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Identification of the Region Encoding Opine Synthesis and of a Region Involved in Hairy Root Induction on the T-DNA of Cucumber-Type Ri Plasmid

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Partial digestion products from a plasmid extending over the entire T-DNA region of the cucumber-type Ri plasmid have been cloned on the wide host range plasmid pRK404. Recombinant plasmids were introduced by triparental mating in *Agrobacterium* strains carrying disarmed Ti plasmids. Two constructs, pMOA3 and pMOA4, were able to incite hairy roots on carrot disks. They were mutagenized by transposon (Tn*7*) insertion, and deletions were created in the T-region. The resulting plasmids were studied for their ability to trigger root formation or opine synthesis. Opine synthesis gene(s) and one region involved in induction of hairy roots on carrot disks were identified.

Additional key words: *Agrobacterium rhyzogenes,* opine synthesis, Ri plasmid, T-DNA

Agrobacterium rhizogenes is responsible for the hairy root disease that results in the appearance of abundant root proliferations on a large number of dicotyledonous plants when they are wounded and inoculated with the bacterium. The pathogenic agent was first identified as *A. tumefaciens* and later given the species name *A. rhizogenes* (Riker *et al.* 1930). The pathogenic determinants in both *A. tumefaciens*, which causes the crown gall disease, and *A. rhizogenes* are borne on large plasmids (Gheysen *et al.* 1985) that have been named respectively Ti and Ri plasmids. The molecular basis for both diseases is the transfer and expression of one or more segments from the pathogenic plasmid into the genome of some plant cells of the wounded area. The transferred segments, called T-DNA, are delimited by 25 bp imperfect repeats called border sequences (Gheysen *et al.* 1985). Hairy roots are easily grown *in vitro* (Tepfer and Tempé 1981, Birot *et al.* 1987) where they generally show better growth than normal roots of the same species. In addition, they exhibit a particular phenotype with a higher rate of lateral root formation than normal roots and lack of geotropism (David *et al.* 1984). Plants cells transformed with hairy root or crown gall T-DNA generally produce new compounds called opines (Tempé *et al.* 1977, Tempé and Schell 1977). Different opines are associated with different plasmids that are therefore named with reference to this property (Guyon *et al.* 1980, Petit *et al.* 1983, Petit and Tempé 1985). Three types of Ri plasmids are known (Costantino *et al.* 1981) to which correspond three opine types (Petit and Tempé 1985). Cucumopine, recently identified as 4,5,6,7-tetrahydroimidazo (4,5-C)pyridine, -4-carboxylic acid, -4-propanoic acid, -6-carboxylic acid, or 4-carboxy-4-(2-carboxyethyl)-spinacin (Davioud *et al.*, unpublished data), is produced in hairy roots elicited by Ri plasmids that are present in *A. rhizogenes* strains isolated from diseased cucumber plants (National Collection of Plant Pathogenic Bacteria catalog edition 1977, Harpenden Herts, UK). Cucumopine-type Ri plasmids have so far been little studied. Cucumopine has been found in every extract from roots incited by cucumber strains of *A. rhizogenes* (Petit and Tempé 1985, unpublished data). An approximately 30-kb segment carrying the T-region of the cucumopine-type Ri plasmid from strain NCPPB2659 has been subcloned in pBR328, its restriction map has been established, and the extent of the T-region is known with reasonable precision (Combard *et al.* 1987).

The transfer of T-regions from Ti plasmids and Ri plasmids to plant cells is dependent on virulence functions carried by these plasmids. These functions can act in *trans* on T-regions carried by the same bacterial host on another plasmid (Hoekema *et al.* 1983, De Framond *et al.* 1983). Binary vector systems that take advantage of this property have been developed for plant genetic engineering. We have inserted in the wide host range plasmid pRK404 (Ditta *et al.* 1980) DNA sequences representing the whole or part of the T-region and used a binary vector strategy to study properties of these constructs and some of their derivatives.

MATERIALS AND METHODS

Bacterial strains are listed in Table 1. For *Escherichia coli* strains, media and culture conditions, conjugation and transformation experiments were as described previously (Hassan and Brevet 1983). *Agrobacterium* strains were grown at 28°C in M63 medium (Miller 1972) with saccharose as carbon source. Strains containing pRK404 derivatives were always grown in the presence of tetracyclin (10 μg/ml for *E. coli* strains and 2.5 μg/ml for *Agrobacterium* strains) since the replicon pRK404 is quite unstable in the absence of selection (Ditta *et al.* 1985).

Preparation and analysis of plasmid DNA from *E. coli* strains were performed as described previously (Ouartsi *et al.* 1985). For analysis of plasmid content of *Agrobacterium* clones, alkaline extraction (Kado and Liu 1981) was conducted at 37°C to minimize degradation of large plasmids.

Cloning of fragments carrying part or all of the T-region from cucumopine-type Ri plasmid in the broad host range vector pRK404. The general procedure to select clones

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containing pRK404 recombinants was as follows: plasmid DNA containing target fragment(s) was digested with appropriate restriction endonucleases and ligated by standard procedures (Maniatis *et al.* 1982) into pRK404 linearized by restriction endonuclease(s) for which a unique site is in the polylinker. The ligation mixture was used to transform *E. coli* JM83. Transformant cells were selected on antibiotic II medium (Difco) containing tetracyclin (10 μg/ml) and 5-bromo-4-chloro-3-indol-β-D-galactoside (X-Gal). White tetracyclin-resistant (Tc^r) colonies were purified by streaking onto the same selective medium as above and checked for plasmid DNA content by agarose gel electrophoresis.

Digestion of pMOA3 by *Spe*I or both *Spe*I and *Nhe*I followed by ligation, transformation of *E. coli*, and selection of Tc^r clones allowed us to obtain the deleted recombinant plasmids pMOA3-10 and pMOA3-11 respectively. The right 8.5-kb *Bgl*II fragment from pMOC3 was inserted into the *Bam*HI site of pRK404 to give pMOA5. The right 4.4 *Hind*III fragment from pMOC3 was ligated into the *Hind*III site of pRK404 to give pMOA6. The right *Bgl*II site used to generate restriction fragments cloned in plasmids pMOA7, pMOA8, and pMOA9 (Table 1, Fig. 1) was the rightmost *Bgl*II site of Tn*7*Δ9 (40 bp from the right border [Gosti-Testu and Brevet 1982, Gosti-Testu *et al.* 1983]) inserted at different locations into pMOA3.

Transposition mutagenesis. Plasmid pMOA3 or pMOA4 was transferred in *E. coli* MODB70 (ColE1::Tn*7*Δ9) via triparental mating with *E. coli* C600 harboring the helper plasmid pRK2013. Transconjugants were selected on Mueller Hinton solid medium (Difco) containing rifampicin (100 μg/ml), trimethoprim (100 μg/ml), and tetracyclin (10 μg/ml). Clones were checked for the presence of both ColE1::Tn*7*Δ9 and pMOA3 or pMOA4. Two selected clones containing either pMOA3 or pMOA4 in addition to ColE1::Tn*7*Δ9 were grown at 30° C in Mueller Hinton broth (Difco), with antibiotics, through three successive hundredfold dilutions. A triparental mating was then performed with *E. coli* MM383 Nal^r as recipient in the presence of *E. coli* C600(pRK2013). Transconjugants were selected on Mueller Hinton solid medium containing nalidixic acid (100 μg/ml), trimethoprim (100 μg/ml), and tetracyclin (10 μg/ml) at 42° C. Because strain MM383 is a polA thermosensitive mutant, ColE1::Tn*7*Δ9 cannot replicate efficiently in this strain at 42° C (Kingsbury and Helinski 1973). Plasmid DNA was extracted from transconjugants by alkaline extraction and submitted to agarose gel electrophoresis to estimate plasmid sizes. Strains containing plasmids larger than the one initially present (pMOA3 or pMOA4) by about the size of Tn*7*Δ9 (7.1 kb)

Table 1. Characteristics of plasmids and bacterial strains

Plasmids	Description[a]	Reference
ColE1::Tn*7*	Tra-, Mob-, Cel, Iel, Tp, Sm/Sp	Lichtenstein *et al.* 1981
ColE1::Tn*7*Δ9	derived from ColE1::Tn*7* after *Xba*I digestion	This work
pRK404	Tc, *LacZ, IncP*; derivative of pRK2	Ditta *et al.* 1985
pRK2013	Km; can mobilize pRK404 and its derivatives	Ditta *et al.* 1980
pMOC4	Tc, Cm; two contigous 8.5 and 21 kb *Bam*HI fragments containing the T-DNA region of pR12659 inserted into pBR328	Brevet and Tempé 1987
pMOC3	Tc, Cm; the larger *Bam*HI fragment (21 kb) of pMOC4 cloned into pBR328	Brevet and Tempé 1987
pMOA2	Tc, the 8.5-kb *Bam*HI from pMOC4 cloned into pRK404	This work
pMOA3	Tc, the 21-kb *Bam*HI fragment from pMOC3 cloned into pRK404	This work
pMOA4	Tc, the two large *Bam*HI fragments (8.5 and 21 kb) of pMOC4 cloned into pRK404	This work
pMOA4-1::Tn*7*Δ9	Tc, Tp, Sm/Sp; transposition of Tn*7*Δ9 onto pMOA4	This work
pMOA3-2::Tn*7*Δ9	Tc, Tp, Sm/Sp; transposition of Tn*7*Δ9 onto pMOC3	This work
pMOA3-3::Tn*7*Δ9	Tc, Tp, Sm/Sp; transposition of Tn*7*Δ9 onto pMOC3	This work
pMOA3-4::Tn*7*Δ9	Tc, Tp, Sm/Sp; transposition of Tn*7*Δ9 onto pMOC3	This work
pMOA3-5::Tn*7*Δ9	Tc, Tp, Sm/Sp; transposition of Tn*7*Δ9 onto pMOC3	This work
pMOA3-6::Tn*7*Δ9	Tc, Tp, Sm/Sp; transposition of Tn*7*Δ9 onto pMOC3	This work
pMOA3-7::Tn*7*Δ9	Tc, Tp, Sm/Sp; transposition of Tn*7*Δ9 onto pMOC3	This work
pMOA3-8::Tn*7*Δ9	Tc, Tp, Sm/Sp; transposition of Tn*7*Δ9 onto pMOC3	This work
pMOA3-9::Tn*7*Δ9	Tc, Tp, Sm/Sp; transposition of Tn*7*Δ9 onto pMOC3	This work
pMOA3-10	derivative of pMOA3 after *Spe*I digestion	This work
pMOA3-11	derivative of pMOA3 after *Spe*I *Nhe*I digestion	This work
pMOA5	the 8.5-kb *Bgl*II fragment of pMOC3 cloned into pRK404	This work
pMOA6	the 4.4-kb *Hind*III fragment of pMOC3 cloned into pRK404	This work
pMOA7	*Bgl*II fragment from pMOA3-7::Tn*7*Δ9 cloned into pRK404	This work
pMOA8	*Bgl*II fragment from pMOA3-8::Tn*7*Δ9 cloned into pRK404	This work
pMOA9	*Hind*III-*Bgl*II fragment from pMOA3-8::Tn*7*Δ9 cloned into pRK404	This work
pLA4404	pTiAch5 deletion lacking T-region	Ooms *et al.* 1982
pGV3850	C58 nopaline Ti plasmid with oncogenes deleted and replaced by pBR322	Zambryski *et al.* 1983
pRi8196	mannopine Ri plasmid	Chilton *et al.* 1982
Escherichia coli strains		
C600	*thr, leu, thi*	Bachmann 1972
W3101	*trp*E, *rec*A13	Kopecko *et al.* 1976
MODB70	W3101 Rif^r, Sm/Sp; with Tn*7dfr*1 inserted onto the chromosome	Hassan and Brevet 1983
JM83	*ara*, delta (*lac-pro*), *strA*, *thi*, Phi80dlacZ del M1	Vieira and Messing 1982
MM383 Nal^r	Nal, *pol*A12, *thy, rha, lac, str*; derived from W3110	Monk and Kinross 1972
Agrobacterium strains		
LBA4404	Rif, contains pAL4404, a deletion of pTiAch5 lacking the T-region	Ooms *et al.* 1982
C58C1	cured derivative of pTiC58	Holsters *et al.* 1980
C58C1 (pGV3850)	contains pGV3850	Zambryski *et al.* 1983
8196	contains pRi8196	Petit *et al.* 1983
C58C1 (8196)	contains pRi8196	Petit *et al.* 1983

[a] Antibiotic resistance is indicated by a two or three letter abbreviation of the drug. Cm = chloramphenicol, Km = kanamycin, Nal = nalidixic acid, Rif = rifampicin, Sm = streptomycin, Sp = spectinomycin, Tc = tetracyclin, Tp = trimethoprim.

were further studied. They were digested with *Bam*HI, and the size of fragments produced was measured to determine the location of Tn7Δ9. Plasmids in which the transposons had inserted into pRi sequences were further analyzed with different restriction endonucleases to map more precisely the insertion site of Tn7Δ9. A series of Tn7Δ9 containing plasmids (Table 1, Fig. 1) were retained for further studies after inoculation of carrot disks.

Preparation of root extracts and high voltage paper electrophoresis were as described (Petit *et al.* 1986).

RESULTS

Cloning of fragments carrying part or all of the T-region from cucumopine-type Ri plasmids in the broad host range vector pRK404. A partial *Bam*HI digest of pMOC4 (Combard *et al.* 1987) was ligated to a complete *Bam*HI digest from pRK404 and the ligation mixture was transformed into *E. coli* JM83. Three clones containing either the 8.5-kb *Bam*HI fragment, the 21-kb *Bam*HI fragment present in pMOC4, or both (named pMOA2, pMOA3, and pMOA4 respectively) were retained for further studies. They were introduced by triparental mating into strains LBA4404 and C58C1 (pGV3850) in which resident Ti plasmid derivatives served as the source of virulence functions. Hairy roots were formed on inverted carrot disks inoculated with transconjugant strains pMOA3 and pMOA4 (Table 2, Fig. 1). The roots were excised and transferred onto culture medium (Petit *et al.* 1983) on which they grew and exhibited the typical hairy root phenotype. Extracts of these roots were analyzed by high voltage paper electrophoresis; they and roots incited with wild type strains contained similar amounts of cucumopine.

Transposon mutagenesis of pMOA3 and pMOA4. Nine insertion mutants with Tn7Δ9 in the Ri plasmid part of pMOA4 and pMOA3 were obtained (Fig. 1). All of these induced roots. They were analyzed for the presence of cucumopine. One of them (pMOA3-6::Tn7Δ9) was found to have lost the capacity to trigger opine synthesis in hairy roots (Table 2).

Deletion of restriction fragments in pMOA3. Two deleted derivatives of pMOA3, plasmids pMOA3-10 and pMOA3-11, were constructed (Fig. 1). These plasmids were introduced in *A. tumefaciens* C58C1 (pGV3850) and C58C1 (pRi8196), and the resulting strains were inoculated to carrot disks. In C58C1 (pGV3850), pMOA3-10 showed very reduced root-inducing capacity, whereas pMOA3-11 did not incite any roots. A relatively high proportion (about 40%) of the roots incited by the two corresponding strains derived from C58C1 (pRi8196) contained cucumopine.

Cloning of pMOA3 restriction fragments into pRK404. Plasmids pMOA5 to pMOA9 were obtained as described (Fig. 1). They were introduced in C58C1 (pGV3850) or C58C1 (pRi8196) and the resulting strains were inoculated to carrot disks. None of the constructs produced roots when the coresident plasmid was the disarmed Ti plasmid pGV3850 (Table 2). Roots were produced when pRi8196 was the coresident plasmid. These roots were analyzed for the presence of cucumopine with the result that those incited with the strains harboring pMOA5, pMOA6, pMOA8, and pMOA9 frequently contained cucumopine. Roots incited with strain C58C1 (pRi8196, pMOA7) did not contain detectable amounts of cucumopine. All the roots contained mannopine, the opine that is specific for pRi8196 induced hairy roots.

DISCUSSION

Both pMOA3 and pMOA4 were equally able to incite hairy roots with a wild type phenotype on carrot disks. Because previous work showed that in all carrot hairy root cultures studied T-DNA is probably of the same size and extends by about 2.6 kb into the 8.5-kb *Bam*HI fragment of pMOA4, this result shows that no essential function for

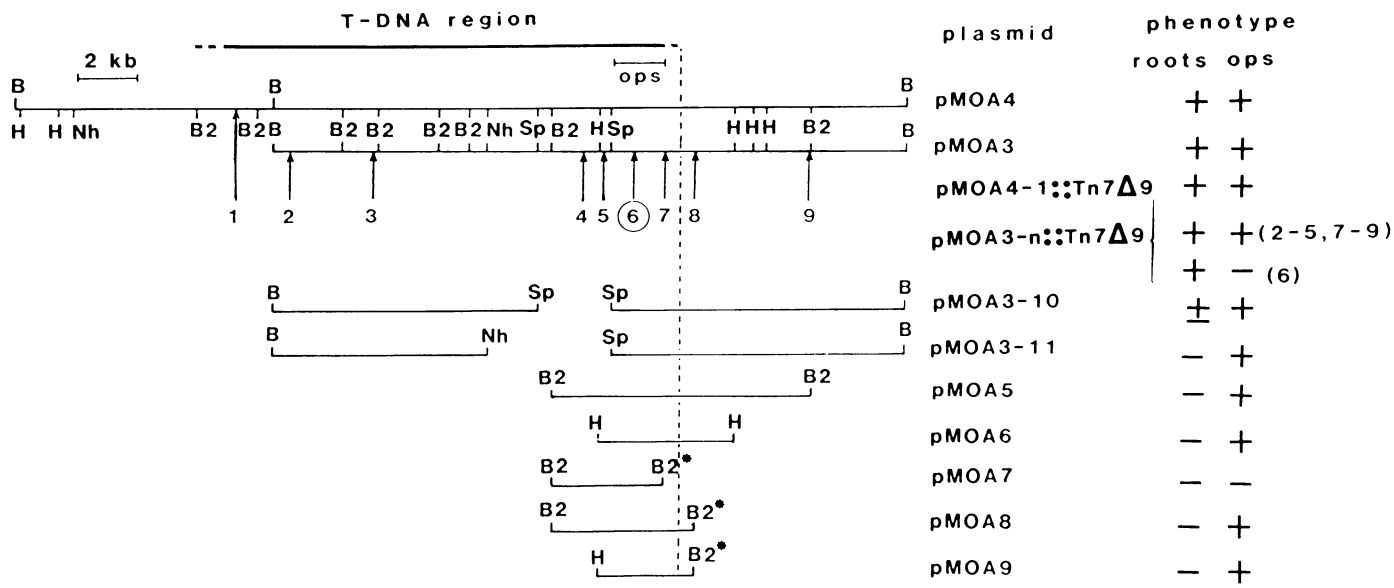

Fig. 1. Maps of recombinant plasmids and corresponding phenotypes with respect to hairy root induction and opine synthesis. B = *Bam*HI, B2 = *Bgl*II, H = *Hind*III, Nh = *Nhe*I, Sp = *Spe*I. B2* = the rightmost *Bgl*II site of Tn7Δ9 (see Table 1). The extent of T-DNA region is shown above the map, dotted ends indicate imprecise location of T-DNA extremities. ops = localization of opine synthetase gene(s). The arrows indicate the different insertion sites of Tn7Δ9, and the numbers under the arrows indicate different mutants. In pMOA3-n::Tn7Δ9 : n replace one of the values from 2 to 9. The vertical dashed line aligns the probable right border of the T-DNA across the different recombinant plasmids. Symbols +, ±, and − describing phenotypes have the same meaning as in Table 2.

pathogenicity in carrot is present in this left portion of the T-region. Thus, as expected, Ti plasmid virulence functions are efficient for transfer of Ri T-DNA region. Our results also indicate that, like for Ti plasmids (Joos *et al.* 1983), the left border of the T-region is not essential for transfer.

None of the insertions with Tn*7*Δ9 affected root inducing properties. One of these, however, pMOA3-6::Tn*7*Δ9 incited roots that did not synthesize cucumopine, suggesting that the insertion had occurred in a gene involved in cucumopine synthesis. Obviously this gene or an essential part of it lies between insertions sites 5 and 7 that incite cucumopine positive roots.

Table 2. Phenotypic analysis of recombinant plasmids

Strains	Roots[a]	Opines[b]		
		Nopaline	Mannopine	Cucumopine
C58C1 (pMOA3)	−	na	na	na
LBA4404 (pAL4404 + pMOA3)	+	−	−	+
C58C1 (pGV3850 + pMOA3)	+	+	−	+
C58C1 (8196 + pMOA3)	+	−	+	+
C58C1 (pMOA2)	−	na	na	na
LBA4404 (pAL4404 + pMOA2)	−	na	na	na
C58C1 (pGV3850 + pMOA2)	−	na	na	na
C58C1 (pMOA4)	−	na	na	na
LBA4404 (pAL4404 + pMOA4)	+	−	−	+
C58C1 (pGV3850 + pMOA4)	+	+	−	+
C58C1 (pGV3850 + pMOA4-1::Tn*7*Δ9)	+	+	−	+
C58C1 (pGV3850 + pMOA3-2::Tn*7*Δ9)	+	+	−	+
C58C1 (pGV3850 + pMOA3-3::Tn*7*Δ9)	+	+	−	+
C58C1 (pGV3850 + pMOA3-4::Tn*7*Δ9)	+	+	−	+
C58C1 (pGV3850 + pMOA3-5::Tn*7*Δ9)	+	+	−	+
C58C1 (pGV3850 + pMOA3-6::Tn*7*Δ9)	+	+	−	−
C58C1 (pGV3850 + pMOA3-7::Tn*7*Δ9)	+	+	−	+
C58C1 (pGV3850 + pMOA3-8::Tn*7*Δ9)	+	+	−	+
C58C1 (pGV3850 + pMOA3-9::Tn*7*Δ9)	+	+	−	+
C58C1 (pGV3850 + pMOA3-10)	+/−	+	−	+
C58C1 (8196 + pMOA3-10)	+	−	+	+
C58C1 (pGV3850 + pMOA3-11)	−	na	na	na
C58C1 (8196 + pMOA3-11)	+	−	+	+
C58C1 (pGV3850 + pMOA5)	−	na	na	na
C58C1 (8196 + pMOA5)	+	−	+	+
C58C1 (pGV3850 + pMOA6)	−	na	na	na
C58C1 (8196 + pMOA6)	+	−	+	+
C58C1 (pGV3850 + pMOA7)	−	na	na	na
C58C1 (8196 + pMOA7)	+	−	+	−
C58C1 (pGV3850 + pMOA8)	−	na	na	na
C58C1 (8196 + pMOA8)	+	−	+	+
C58C1 (pGV3850 + pMOA9)	−	na	na	na
C58C1 (8196 + pMOA9)	+	−	+	+

[a] + denotes formation of roots on inoculated carrot disks, ± denotes occasional formation of scarce roots, − denotes absence of roots, + denotes presence of the corresponding opine, − denotes absence of the corresponding opine, na = not analyzed.

None of the subclones of pMOA3 represented by plasmids pMOA5 to pMOA9 was able to incite root formation when the coresident plasmid was pGV3850. All of them except pMOA7 could trigger cucumopine formation in roots when the coresident plasmid was 8196, confirming that when two T-DNAs are present in the same strain the frequency of cotransformation is high (Depicker *et al.* 1985, Petit *et al.* 1986, Simpson *et al.* 1986). Therefore pMOA5, -6, -8, and -9 must carry all the genetic information necessary for cucumopine synthesis and a functional right border. Furthermore, because deletion mutants pMOA3-10 and pMOA3-11 still encode cucumopine synthesis, we can conclude that the cucumopine synthesis gene(s) is located between the rightmost *Spe*I site and the right T-region border. The fact that pMOA7 failed to trigger opine synthesis can be explained by assuming that the right border sequence in the T-region is located to the right of the transposon insertion site in pMOA3-7::Tn*7*Δ9. As a result, pMOA7 would have no T-region border, preventing transfer to plant cells. This confirms the previous localization of this border (Combard *et al.* 1987).

With respect to opine synthesis functions, it is remarkable that in several cases these functions map closest to the right T-region border. This is for instance the case for octopine synthesis in octopine Ti plasmids, for nopaline synthesis in nopaline Ti plasmids (Gheysen *et al.* 1985), and also most probably for mannopine synthesis in pRi8196 (Lahners *et al.* 1984), as well as for agropine synthesis on the Tr-DNA of agropine-type Ri plasmids (Jouanin 1984, Huffman *et al.* 1984). We wonder whether this feature reflects some evolutionary constraint for *Agrobacterium* pathogenic plasmids.

The deletion in pMOA3-10 resulted in very reduced root-inducing ability, compared with pMOA3, and the one in pMOA3-11 completely abolished root induction on carrot disks. The deletions had no effect on the virulence of pRi8196 when this was present in *trans* in the same bacterial cell; numerous mannopine-containing hairy roots were obtained when a strain with this plasmid combination was inoculated. Many of these roots also contained cucumopine, demonstrating that the deleted T-region was efficiently transferred. When tested on tobacco leaf disks, pMOA3-10 and pMOA3-11 still incited root formation although less efficiently than pMOA3 (F. Vilaine, personal communication). The number of roots per disk was lower for pMOA3-11 than for pMOA3-10, which is coherent with the results observed in carrot disk inoculations. These observations are in keeping with recent results (Cardarelli *et al.* 1987) showing that in pRi1855 a segment similarly located and homologous to the deleted region in pMOA3-10 or -11 is necessary for root formation in carrot but not in tobacco. More work is obviously needed to analyze the role of the deleted region and to understand what is the genetic or physiological basis for the different responses of tobacco and carrot cells.

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