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Agrobacterium rhizogenes T-DNA genes capable of inducing hairy root phenotype

M. Cardarelli[1], D. Mariotti[1], M. Pomponi[2], L. Spanò[2], I. Capone[2], and P. Costantino[2,*]

[1] Centro per lo Studio degli Acidi Nucleici, CNR, I-00185 Rome, Italy

[2] Dipartimento di Genetica e Biologia Molecolare, Università di Roma "La Sapienza", P. le Aldo Moro 5, I-00185 Rome, Italy

Summary. Segments of the TL-DNA of the agropine type Ri plasmid pRi 1855 encompassing single and groups of open-reading frames were cloned in the Ti plasmid-derived binary vector system Bin 19. Leaf disc infections on *Nicotiana tabacum* led to transformed plants, some of which showed typical hairy root phenotypes, such as the wrinkled leaf morphology, excessive and partially non geotropic root systems and the ability of leaf explants to differentiate roots in a hormone-free culture medium. Particularly interestingly, most of these traits were shown by plants transformed with a TL-DNA segment encompassing the single ORF 11, corresponding to the *rol*B locus. Hairy root can be induced by this latter T-DNA segment on wounded stems of tobacco plants; hairy root induction on carrot discs requires, on the contrary, a more complex complement of TL-DNA genes.

Key words: *Agrobacterium* – Hairy root – T-DNA – Transgenic tobacco

Introduction

The hairy root syndrome, caused by *Agrobacterium rhizogenes* on dicotyledonous plants, characteristically consists of an abundant proliferation of adventitious roots at the site of bacterial infection (Elliot 1951). The neoplastic roots have been shown to be transformed by a fragment (T-DNA) of large *A. rhizogenes* plasmids (Ri plasmids) (Chilton et al. 1982; White et al. 1982; Willmitzer et al. 1982; Spanò et al. 1982); it is the T-DNA that directs the synthesis of opines (Petit et al. 1983; Petit and Tempé 1985) and the differentiation and growth of the plant cells. The presence of this exogenous DNA segment does not prevent the regeneration of whole fertile plants from hairy roots; hairy root regenerants from various plant species have been obtained in several laboratories (Chilton et al. 1982; Ackerman 1977; Spanò and Costantino 1982; Tepfer 1984), and a number of distinctive morphological traits have been linked to the presence and expression of the T-DNA. These include, among those common to all the plant species analyzed, wrinkled leaves (Tepfer 1984), a very abundant and only partially geotropic root system (Spanò et al. 1987), short internodes and reduced apical dominance as compared with normal plants (Tepfer 1984; Spanò et al. 1987). In addition, leaf explants from hairy root plants rapidly and characteristically differentiate roots on a hormone-free culture medium (Benvenuto et al. 1983). Very little is known to date on the nature of the Ri plasmid T-DNA genes that are capable of directing root differentiation upon infection of host plants and of affecting the morphology of hairy root plants.

In the case of agropine-type Ri plasmids, it has been shown that the T-DNA actually consists of two distinct transforming elements, designated TL- and TR-DNA (De Paolis et al. 1985; White et al. 1985). Genes encoding auxin synthesis – homologous to the equivalent genes on Ti plasmid T-DNA – have been localized on the TR-DNA (Cardarelli et al. 1985; Huffmann et al. 1984). These genes have been demonstrated not to play a crucial role in hairy root induction: they provide auxin that triggers differentiation of auxin-responsive TL-DNA-containing cells (Cardarelli et al. 1987), but the same role can be played by plant auxin in the case of hairy roots induced by mannopine and cucumopine-type *A. rhizogenes* strains which are naturally devoid of *aux* genes, or in the case of *aux*$^-$ mutants of agropine Ri plasmids (Cardarelli et al. 1987; Cardarelli et al. 1985). Neither are *aux* genes responsible for the altered morphology of hairy root regenerants: plants not containing such genes show the full hairy root phenotype described above (Spanò et al. 1987; Taylor et al. 1985; Durand-Tardif et al. 1986). It is even likely that, at least in some plant species, the presence of *aux* genes is counterselected for in the process of regeneration, since several agropine-type hairy root regenerants analyzed by different authors appear to be at least partially defective in this T-DNA locus (Spanò et al. 1987; Taylor et al. 1985; Durand-Tardif et al. 1986; Costantino et al. unpublished results). Thus, all the relevant hairy root genes seem to reside on the TL-DNA.

In the work presented here, we have cloned several segments of the TL-DNA of the agropine type Ri plasmid pRi 1855 in the binary vector system Bin 19 (kindly provided by Dr. M. Bevan). These constructions were assayed both for their capacity to alter morphological traits in tobacco regenerants from leaf disc infections and for their ability to induce hairy roots on tobacco stems and carrot discs.

Offprint requests to: P. Costantino

Abbreviations: YMB, yeast mannitol broth; MS, Murashige and Skoog medium; 6-BAP, 6-benzylaminopurine; NAA, naphthalene acetic acid; Km, kanamycin; Cb, carbenicillin

Materials and methods

Bacterial strains and media. Agrobacterium strains were grown at 28° C in YMB (Hooykass et al. 1977). *Escherichia*

coli strains were grown in LB. Media for strains harbouring Bin 19 and constructions thereof were supplemented with 100 μg/ml Km (YMB) or 30 μg/ml Km (LB).

Cloning of TL-DNA fragments in Bin 19. Plasmid DNA from pBR322 clones of pRi 1855 (pMP clones; Pomponi et al. 1983) and from Bin 19 (Bevan 1984) was isolated by a large scale alkaline lysis procedure (Ish-Horowicz and Burke 1981) followed by CsCl/ethidium bromide gradient purification. DNA from clones pMP30, pMP114 and pMP101 (Pomponi et al. 1983) was digested with appropriate restriction endonucleases and the relevant TL-DNA fragments (see Fig. 1) were extracted from agarose gels by electroelution onto DEAE paper (Dillon et al. 1985).

The restriction fragments were inserted into the polylinker of the vector Bin 19 (Bevan 1984) by standard cloning methods, *E. coli* HB101 was transformed and selection was on LB containing 30 μg/ml Km. Transformants, screened by colony hybridization (Maniatis et al. 1982), were checked by appropriate restriction endonuclease digestion. Recombinant Bin 19 plasmids with the correct structure were transferred to *Agrobacterium* LBA 4404 (Ooms et al. 1982) via triparental mating with HB101 harbouring the helper plasmid RK 2013. Conjugations were carried out as previously described (Cardarelli et al. 1985); transconjugants were selected on YMB containing 20 μg/ml rifampicin and 100 μg/ml Km and checked for the presence of the resident deleted Ti plasmid and Bin 19 construction by agarose gel electrophoresis.

Plant inoculation. Nicotiana tabacum, var. Petite Havana (SR1; Maliga et al. 1973) plantlets were grown aseptically in vitro on minimal MS medium (Murashige and Skoog 1962). Stems were punched with a microscalpel and wounds inoculated with 48 h solid cultures of the appropriate *Agrobacterium* strain. For leaf disc transformation (Horsch et al. 1985), leaves from 5-weeks-old plants were punched to obtain 6 mm discs; these latter were incubated 3–5 min in exponentially growing cultures of the appropriate *Agrobacterium* strains, blotted dry and transferred onto agar plates of MS medium supplemented with 1 mg/l 6-BAP, 0.1 mg/l NAA and 300 μg/ml Km. After 3 days the discs were transferred onto fresh medium with the same hormonal balance supplemented with 300 μg/ml Km and 500 μg/ml Cb. Kanamycin resistant shoots regenerated from the callus around the discs after 8–10 weeks and were transferred to hormone-free MS medium.

Carrot discs were prepared and inoculated as described previously (Cardarelli et al. 1987), stored at 25° C in the dark and scored for rooting after 4–6 weeks. Individual roots were explanted and cultured on solid hormone-free MS medium supplemented with 300 μg/ml Cb.

Southern-blot hybridizations. Plant DNA extraction, restriction endonuclease digestion, transfer onto nitrocellulose filters and Southern blot hybridizations were as previously described (Spanò et al. 1982).

Results

Cloning of pRi1855 TL-DNA segments in Bin19

A detailed restriction map of the TL-DNA of pRi1855 was constructed in our laboratory (Pomponi et al. 1983); subsequently, data on pRiA4 (Huffman et al. 1984) and pRiHRI (Jouanin 1984) showed that all agropine-type Ri plasmids are virtually identical. More recently, the complete nucleotide sequence of the TL-DNA was derived from clone banks of pRiA4 and pRiHRI, leading to the identification of 18 open-reading frames (ORFs) in this transforming DNA element (Slightom et al. 1986). We thus utilized the data on the position of the various ORFs relative to the restriction endonuclease sites (see Fig. 1) to clone relevant segments of the TL-DNA of pRi1855 in the binary plant vector system Bin 19 (Bevan 1984). The restriction fragments *Hin*dIII 23 (encompassing ORFs 8 and 9), *Hin*dIII 37b (ORF 11), *Eco*RI 15 (ORFs 10, 11 and 12), *Eco*RI 36 (ORF 13), *Eco*RI 37a (ORF 14) and *Kpn*I 13 (ORFs 14, 15, 16, 17 and 18) were individually extracted from suitable pRi1855 pBR322 clones previously constructed in our laboratory (pMP clones; Pomponi et al. 1983), cloned in the polylinker of Bin 19 (Bevan 1984) in *E. coli* HB101 and transferred to *Agrobacterium* strain LBA 4404 (Ooms et al. 1982) via triparental mating as described in Materials and methods. Figure 1 shows the restriction map of the TL-DNA of pRi1855, the restriction fragments cloned in Bin 19, their positions relative to the ORFs and the pMP clones from which they were extracted. *Eco*RI fragments 36 and 37a were also cloned as a single insert in Bin 19 starting from a partial digest of pMP101. All constructions were checked both in *E. coli* and in *Agrobacterium* by restriction/gel analysis.

Tobacco leaf disc infections and regeneration of transformed plants

Owing to the presence of functional *vir* genes in its T-DNA-devoid Ti plasmid, *Agrobacterium* LBA 4404 is capable of directing transfer in plant cells of inserts cloned in vectors such as Bin 19 (Ooms et al. 1982). Expression of an insert in the plant host depends on the presence of suitable promoters, Bin 19 itself only providing expression in plants of the kanamycin resistance gene, neomycin phosphotransferase (Bevan 1984). We trusted that expression in plant cells of our T-DNA segments would be directed by whichever ORF-specific promoters allow expression in the natural phytopathogenic process. *Agrobacterium* LBA 4404 strains, containing the various Bin 19 constructions described above, were utilized to infect tobacco leaf discs as described in Materials and methods and kanamycin resistant plants were obtained from all infections. Among these plants, the ones derived from infection with T-DNA fragments *Eco*RI 15 and *Hin*dIII 37b showed some interesting morphological traits, as compared with the isogenic untransformed SR1 tobacco. Thus, plants derived from infections with *Agrobacterium* LBA 4404 (Bin 19::*Eco*RI15), designated TE 15 plants, showed most of the typical morphological markers associated with hairy root plants (Tepfer 1984; Spanò et al. 1987). TE15 tobacco regenerants have in fact markedly wrinkled leaves, darker green than normal (Fig. 2C) and abnormally abundant and rather superficial roots.

Leaf explants from these regenerants produce roots from the cut veins at the wound edges upon transfer onto a hormone-free culture medium, a typical response of plants regenerated from hairy roots induced by wild-type *A. rhizogenes* 1855 (Benvenuto et al. 1983). In the case of TE15 leaf explants, rooting is, however, retarded – roots are evident 2 weeks rather than 1 week after explantation – and

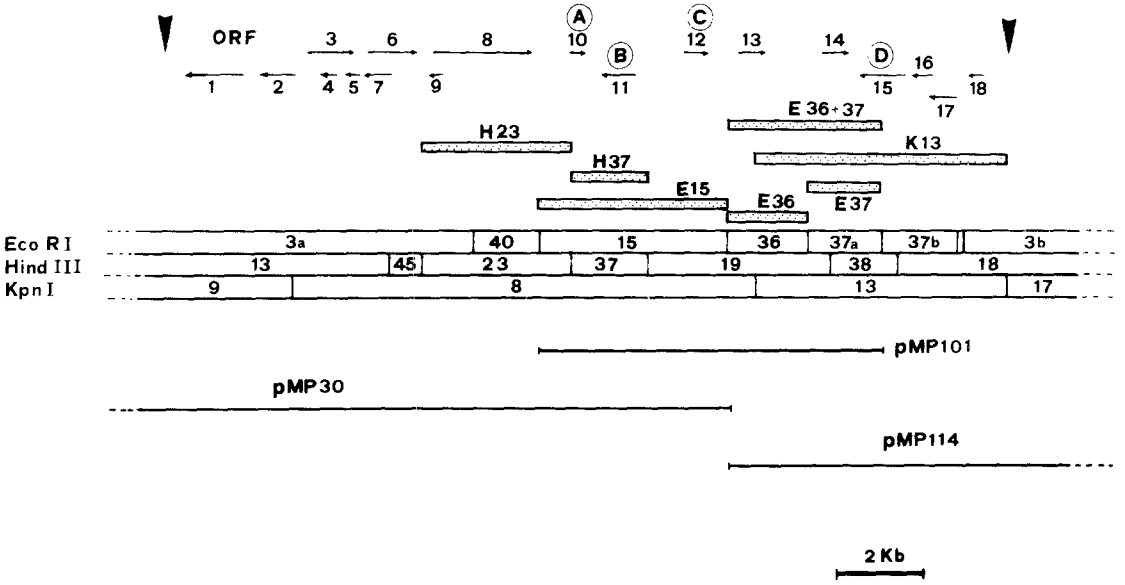

Fig. 1. Restriction endonuclease map of the TL-DNA of pRi 1855 (spanning between vertical arrows), showing the position and lenght of the open-reading frames (Slightom et al. 1986) (ORF, *numbered arrows*) and the segments cloned in Bin 19 (*dotted boxes*). *Letters* (A, B, C, D) above ORF 10, 11, 12 and 15 respectively, indicate *rol* loci (White et al. 1985; Slightom et al. 1986). The pMP clones (Pomponi et al. 1983) utilized to extract the restriction fragments to be cloned in Bin 19 are indicated as *bars* below the map

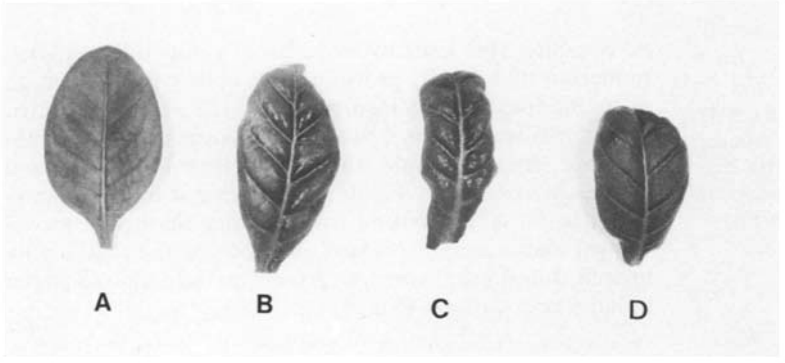

Fig. 2A–D. Leaves from SR1 tobacco plants.
A Untransformed control; **B** Transformed by pRi 1855 full-length TL-DNA (Costantino et al. 1984);
C Transformed by *rol* A, B, C (Bin 19::*Eco*RI15);
D Transformed by *rol* B (Bin 19::*Hin*dIII 37)

roots are somewhat less abundant. No rooting was ever observed from untransformed tobacco leaf explants under the same hormone-depleted conditions (Benvenuto et al. 1983; this work).

The same morphological traits, wrinkled, dark leaves (Fig. 2D) and excessive, partially nongeotropic roots were shown by plants derived from leaf disc infection with Bin 19 harbouring *Hin*dIII fragment 37b (TH37 plants), which encompasses the single ORF11 of the TL-DNA of pRi1855. The presence of this T-DNA segment in TH37 tobacco regenerants was checked by hybridizing ^{32}P-labelled *Hin*dIII fragment 37b against a Southern blot of *Hin*dIII-digested total TH37 DNA (Fig. 3). Thus, a single T-DNA gene is capable of conferring on tobacco plants these most readily recognizable traits of the hairy root phenotype. However, leaf explants from TH37 regenerants would not produce roots in vitro on hormone-depleted medium.

Transgenic tobacco plants regenerated from leaf disc infections with TL-DNA segments, other than fragments *Eco*RI 15 and *Hin*dIII 37b, do not show appreciable morphological modifications, as compared to normal SR1 plants.

Tobacco stem infections

Agrobacterium LBA 4404 strains harbouring Bin 19 constructions were also utilized to infect wounded stems of SR1 tobacco plantlets. Infections were carried out with all the Bin 19 recombinant plasmids described above harbouring, individually, *Hin*dIII fragment 23, *Eco*RI 15, *Hin*dIII 37b, *Eco*RI 36, *Eco*RI 37a, *Eco*RI 36+37a and *Kpn*I 13 (see Fig. 1). Hairy root symptoms – thick, curly, non geotropic roots originating directly from the wound – could be observed only in plants infected with Bin 19 carrying *Eco*RI fragment 15 and, more intriguingly, fragment *Hin*dIII 37b comprising the single ORF11 (see Fig. 4A).

Carrot disc infections

Discs cut from carrot tap-roots were infected on their apical surface (facing the root tip) with the Bin 19 constructions described above. In no case was rooting observed from the discs when strains were individually inoculated onto the cut surface. We thus proceeded with a series of co-infections, by mixing appropriate bacterial cultures immedi-

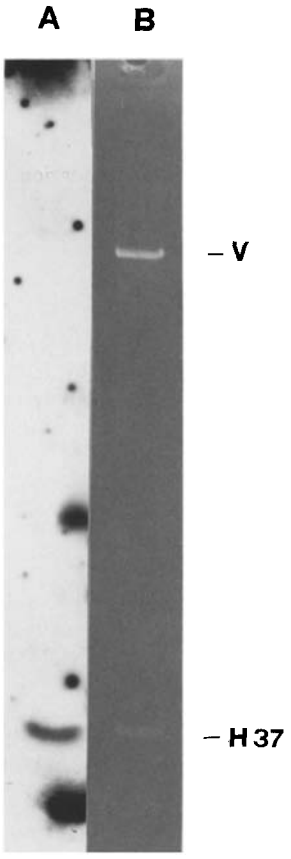

Fig. 3. Southern blot hybridization between *Hin*dIII fragment 37 of pRi 1855 TL-DNA and *Hin*dIII-digested total DNA from a tobacco regenerant derived from leaf disc transformation with Bin-19::*Hin*dIII 37 (Lane A). Lane B: agarose gel electrophoresis of Bin 19::HindIII 37 digested with HindIII. *V* indicates the vector Bin 19; *H37* indicates *Hin*dIII fragment 37

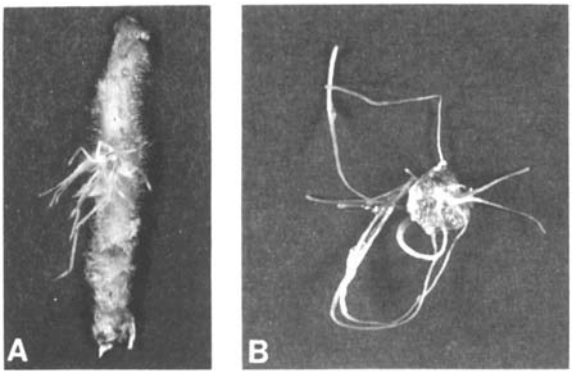

Fig. 4A. Hairy roots induced on tobacco stem by inoculation with *Agrobacterium* LBA 4404 (Bin 19::*Hin*dIII 37). **B** Callus derived from carrot disc co-inoculation with LBA 4404 (Bin 19::*Eco*RI 15) and LBA 4404 (Bin 19::*Eco*RI 36+37a) growing on hormone-free MS medium. Pictures were taken 1 month after bacterial inoculum (**A**) and transfer onto culture medium (**B**)

ately prior to inoculation on the discs. Rooting was then observed, although less pronounced and delayed as compared with wild-type *A. rhizogenes* 1855 infections, on discs doubly infected with the strain containing fragment *Eco*RI 15 and the strain containing *Eco*RI 36+37a. Discs from these double infections produced few roots and abundant callus; this kanamycin resistant callus differentiated numerous and rapidly growing roots upon explantation on hormone-free culture medium (Fig. 4B). Untransformed callus growing on the apical side of carrot discs did not produce any roots under the same culture conditions and eventually necrotized. Roots from discs and from kanamycin resistant calli resulting from the double infections described above were explanted and showed the fast, highly branched and non geotropic growth pattern characteristic of hairy root cultures (Fig. 5).

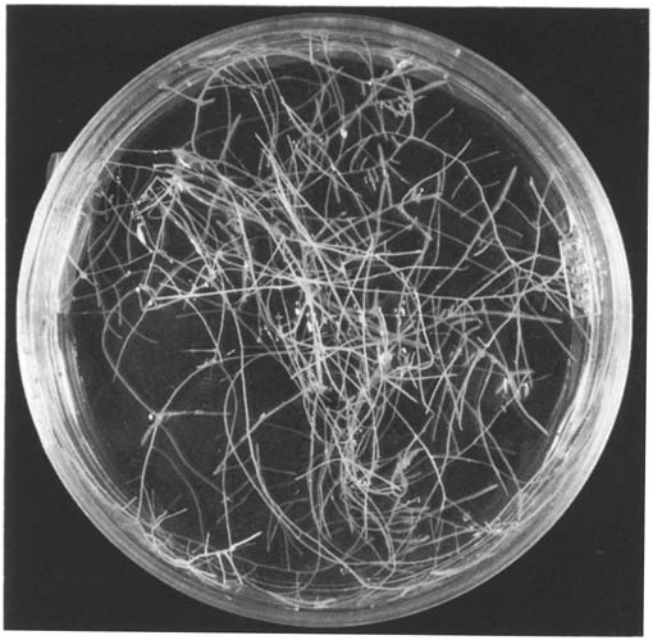

Fig. 5. In vitro culture of roots induced on carrot discs by co-inoculation with LBA 4404 (Bin 19::*Eco*RI 15) and LBA 4404 (Bin 19::*Eco*RI 36+37a)

Discussion

An extensive genetic analysis of the TL-region of agropine-type Ri plasmids has revealed four genetic loci, designated *rol* A, B, C and D, which affect the virulence properties of *A. rhizogenes* on various host plants (White et al. 1985). Subsequently, the complete nucleotide sequence of the same T-region led to the identification of 18 open-reading frames (ORFs) (Slightom et al. 1986), 4 of which (ORFs 10, 11, 12 and 15 respectively) coincide with the *rol* loci.

In the work presented here we have cloned in the binary vector system Bin 19 (Bevan 1984) various segments of the TL-DNA of the agropine-type Ri plasmid pRi1855, covering single and groups of open-reading frames. The portion of the TL-region covered by our analysis (from ORF 8 to the right border of TL-DNA) was shown by Durand-Tardif et al. (1986) to be responsible for the characteristic and inheritable morphological traits of hairy root regenerants in tobacco and several other species; specific transcripts were assigned by the same authors to ORFs 8, 11, 12, 13 and 15 in tobacco regenerants (Durand-Tardif et al. 1986). Leaf disc infections on tobacco with our Bin 19 constructions led to regenerants transformed by different subsets of TL-DNA genes. Of particular interest, plants containing the single ORF11 (*rol*B) show the typical hairy root pheno-

type, consisting of dark wrinkled leaves and extremely abundant and only partially geotropic roots. Moreover, we show here that the Bin 19 construction carrying the single ORF11 (*rol*B) is capable of inducing hairy roots upon infection of wounded tobacco stems. In the transposon-insertion mutagenesis previously published by White et al. (1985), *rol*B was identified as the only TL-DNA locus whose inactivation led to avirulence of agropine-type *A. rhizogenes* on *Kalanchoe daigremontiana* leaves. Undoubtedly therefore, *rol*B represents a key morphogenic locus in agropine-type *A. rhizogenes* T-DNA and the characterization of its function may provide interesting clues on basic mechanisms controlling leaf morphology and root differentiation.

A Bin 19 construction carrying *Eco*RI fragment 15 – comprising ORF 10 (*rol*A) and ORF 12 (*rol*C) in addition to *rol*B – is also capable of inducing hairy roots on tobacco stems; regenerants obtained from leaf disc infections with this construction showed the characteristic hairy root morphology. Interestingly, leaf explants from these regenerants produced roots upon transfer to hormone-free culture medium, a behaviour typical of hairy root regenerants containing full length TL-DNA (Benvenuto et al. 1983; Costantino et al. 1984), which is not shown by plants transformed by *rol*B alone.

An interesting additional finding reported in this work and currently under further investigation in our laboratory, is that a more complex set of TL-DNA genes is required for hairy root induction on carrot discs than on tobacco stems. We have previously shown that the role of TL-DNA is to confer on the cells of the carrot disc surface the ability to differentiate roots upon stimulation by auxin (Cardarelli et al. 1987); this hormone was shown to be incapable per se of directing differentiation of untransformed carrot disc cells (Cardarelli et al. 1987). On the contrary, roots can be induced on wounded stems of tobacco plantlets by local addition of auxin (unpublished results). Cells directly differentiating roots upon stimulation by auxin have been called "prerhizogenic" (J. Bercetche, D. Chriqui, S. Adam and C. David; submitted for publication). Thus, cells on tobacco stems, but not those on carrot discs, could be regarded as prerhizogenic; we can then regard the overall effect of the TL-DNA as conferring a prerhizogenic state on transformed cells. Accordingly, we show here that neither inoculations with the strain carrying only *rol*B nor with the strain carrying *rol*A, B and C, are sufficient to elicit rooting on carrot discs; co-inoculation of this latter strain, together with one carrying ORFs 13 and 14 is needed. T-DNA analysis of cultures derived from individual roots elicited by these double infections will reveal whether, in order to direct differentiation, all of the 5 TL-DNA genes (*rol*A, B, C and ORF 13, 14) must be present in the same cell, or whether cells transformed by different TL-DNA subsets can influence the morphogenic potential of each other.

Much further work is obviously needed to draw any firm conclusion on the nature and interplay of the genes involved in hairy root induction, but the present analysis, pinpointing *rol*B as a major genetic determinant in controlling morphological features of hairy root plants and as capable alone of inducing hairy roots on tobacco stems, represents a significant step forward in the comprehension of this intriguing biological system. Furthermore, our analysis has narrowed down considerably the number of TL-DNA genes actively involved in conferring differentiative competence to normally incompetent plant cells, such as those of carrot discs.

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