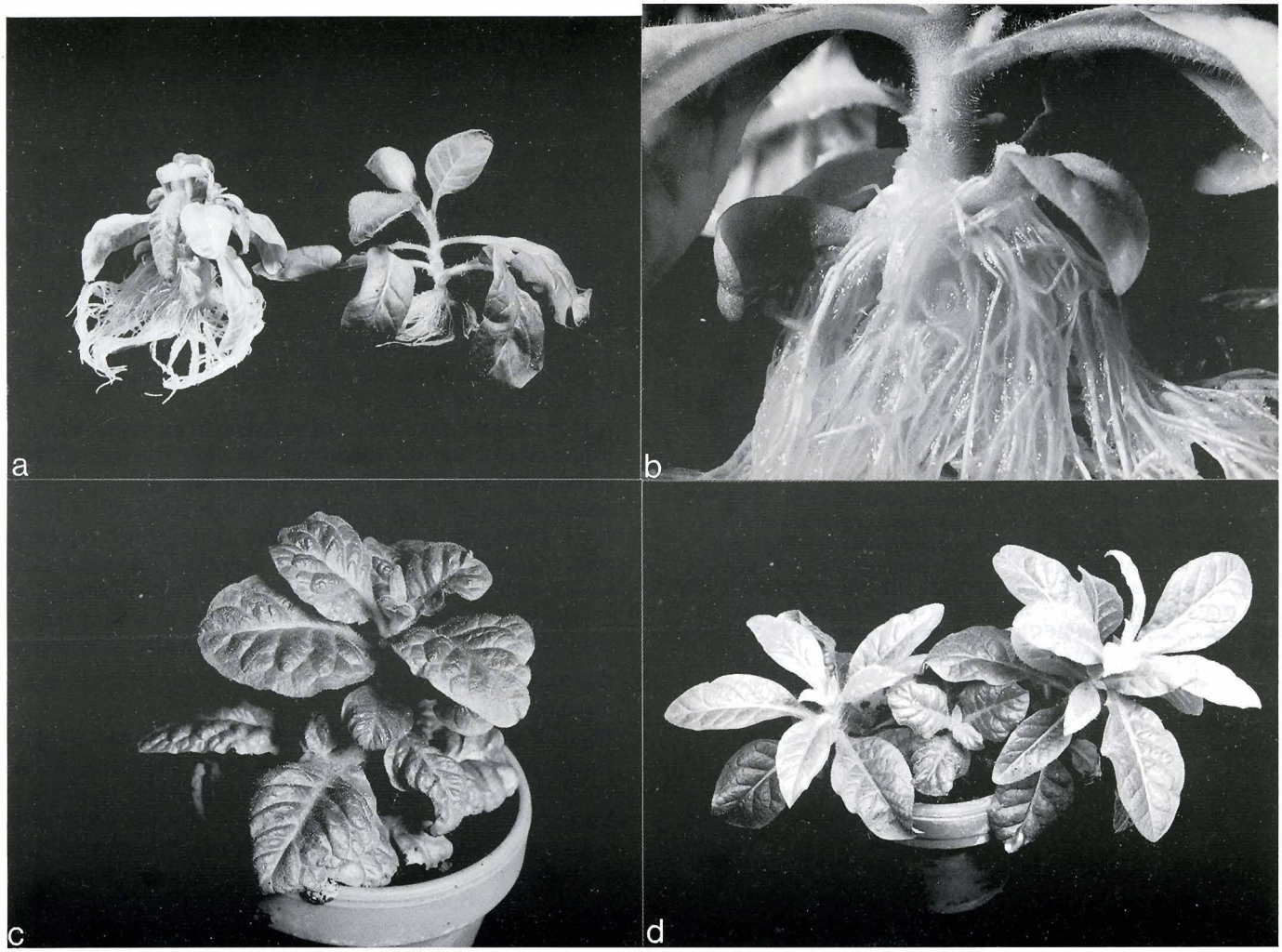


Fig. 6. (**a**) The hairy root (hr) syndrome. Left: Transgenic plant clone (ABC1-II3) regenerated from a root forming callus showed the typical symptoms of the hairy root syndrome. The plant was also displaying symptoms of senescence (i.e. depigmentation) which were reversed when the plant was shifted from in vitro culture conditions to the greenhouse. Right: normal SR1 tobacco plant. (**b**) hr symptoms: Adventitious root formation in a transgenic tobacco plant (clone ABC1-II). (**c**) hr symptoms: Wrinkled leaves with increased width to length ratio in a hr plant (clone ABC1-II). (**d**) T′/T switch. Transgenic plant clone ABC1-alpha displaying the hr syndrome (middle) has generated two lateral branches which show less pronounced alterations.

to trigger root formation on kalanchoe leaves. For infected kalanchoe leaves to produce roots they had to be co-infected with an *A.tumefaciens* strain harbouring a Ti plasmid deficient for the *ipt* gene but which still has the auxin producing genes (pGV3297; Joos *et al.*, 1983). Kalanchoe leaves infected with *A.tumefaciens* strain pGV3297 only did not form roots. When coinfected with pGV3297, constructions containing ORF 11 (*rol B* coding region), either under the control of its own regulatory regions (e.g. -ABC, -AB, -B1100; Figure 1) or under the control of the cauliflower mosaic virus 35S promoter (e.g. -CaMVBT, -CaMVBT+C; Figure 1) all produced roots, albeit at different frequencies. The fastest and most pronounced response was obtained with pPCV002-ABC and the slowest and weakest with pPCV002-CaMVBT. pPCV002-AB and pPCV002-B1100 gave intermediate responses, with -B1100 having a stronger effect when associated with *rol A* (pPCV002-AB). Moreover the chimeric construction CaMVBT elicited a root-inducing response weaker than CaMVBT plus *rol C*. Twelve weeks after inoculation neither pPCV002-B300 nor pPCV002-C had elicited root formation. Also pPCV002-A and pPCV002-AC were not able to induce root formation on kalanchoe leaves. These results point to a primary role for the *rol B* (ORF 11) locus in the hairy root phenomenon but also indicate an ancillary role for both the *rol A* and the *rol C* loci. It is interesting to note that, also in kalanchoe, the CaMVBT chimeric gene, where the ORF 11 (*rol B*) is positioned under the control of a strong promoter, is eliciting a response weaker than -B1100, where ORF 11 is controlled by its own, weaker 5′ promoter sequences.

The auxin requirement satisfied by the co-infecting *A.tumefaciens* pGV3297 strain is not surprising considering the auxilliary role of auxin in the hairy root phenomenon (Cardarelli *et al.*, 1987).

Establishment of the hairy root phenotype in plants

When plants were regenerated from cloned tobacco calli transgenic for the *rol A, B* and *C* loci, most of them showed alterations which are characteristic of the hairy root syndrome (Figure 6a, b and c). Whereas root-forming calli usually generated transgenic plants displaying the transformed phenotype (e.g. clone ABC1-II; Figure 6a, b and c), non-rooting calli (e.g. ABC1-I, ABC1-IX) generated normal looking plants.

The availability of transgenic plants raised the possibility of correlating the transformed phenotype with the expression of the *rol* genes. Figure 7 shows a Northern blot analysis of poly(A)$^+$

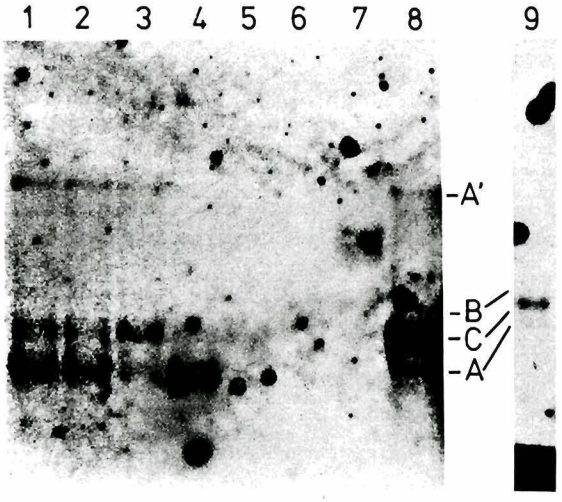


Fig. 7. Transcription of *rol* loci in transgenic plants. Poly(A)$^+$ RNA (1.7 μg) was purified from leaves of plant regenerants then fractionated in a 1.5% agarose-formaldehyde gel, blotted onto nitrocellulose filters and hybridized to *Eco*RI fragment 15. **Lane 1**, plant ABC2-e1 (hr); **lane 2**, plant ABC2-e2 (hr); **lane 3**, plant ABC1-II1 (hr); **lane 4**, plant ABC1-II3 (hr); **lane 5**, plant ABC1-II5; **lane 6**, plant ABC1-I1; **lane 7**, plant ABC1-IX1; **lane 8**, plant ABC1-X1 (hr); **lane 9**, plant ABC1-II3 (hr) later stage of development. (hr): plants showing the hairy root syndrome.

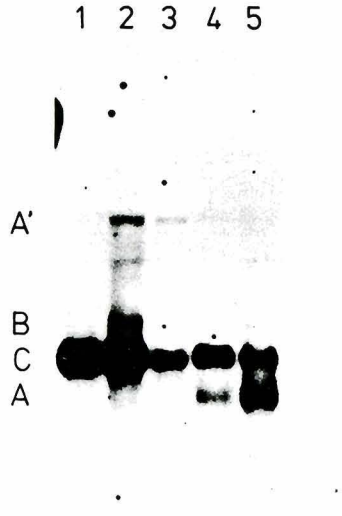


Fig. 8. Organ-specific expression of the *rol* loci in transgenic plants. Poly(A)$^+$ RNA (1.5 μg) extracted from roots (**lane 1**), stems (**lane 2**) and leaves (**lane 3**) of plant clone ABC2-N were fractionated on a 1.5% agarose–formaldehyde gel, transferred to filter membrane and hybridized to the *Eco*RI fragment 15. **Lanes 4** and **5** show the hybridization pattern obtained with poly(A)$^+$ RNA extracted from roots and stems of plant clone ABC2-e2.

RNA extracted from leaves of transgenic plants. Lanes 6 and 7 correspond to RNA extracted from normal looking plants (ABC1-I and ABC1-IX). Despite the fact that ~10 times more poly(A)$^+$ RNA was loaded in these two lanes, we could not detect any transcript. The lack of detectable transcripts of the *rol* loci in plant clones ABC1-I and ABC1-IX is not due to gross DNA rearrangements as indicated by Southern blot analysis (data not shown). The plants were resistant to a concentration of 50 mg/l of kanamycin and the antibiotic resistance was transmitted to the progeny as a single dominant locus in genetic crosses (data not shown). On the same Northern blot the hybridization pattern obtained with RNA extracted from leaves of transgenic plants displaying the transformed phenotype (plant clones ABC2-e1, -e2, ABC1-II1, -II3, -X1) clearly demonstrates expression of the *rol* loci albeit at different relative levels (lanes 1, 2, 3, 4, and 8 respectively). While in plant clones -II1 and -X1 (lanes 3 and 8), the *rol C* transcript of 850 nucleotides is the most abundant, in other plant clones (-II3, -e1, -e2), it is the *rol A* transcript of 650 nucleotides which is the most abundant. The *rol B* transcript was barely detectable in most of the mRNA extracted from leaves. Interestingly, RNA extracted from plant clone ABC1-II5 (lane 5) does not contain any detectable transcript. This plant was regenerated from the same root-forming callus (clone ABC1-II) as the other two regenerants (II1 and II3), but it did not show the hr syndrome whilst the other two plant clones displayed the full transformed state.

Moreover we have observed variation in the relative level of expression of the *rol* transcripts not only amongst different regenerants but also during different growth stages of a single transgenic plant. This was observed with plant ABC1-II3b, a clone propagated vegetatively from the original regenerant ABC1-II3 and displaying the same abnormal phenotypic traits, which showed a different hybridization pattern (compare lane 9 with lane 4 of Figure 7) when analysed at a later stage of development (i.e. five weeks after the vegetative propagation).

Expression of the rol A, B and C loci in roots, stems and leaves of transgenic plants

Figure 8 shows a Northern blot analysis of poly(A)$^+$ RNA extracted from roots, stems and leaves of one and the same transgenic plant transformed with pPCV002-ABC2 and displaying the hr syndrome. The *rol A* transcripts (A+A′) show considerable variation amongst different individuals and are less expressed in roots than in stems and leaves. In contrast significant amounts of *rol B* transcripts were reproducibly observed in stems, giving rise to adventitious roots but not in leaves and much less in separated roots (plant ABC2-N, lanes 1–3). A similar organ specific pattern was observed not only for plant ABC3-e2 (lanes 4 and 5) but also for plant ABC2-e1 (data not shown).

Interestingly, a plant showing a very pronounced transformed phenotype may generate branches with a less pronounced abnormal morphology (Figure 6d). This phenomenon, called T′/T switch, was previously described for hr transgenic plants regenerated from wild-type hairy roots and supposed to be epigenetic (Tepfer, 1984).

Discussion

The transferred T_L-DNA of *A. rhizogenes* Ri plasmids codes for at least three genes, *rol A, B* and *C*, each of which is individually capable of stimulating root formation in tobacco. These three genes differ in their capacity to stimulate root differentiation in different hosts and their effects are cooperative. In tobacco *rol B* is more efficient than *rol A* or *rol C* in stimulating root formation and in another host, kalanchoe, it is the only one of these *rol* genes to be able to stimulate root formation by itself, provided auxins are independently supplied.

These general conclusions were reached by studying the morphogenic phenotypes resulting from the transfer of various fragments of the T_L-DNA of Ri plasmids into either tobacco or kalanchoe. We concentrated our attention on the loci carried by the T_L-DNA for the following reasons: although Ri plasmids contain two different transferable segments (the so-called T_L- and T_R-DNA's, see introduction), their role in root-induction might well be based on different mechanisms. Indeed the T_R-

DNA has been shown to contain sequences homologous to the auxin biosynthetic genes *iaaM* and *iaaH* of Ti plasmids (Inze *et al.*, 1984; Schroeder *et al.*, 1984; Tomashow *et al.*, 1984; Yamada *et al.*, 1984) and this explains why on some organs of a number of plants, T_R-DNA transfer can result in root-formation. In contrast circumstantial evidence points to the possibility that genes carried by the T_L-DNA influence root-induction not by controlling auxin synthesis but by rendering transformed cells sensitive to auxin-action (Cardarelli *et al.*, 1987). The analysis of T_L-DNA genes was therefore attractive since the products of some of these genes might play a role in plant hormone signal transduction.

A genetic analysis of the T_L-DNA segment of the Ri plasmid of strain A4 (White *et al.*, 1985) has identified a number of loci involved in the control of hairy root induction. Whereas insertional inactivation of the *rol B* locus led to avirulence, inactivation of *rol A, C* and *D* loci led to attenuation of root-induction on kalanchoe leaves. Transcriptional analysis of Ri transformed plant tissues, showed that the T_L-DNA segment was transcribed into a number of RNAs of different sizes (Durand-Tardif *et al.*, 1985; Taylor *et al.*, 1985; Ooms *et al.*, 1986). Gene transfer vector were therefore constructed to try to define which of these *rol* loci would be sufficient to stimulate root formation in two different hosts.

On tobacco as well as kalanchoe, a fragment covering *rol A, B* and *C* loci (Figure 1) was sufficient to induce efficient root formation. In different transgenic calli root formation correlated well with transcription of the different *rol* loci. Subfragments separating these three *rol* loci (Figure 1), so that each *rol* locus could be transferred separately or in combination with one of the other loci, gave somewhat different results on the two hosts (see Tables I and II).

In tobacco *rol B*, *rol A* and *rol C* could each separately promote root formation but with different efficiencies, *rol B* being the most effective. In kalanchoe leaves only *rol B* locus was able to stimulate root formation and no effect of *rol A* or *rol C* by themselves could be observed. In both hosts it was however clear that the different *rol* loci had a synergistic effect. In both cases root induction was most efficient when all three loci were introduced and combinations *B + A* or *B + C* were also more effective than *rol B* alone. Also the combination of *rol A + rol B* was more effective than either of these *rol* loci alone in tobacco. These results are therefore consistent with those obtained by insertional mutagenesis of the T_L-DNA of Ri plasmid A4 and tested on kalanchoe leaves, indicating that insertions in the *rol A* or *rol C* or *rol D* loci gave attenuated root induction (White *et al.*, 1985).

In a further attempt to study the role of *rol* loci in root induction, chimeric genes were constructed and transferred to tobacco or kalanchoe such that expression of either *rol B* or *rol C* would be under the control of a foreign, strong unregulated promoter, i.e. the 5′ flanking sequences of the cauliflower mosaic virus 35S transcript (35S promoter), whereas in tobacco calli containing the *rol C* locus under control of its own 5′ flanking sequences, no root formation could be observed. The same *rol C* locus under control of the 35S promoter did result in delayed but reproducible root induction. Yet on kalanchoe leaves this 35S-*rol C* construct was not able to induce root formation.

On the other hand the *rol B* locus driven by the 35S promoter appeared to be less efficient in promoting root induction in tobacco calli than the same *rol B* locus driven by 1100 bp of its own 5′ flanking sequences. It is possible that overexpression of the *rol B* transcript is not conducive to root induction since a transgenic callus with a high level of *rol B* transcript (*rol B* 300−10) did not show any root formation, whereas usually lack of root formation by *rol B* containing tobacco calli correlated with lack of detectable *rol B* transcripts and actual root formation correlated well with detection of the *rol B* transcript (Figure 5).

The fact that the *rol B* locus under the control of 1100 bp of its 5′ flanking sequence elicited a stronger response both in tobacco and on kalanchoe leaves than when driven by 300 bp only of its proximal 5′ flanking sequences, could be explained if *cis*-regulatory sequences necessary to optimally modulate *rol B* expression would be located between −300 and −1100. Whatever the promoter sequence driving the *rol B* expression, the root response was greater when combined with *rol C*.

The observation that the *rol A* and *rol C* products can by themselves stimulate root formation in tobacco but not in kalanchoe leaves indicates that the products of these *rol* loci are not only synergistic with that of the *rol B* locus but also must interact with plant factors (targets?) controlling organ differentiation. If these factors were similar or identical to those responsible for the effects of growth hormones in plants, it is understandable why they could be different in different host species.

One of the most important observations made in this work is that in clonal transgenic tobacco calli, actively transcribing the *rol* loci, not all the transformed cells form root primordia (as can be easily appreciated from Figures 2 and 3a). This could mean that activation of expression of *rol* loci can remain cryptic with little or no effect on organogenesis in most cells constituting a callus or an organ and that only a subset of cells is competent to react to the *rol* gene products. A similar dependance from a specific cellular context has also been reported for the action of mammalian oncogenes (Land *et al.*, 1983; Zarbl *et al.*, 1985; Quintanilla *et al.*, 1986). In mammalian somatic tissues for example, oncogenes such as c-*myc* and v-Ha-*ras*, can be active with little apparent effect on the organism (Sinn *et al.*, 1987).

The hr syndrome in transformed plants is established by the rol A, B and C loci

Tobacco plants transgenic for *rol A, B* and *C* display the full hr syndrome consisting of high growth rate of roots, reduced apical dominance in roots and stems, wrinkled leaves with altered width to length ratio and plagiotropic roots. Transgenic plants containing either the *rol B* or the *rol C* locus alone do not show all the typical traits of hairy root plants (Schmülling *et al.*, in preparation). However, some of these transgenic plants show some alterations such as adventitious root formation and/or moderately altered leaf morphology (Schmülling *et al.*, in preparation). Tobacco plants transgenic for double combinations (i.e. -AB, -CaMVBT+C) show more severe alterations, however they do not show the complete hairy root syndrome (Schmülling *et al.*, in preparation). Therefore, the available data show that the hairy root syndrome is fully established only in plants transformed with the *rol A, B* and *C* loci and suggest that it is due to the effects of their synergistic action.

Here we have reported variability in the expression of the *rol* genes, superimposed on an organ-specificity of expression. Different patterns of expression of the *rol A, B, C* transcripts have also been observed in potato plants (Ooms *et al.*, 1986) and in transformed *Nicotiana glauca* calli (Taylor *et al.*, 1985). Moreover in our transgenic plants we have observed the same somatic variability of the severity of the phenotypic traits previously reported by Tepfer and Durand-Tardif *et al.* and called T′/T switch (Tepfer, 1984; Durand-Tardif *et al.*, 1985). It is tempting to speculate that changes in the level of expression of the

rol genes could be responsible for the different penetrance of the hr phenotypic traits, and be due to variation in the level of plant growth substances in different tissues and/or at different stages in the life of the plant (Lachaud and Bommerain, 1984).

When taken together our results along with previously published work, indicates that the products of the *rol A, B* and *C* loci somehow interfere or interact with the normal growth hormone controlled differentiation mechanisms. Some recent preliminary and unpublished results from our laboratory further support this notion.

Cytokinins such as benzylaminopurine (BAP) completely inhibit or severely delay root formation by stem cuttings of tobacco grown in tissue culture. Stem cuttings from transgenic tobacco plants containing the *rol A, B, C* loci will however form roots on such media indicating that some of these *rol* gene products might have an action which antagonizes the cytokinin effect.

Our results have clearly demonstrated synergistic action of the different *rol* loci in determining root induction in tobacco calli and kalanchoe leaves and the hairy root syndrome in transgenic tobacco plants. This is a further strong analogy between some T-DNA loci of Ti and Ri plasmids and some of the oncogenes in mammalian systems (Schwartz *et al.*, 1986; Sinn *et al.*, 1987).

Materials and methods

Bacterial strains and cultures

The *E.coli* strains used were HB101 (Boyer and Roulland-Dussoix, 1969) and SM10 (Simon *et al.*, 1983). *A.tumefaciens* strain GV3101 (pMP90RK) is a derivative of the nopaline strain C58 deleted of its T-DNA (Koncz and Schell, 1986). *E.coli* strains were grown at 37°C in LB medium (Miller, 1972). *Agrobacterium* strains were grown at 28°C in YEB medium supplemented with 5 mM $MgCl_2$ (Miller, 1972).

Construction of plasmids

Standard techniques were used for the construction of recombinant DNA plasmids (Maniatis *et al.*, 1982). Plasmid pPCV002-ABC1 and pPCV002-ABC2 were obtained by inserting in both orientations the *Eco*RI fragment 15 of 4374 bp in the unique *Eco*RI site of the binary vector pPCV002 (Koncz and Schell, 1986). Similarly pPCV002-B300 was constructed by subcloning the *Hin*dIII fragment 30a of 1773 bp. pPCV002-C was obtained by subcloning an *Eco*RI–*Hin*dIII fragment of 1861 bp spanning the entire *rol C* coding region and 872 bp of its 5′ flanking sequence. pPCV002-B1100 and pPCV002-AB were respectively subclones of the *Sma*I–*Hpa*I and the *Hpa*I–*Eco*RI fragments (Figure 1). pPCV002-A was constructed by subcloning the *Eco*RI–*Nru*I fragment of 1891 bp to the pPCV002 vector. pPCV002-AC contains the *Eco*RI–*Nru*I and the *Eco*RI–*Hin*dIII fragments spanning the *rol A* and the *rol C* loci respectively. The chimeric gene CaMVBT has the ORF 11 positioned under the control of the cauliflower mosaic virus 35S promoter (Pietrzak *et al.*, 1986). A *Bal*31 derived fragment containing the entire coding region of the ORF 11 and 39 bp of its untranslated leader region was used in this construction. The chimeric gene retains 693 bp of 3′ flanking sequences and Camv 35S terminator too. pPCV002-CaMVBT+C contains the CaMVBT chimeric gene and the *rol C* containing *Eco*RI–*Hin*dIII fragment of 1861 bp. CaMVC has the orf 12 (*Hpa*I–*Eco*RI fragment) positioned under the control of the cauliflower mosaic virus 35S promoter. These constructions were transferred from *E.coli* strain SM10 to *A.tumefaciens* strain GV 3101 as described (Koncz and Schell, 1986).

Plant tissue culture and transformation

Inoculation of *Kalanchoe diagremontiana* leaves was carried out as described (Boulanger *et al.*, 1986) and plants were analysed for 12 weeks after inoculation. Leaf protoplasts of *Nicotiana tabacum* cv. Petit Havana SR1 (Maliga *et al.*, 1973) were isolated from sterile shoot cultures grown on MS medium according to Nagy and Maliga (1976). Protoplasts co-cultivation with *A.tumefaciens* GV 3101 strains harbouring the different constructions and regeneration of transformed plants were done according to established methods (Marton *et al.*, 1982; De Block *et al.*, 1984; Horsch *et al.*, 1984; Marton, 1984). Kanamycin-resistant transformants were selected on MS medium (Murashige and Skoog, 1962), 1 mg/l of naphthyl acetic acid (NAA), 0.2 mg/l kinetin containing 50 mg/l of kanamycin sulphate. Callus cultures were further grown on solid MS medium (Murashige and Skoog, 1962) supplemented with 50 mg/l of kanamycin and three different hormone complements: 0.6 mg/l of naphthyl acetic acid (NAA) and 0.2 mg/l of kinetin (Kin) MS1), or 0.1 mg/l NAA and 0.2 mg/l Kin (MS2), or 0.5 mg/l benzylaminopurine (BAP) (MS3). Regeneration of plants was usually carried out on MS medium containing 0.5 mg/l benzylaminopurine (BAP). Shoots were rooted on hormone-free medium. The constructions were tested for their ability to induce root formation on tobacco leaf discs on hormone free MS medium. 25 ml of an overnight culture were grown at 28°C in LB medium supplemented with 5 mM $MgSO_4$ and the appropriate antibiotics. The bacteria were harvested by centrifugation, washed with K3 0.4 M at a concentration of 10^6 cells/ml. Tobacco leaf discs were incubated for 5 min with the bacterial solutions, placed for 2 days on hormone free MS medium and then transferred to MS medium supplemented with claphoran (500 mg/l) and kanamycin (50 mg/l). Root formation was checked up to 4 weeks.

DNA and RNA analysis

DNA and RNA were extracted from plant material as described (Taylor and Powell, 1983). Poly(A)$^+$ RNA was selected by chromatography on oligo(dT) cellulose according to the manufacturer (Boehringer Mannheim) and then separated on 1.5% agarose–formaldehyde gel, transferred to nitrocellulose filters and hybridized to radioactive probes. Purified DNA fragments were labelled using the BRL nick-translation kit. Single strand probes, spanning the entire coding region of the *rol B* locus and 693 bp of its 3′ flanking sequences, were prepared according to Messing (1983). Hybridizations were carried out in 50% formamide, 5 × SSPE, 10 × Denhardt's, 100 μl/ml denatured salmon sperm DNA. Washes were performed first in 2 × SSPE, 0.1% SDS at room temperature and then in 0.2 × SSPE, 0.1% SDS at 65°C. In order to check that approximately equal amounts of poly(A)$^+$ RNA were loaded on each slot, the nitrocellulose filters were hybridized to a soybean actin probe. In this case washes were in 1 × SSPE, 0.1% SDS at 60°C. DNA blots were performed according to established methods (Maniatis, 1982).

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The capability of the *rol B* locus [Cardarelli *et al.* (1987) *Mol. Gen. Genet.*, **209**, 475–480] and of the *rol A* locus [Vilaine *et al.* (1987) *Mol. Gen. Genet.*, **210**, 411–415] to induce root formation in tobacco was independently reported.