MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 2, pp. 163-172, 1991

Role of T-Region Borders in *Agrobacterium* Host Range

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Received 24 August 1990. Accepted 3 December 1990.

The limited host range AB3 strain of *Agrobacterium tumefaciens* induces tumors by transferring two T-regions, TA and TB. TA is a deleted version of the well-known biotype I octopine TL-region that lacks the *iaa* and *ipt* genes, but carries an intact oncogene, gene 6b, and typical left and right border sequences. TB carries two *iaa* genes that together code for the synthesis of indoleacetic acid. Gene 6b and the *iaa* gene act synergistically when transferred in a coinoculation experiment. The TA-region of the limited host range isolate Ag57 is related to the TA-region of AB3, but differs from it at several positions. The most significant difference is the absence of the right border region. In spite of this, Ag57 and the exconjugant strain C58C9(pTiAg57) induce normal tumors on *Nicotiana rustica* and *Vitis vinifera*. Various experiments indicate that gene 6b of the Ag57 TA-region is active and transferred in spite of the absence of the right border. On *N. tabacum*, C58C9(pTiAg57) is nononcogenic but becomes oncogenic when the pTiAg57 TA-region is restored by the right TA border sequence of pTiAB3. Thus, the right TA border sequence of the biotype III limited host range strains is required for tumor induction on some hosts, but not on others.

Additional keywords: oncogenes, Ti plasmid evolution.

Agrobacterium tumefaciens (Smith and Townsend) Conn is well-known for its tumor-inducing (Ti) plasmid and its capacity to transfer DNA into plant cells (Hooykaas and Schilperoort 1984; Nester *et al.* 1984; Zambryski *et al.* 1989). Currently, many studies are being aimed at the determination of the basic molecular mechanisms that govern this remarkable interaction. These studies involve only a few, well-characterized strains, mainly A6 (very similar to Ach5, B6S3, and 15955) and C58. However, several *Agrobacterium* isolates have been described that carry other types of Ti plasmids, and it remains to be seen whether these strains use the same basic plant cell transformation system as A6 or C58. Strains of *Agrobacterium* have been classified according to chromosomal characteristics into three biotypes or biovars. A6 is an octopine biotype I strain, and C58 is a nopaline biotype I strain. Biotype III strains have mainly been isolated from grapevine, and constitute the major strain type on this plant (Panagopoulos and Psallidas 1973; Thomashow *et al.* 1980, 1981; Szegedi 1985; Paulus *et al.* 1989a, 1989b). Within the biotype III group, octopine/cucumopine, nopaline, and vitopine Ti plasmids have been described (Szegedi 1985; Paulus *et al.* 1989a). Octopine/cucumopine Ti plasmids have only small areas of DNA homology with the Ti plasmids of the biotype I octopine strains. Among the octopine/cucumopine strains, wide and limited host range strains have been defined (Thomashow *et al.* 1980). The limited host range strains Ag162 (Knauf *et al.* 1982, 1984; Yanofsky *et al.* 1985a), Ag63 (Buchholz and Thomashow 1984a), and AB3 and Ag57 (Paulus *et al.* 1989a) have two T-regions, TA (a deleted version of the biotype I octopine TL-region) and TB (with two *iaa* genes, *iaaH* and *iaaM*). Host range limitation is mainly due to the lack of the cytokinin gene *ipt*, as shown by enlargement of the host range with an intact *ipt* gene (Hoekema *et al.* 1984; Buchholz and Thomashow 1984b; Yanofsky *et al.* 1985b), but also involves the *virC* operon (Yanofsky *et al.* 1985b). Hybridization studies indicated that whereas pTiAg162, pTiAg63, and pTiAB3 are very similar, pTiAg57 is different. In this study the structure and function of the TA-regions of Ag57 and AB3 are compared.

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Nucleotide sequence data are to be submitted to GenBank, EMBL, and DDBJ as accession numbers JO3690, JO3693, and JO3694.

MATERIALS AND METHODS

Strains and plasmids. Bacterial strains and plasmids used in this study are described in Table 1.

Cloning, mapping, and sequencing procedures. Cloning, mapping, and sequencing procedures were as described by Sambrook *et al.* (1989). Enzymes were used as indicated by the supplier. Nucleotide sequences were determined by the dideoxy chain termination method (Sanger *et al.* 1977) on fragments cloned in the pBluescript vectors KS− and KS+ (Stratagene, La Jolla, CA). Sequences were analyzed with a MicroVAX computer with the GCG sequence analysis software package (Devereux *et al.* 1987). Homology mapping was conducted with labeled probes hybridized to total or plasmid DNA size-separated on agarose gels and blotted onto nitrocellulose.

Growth of bacteria. Strains of *Escherichia coli* were grown in Luria broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.4). Strains of *A. tumefaciens* were grown in yeast extract broth (5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 0.5 g/L $MgSO_4 \cdot 7H_2O$, pH 7.2). Antibiotics were added at the following concentrations: kanamycin, 25 mg/L; streptomycin, 15 mg/L; ampicillin, 100 mg/L; rifampicin, 100 mg/L;

Table 1. Bacterial strains and plasmids used in this study

Designation	Description[a]	Reference
Escherichia coli		
GJ23	Mobilizing strain, JC2926-(pGJ28)(R64drd11). pGJ28 (Km^r) providing mobilization functions; R64drd11 (Tc^r, Sm^r) providing transfer functions	Van Haute *et al.* 1983
Agrobacterium tumefaciens		
AB3	Biotype III, octopine/-cucumopine limited host range	Szegedi 1985
Ag57	Biotype III, octopine/-cucumopine limited host range	Panagopoulos and Psallidas 1973
C58C9(pTiAg57) (=LBA649)	C58C9 derivative carrying pTiAg57	Hoekema *et al.* 1984
GV3101(pPM6000)	C58C1 derivative carrying disarmed pTi	Bonnard *et al.* 1989a
Plasmids		
pBluescript KS+, KS−	Sequencing vectors	Stratagene[b]
pGP100	Km^r pBR325 derivative	Otten *et al.* 1985
pKC7	Km^r pBR322 derivative	Rao and Rogers 1979
pPM40	2.5-kb *Eco*RI fragment with pTiAg57 gene 6b in pGP100	This study
pPM3064	8.6-kb *Hin*dIII partial fragment with pTiAB3 TB-*iaa* region in pKC7	This study
pPM3091	11.2-kb *Bam*HI fragment with pTiAB3 TA-region in pKC7	This study
pPM3100	2.9-kb *Cla*I fragment with pTiAB3 gene 6b in pKC7	This study
pPM3104	2.2-kb *Hin*dIII fragment with pTiAB3 gene 6b in pKC7	This study
pPM3118	Internally deleted 11.2-kb *Bam*HI fragment with pTiAB3 TA-region in pKC7	This study
pPM3119	pTiAg57 with 2.9- and 0.3-kb *Hin*dIII TA fragment deletion	This study
pPM3125	2.8-kb *Hin*dIII-*Eco*RI fragment with pTiAB3 right TA border in pKC7	This study
pPM3197	pTiAg57 with TB-*iaa* deletion	This study
pPM4004	Internally deleted pTiTm4 TB-*iaa* region clone	Huss *et al.* 1990

[a] Km, kanamycin; Tc, tetracycline; Sm, streptomycin; r, resistant; and kb, kilobase.
[b] La Jolla, CA.

tetracycline, 10 mg/L; and chloramphenicol, 25 mg/L (Leemans *et al.* 1983).

Introduction of genes into disarmed Ti plasmids and modification of wild-type plasmids. Genes were introduced into Ti plasmids in several steps. They were first cloned in pKC7, a pBR322-derived vector with a kanamycin resistance gene. The recombinant plasmid (intermediate vector) was transformed into *E. coli*, and the resulting strain was conjugated with *E. coli* GJ23. GJ23 contains the R64drd11 plasmid with transfer functions and the pGJ28 plasmid with mobilization functions. Upon conjugation, both helper plasmids entered the strain that contained the intermediate vector. The resulting strain with the intermediate vector and the two helper plasmids was then conjugated with an *Agrobacterium* strain containing a disarmed Ti plasmid with pBR322 sequences between transferred DNA (T-DNA) borders. Since the intermediate vectors are unable to replicate in *Agrobacterium*, recombinants between the intermediate vector and the Ti plasmid can be easily selected on kanamycin (Van Haute *et al.* 1983). Double recombinants were obtained by screening for the loss of the relevant marker gene. All recombinant Ti plasmids were verified by Southern analysis of total bacterial DNA with appropriate DNA fragments as probes (Dhaese *et al.* 1979).

Plants. Seeds of *Nicotiana tabacum* L. cv. Samsun and *N. rustica* L. were obtained from the Tobacco Research Station at Bergerac, France. Vegetatively propagated *Vitis vinifera* L. cv. Cabernet Sauvignon plants were obtained from the French National Agronomic Research Institute at Colmar, France.

Infection of plants. Two-month-old tobacco plants (15 internodes) grown in the greenhouse from seeds were infected by puncturing the stems at 2-cm intervals along the stem (eight infection points per plant) with an 18-gauge needle dipped into a fresh colony. Grapevine stems were surface-sterilized, and 2-cm-long stem fragments were placed apical side up in agar and infected with agrobacteria grown on solid medium as described by Huss *et al.* (1990). Tumor growth was scored after 1 to 2 months.

RESULTS

Cloning and mapping of the T-regions of the limited host range Ti plasmids pTiAB3 and pTiAg57. The TA- and TB-regions of pTiAB3 and pTiAg57 were cloned and restriction maps were established. The region between the TA- and TB-regions is about 30 kilobases (kb) (F. Paulus, unpublished), as was also found for Ag162 (Yanofsky *et al.* 1985a). Previously, homology maps had been made for pTiAB3 with DNA fragments of the biotype I octopine strain Ach5, allowing the identification of gene 6b and an octopine synthase gene on the pTiAB3 TA-region, and of the two *iaa* genes, *iaaH* and *iaaM*, on the TB-region (Paulus *et al.* 1989a). Similar data were obtained in this study for pTiAg57. The maps are presented in Figure 1A and B. pTiAB3 and pTiAg57 are clearly related, since they have a large number of restriction sites in common. Sequence data (see below) showed that the common areas have identical sequences.

Outside the common areas a number of differences can be noted. The TA-region of pTiAB3 carries two bacterial

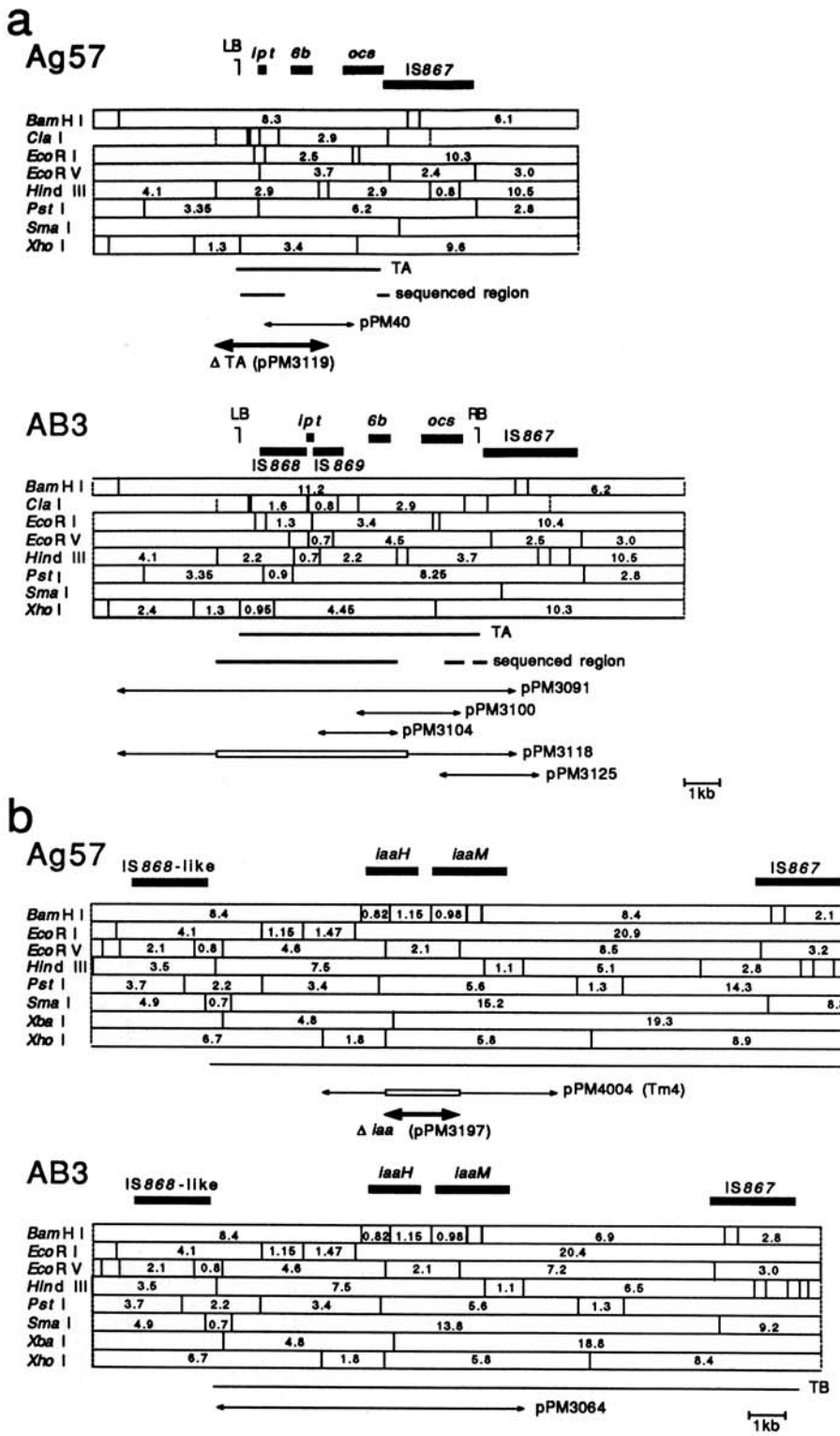

Fig. 1. T-region maps of pTiAg57 and pTiAB3. **A**, TA-regions of pTiAg57 and pTiAB3 (defined for pTiAB3 by border sequences identified in this study and for pTiAg57 by comparative restriction mapping). **B**, TB-regions of pTiAg57 and pTiAB3 (as defined by Yanofsky *et al.* 1985a). T-DNA genes and IS elements were located by sequencing (IS*868*, *ipt*, IS*869*, gene 6b of AB3, and *ipt* of Ag57), homology mapping (IS*867*, *iaaH*, *iaaM*, IS*868*-like sequence of AB3 and Ag57, and *ocs* of AB3), or by comparison of restriction maps (gene 6b and *ocs* of Ag57). Sizes of restriction fragments are indicated in kilobases (kb). LB and RB indicate left and right borders. Dotted vertical lines indicate limits of the restriction maps for different enzymes. White rectangles show deleted regions. Heavy arrows designate deletions in mutant Ti plasmids. The pPM numbers indicate the plasmids used in this study.

insertion sequences, IS*868* and IS*869*, which are characterized by inverted repeats and target site duplications (F. Paulus, unpublished, EMBL accession numbers X55075 and X53945, respectively). These elements are not found in the pTiAg57 TA-region. Furthermore, compared with pTiAB3, pTiAg57 has a 0.6-kb deletion in the right TA border region. Immediately to the right of the TA-region, both pTiAB3 and pTiAg57 carry an IS*867* element. IS*867* is very similar to the previously described IS*866* element from pTiTm4 (Bonnard *et al.* 1989b): partial sequence data (F. Paulus and A. Herrmann, unpublished) show about 80% homology. The pTiAg57 IS*867* element has a 0.1-kb internal deletion. Although the TB-regions of pTiAg57 and pTiAB3 are very similar, pTiAg57 contains an additional 1.4-kb sequence to the right of the *iaa* genes, whereas pTiAB3 carries an additional 0.7-kb sequence to the right of the TB-associated IS*867* element. The restriction maps of the region containing the TB-*iaa* genes are identical for both strains. To establish the role of the pTiAB3 and pTiAg57 T-regions, we first investigated the pTiAB3 system. AB3 was chosen as the reference strain since it represents a widely distributed strain type, whereas Ag57 is unique (Paulus *et al.* 1989a, 1989b). Moreover, the pTiAg57 TA-region seemed to be a deleted version of the pTiAB3 TA-region. The AB3 results were subsequently used to study the role of the pTiAg57 T-regions.

Tumor induction with isolated pTiAB3 oncogenes. The limited host range Ti plasmid pTiAg162 (probably identical to pTiAB3, Paulus *et al.* 1989a) has been reported to be oncogenic on its natural host, *V. vinifera*, and on *N. rustica*, but to induce little or no tumor growth on *N. tabacum* (Thomashow *et al.* 1981). However, we found that limited host range strains reliably induce tumors on *N. tabacum* when stems are infected with a needle dipped in a bacterial colony.

Tumor induction results from the transfer and activity of oncogenes, which in the case of AB3 and related strains are most likely gene 6b of the TA-region and the *iaa* genes of the TB-region.

This assumption was tested by infection of plants with well-defined pTiAB3 T-region fragments carried by the pTiAch5-derived disarmed Ti vector pPM6000 (Bonnard *et al.* 1989a). The complete pTiAB3 TA-region was cloned into the pBR322-derivative pKC7 as an 11.2-kb *Bam*HI fragment (Fig. 1A), yielding pPM3091. Gene 6b of the TA-region was cloned as a 2.2-kb *Hin*dIII fragment and as a 2.9-kb *Cla*I fragment (Fig. 1A) into pKC7, yielding pPM3104 and pPM3100, respectively. These fragments have a 1.1-kb region in common that only contains gene 6b and its 5′ and 3′ noncoding regions (F. Paulus, unpublished). pPM3091, pPM3104, and pPM3100 were inserted into pPM6000 (carried by the C58C1 derivative GV3101) by recombination, yielding GV3101(pPM6000::pPM3091), GV3101(pPM6000::pPM3104), and GV3101(pPM6000::pPM3100). The TB-*iaa* genes were cloned as an 8.6-kb fragment composed of two *Hin*dIII fragments (Fig. 1B)

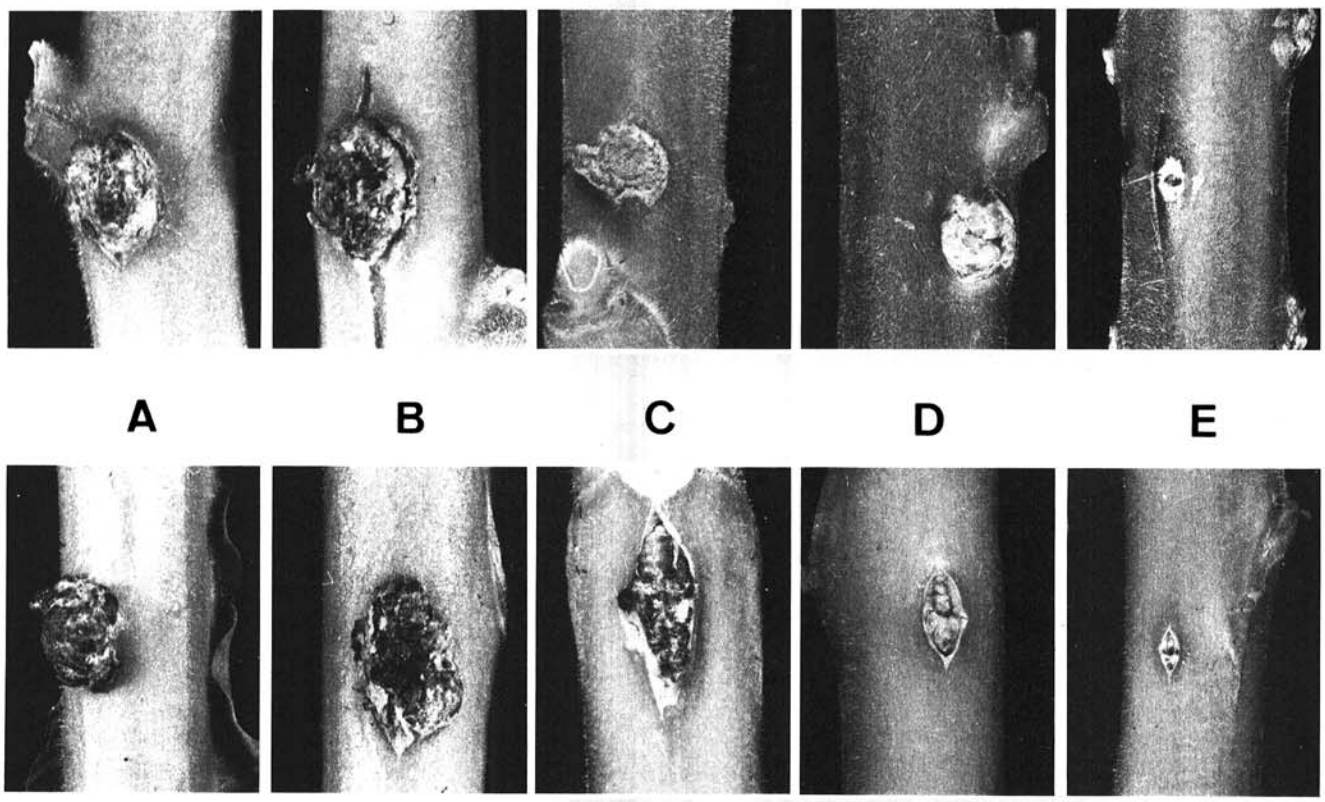

Fig. 2. Functional analysis of pTiAB3 oncogenes. Stems of *Nicotiana rustica* (top) and *N. tabacum* (bottom) were infected with strains carrying different genes and are shown 2 months after infection. **A**, AB3. **B**, GV3101(pPM6000::pPM3104) and GV3101(pPM6000::pPM3064) carrying gene 6b and TB-*iaa*, respectively. **C**, GV3101(pPM6000::pPM3104), gene 6b. **D**, GV3101(pPM6000::pPM3064), TB-*iaa*. **E**, GV3101(pPM6000), disarmed vector strain.

into pKC7 and inserted into pPM6000, yielding GV3101 (pPM6000::pPM3064).

Stems of *N. tabacum* and *N. rustica* plants were infected with AB3, the TA-region strain, the two 6b strains, the TB-*iaa* strain, and a 1:1 mixture of the 6b and TB-*iaa* strains. The results (Fig. 2C) show that gene 6b alone induces small, necrotic calli on *N. tabacum* and small, greenish calli on *N. rustica* (pPM3104 and pPM3100 yielded the same result: only the reaction obtained with the smallest construct, pPM3104, is shown). The same reaction as with gene 6b was obtained with the 11.2-kb TA-region clone (results not shown). Thus, gene 6b acts as an oncogene, as has also been found for gene 6b of the wide host range strains Ach5, C58, and Tm4 (Hooykaas *et al.* 1988; Tinland *et al.* 1989), and is the main oncogenic determinant of the TA-region. The TB-*iaa* genes induce small outgrowths on *N. rustica* and very small, but significant, knoblike structures on *N. tabacum* (Fig. 2D). Whereas AB3 induces large, necrotic tumors (Fig. 2A), the TA-region and gene 6b induce small tumors. An AB3-like response can, however, be obtained by a mixed infection with the 6b and the TB-*iaa* strains (Fig. 2B). From these experiments we conclude that gene 6b of the TA-region and the *iaa* genes of the TB-region act synergistically and constitute the major oncogenes of the AB3 strain.

Earlier experiments in our laboratory showed that the Ag57 exconjugant strain C58C9(pTiAg57) induced large necrotic tumors on *N. rustica*. In view of the results obtained with the pTiAB3 genes, this observation indicated that pTiAg57 might be able to transfer its gene 6b to the plant cells, in spite of its modified TA right border region. To establish the precise structure of the right TA border region of pTiAg57, clones from this region and from the corresponding pTiAB3 region were sequenced.

Sequence of the right border region of pTiAg57 and pTiAB3. A 0.6-kb *Cla*I-*Eco*RI fragment of the right border of the pTiAg57 TA-region (Fig. 1A) was cloned in the sequencing vector pBluescript KS− and sequenced starting from the *Cla*I site. On the basis of this sequence, a synthetic oligonucleotide was made to obtain the sequence of the complementary strand. The AB3 sequence was obtained by using an oligonucleotide derived from the Ag57 *ocs* sequence and another oligonucleotide derived from the Ag57 TA-associated IS*867* sequence. These sequences were used to synthesize oligonucleotides that permitted the sequencing of the opposite stands. The sequences of both regions were compared with the sequence of the right border region of the TL-DNA of biotype I octopine Ti plasmid pTi15955 and are shown in Figure 3A. At the same relative position as in pTi15955, pTiAB3 carries a 25-base pair (bp) T-region border repeat, T**GACAGGATATAT**ACCGT**TGTAA**TT (sequences corresponding to the consensus sequence according to Van Haaren *et al.* [1988] are boldfaced), which has 24 of 25 nucleotides in common with the pTi15955 right TL border repeat (T**GGCAGGATATAT**ACCGT**TGTAA**TT, Barker *et al.* 1983). We also determined the sequence of the left border repeat of the pTiAB3 TA-region. This border, C**GGCAGGATATAT**TGAAT**TGTAA**AT, has 23 of 25 nucleotides in common with the pTi15955 left TL border repeat C**GGCAGGATATAT**TCAAT**TGTAA**AC, and 18 of 25 nucleotides in common with the pTiAB3 right TA border repeat. To the right of the right border repeat, pTiAB3 carries a well-conserved 24-bp *overdrive* sequence (Peralta *et al.* 1986), with only a difference of 1 bp with the pTi15955 *overdrive* sequence and situated at the same relative position as in pTi15955, that is at 14 bp from the border sequence. In pTi15955, the *overdrive* sequence is followed by a 32-bp sequence that has been identified as the inverted repeat of a truncated IS*426* element (De Meirsman *et al.* 1987). The AB3 sequence does not contain this IS*426* fragment but diverges at this point and continues 96 bp up to the 27-bp inverted repeat of the IS element IS*867* (**GTATGCGGCGTCCACGCCCCATTGATT**), which has 24 bp in common with the right inverted repeat of the related IS*866* element (homologous sequences boldfaced, underlined in Fig. 3), **GTATGCGGCGTC**TCCGTCC-**CATTGATT** (Bonnard *et al.* 1989b). Figure 3B summarizes the right border region structures of pTi15955, pTiAB3, and pTiAg57.

In pTiAg57, the DNA sequence of the right border region shows a 0.6-kb deletion that starts within the octopine synthase gene and ends at the start of the inverted IS*867* repeat. Analysis of C58C9(pTiAg57)-induced grapevine tumors showed no traces of octopine or of octopine synthase activity (results not shown), confirming the inactivation of the octopine synthase gene. The TA-region of pTiAg57 also lacks the right TA border sequence, as well as the *overdrive* sequence. The pTiAg57 sequences around the deleted region are identical to the pTiAB3 sequences (225 bp sequenced). Since the left part of the pTiAg57 TA-region shows the same restriction sites as the pTiAB3 region that contains an intact left TA border sequence, the left TA border of pTiAg57 is most probably intact. The effect of the pTiAg57 TA right border deletion on tumor induction was studied by infecting plants with pTiAg57 and with a pTiAg57 construct in which the right TA border of pTiAB3 was inserted to the right of the deleted pTiAg57 TA-region.

Insertion of the pTiAB3 right TA border into the pTiAg57 TA right border region increases oncogenicity on *N. tabacum*. A 2.8-kb *Hin*dIII-*Eco*RI TA fragment of pTiAB3 (Fig. 1A), which carries the right border sequence, was cloned into the pBR322-derivative pKC7 (yielding pPM3125) and inserted into the TA-region of pTiAg57 by homologous recombination. Southern analysis of the recombinant Ti plasmid, pTiAg57::pPM3125, showed that recombination had taken place at the right end of the 2.2-kb *Hin*dIII-*Eco*RI pTiAg57 fragment, that is to the right of the pTiAg57 TA deletion.

C58C9(pTiAg57::pPM3125) and the control strain C58C9(pTiAg57) were tested on *N. rustica*, *V. vinifera*, and *N. tabacum*. On *N. rustica* or *V. vinifera*, both strains induce the same type of tumors (results not shown). On *N. tabacum*, C58C9(pTiAg57) induces very little, but significant callus growths (probably due to the activity of the TB-*iaa* genes) (Fig. 4A), whereas the strain with the right TA border of AB3 induces large, necrotic tumors (Fig. 4B). Thus, the absence of the right border sequence in the pTiAg57 TA-region leads to diminished oncogenicity on *N. tabacum*, but not on *V. vinifera* or *N. rustica*.

Gene 6b of pTiAg57 is required for induction of large

tumors on *N. rustica*. Since it could not be excluded that on *V. vinifera* or *N. rustica* a cryptic, pTiAg57-specific oncogene would compensate for a nontransferred gene 6b, we deleted the TA-region from pTiAg57 and tested the mutants on *N. rustica*. The TA-region of pTiAg57 was deleted by double recombination with the intermediate vector pPM3118, obtained by cloning the 11.2-kb *Bam*HI fragment of the pTiAB3 TA-region (Fig. 1A) into pKC7 and deleting the internal 2.2-, 2.2-, 0.7-, and 0.3-kb *Hin*dIII fragments. pPM3118 retains a 2.9-kb *Bam*HI-*Hin*dIII frag-

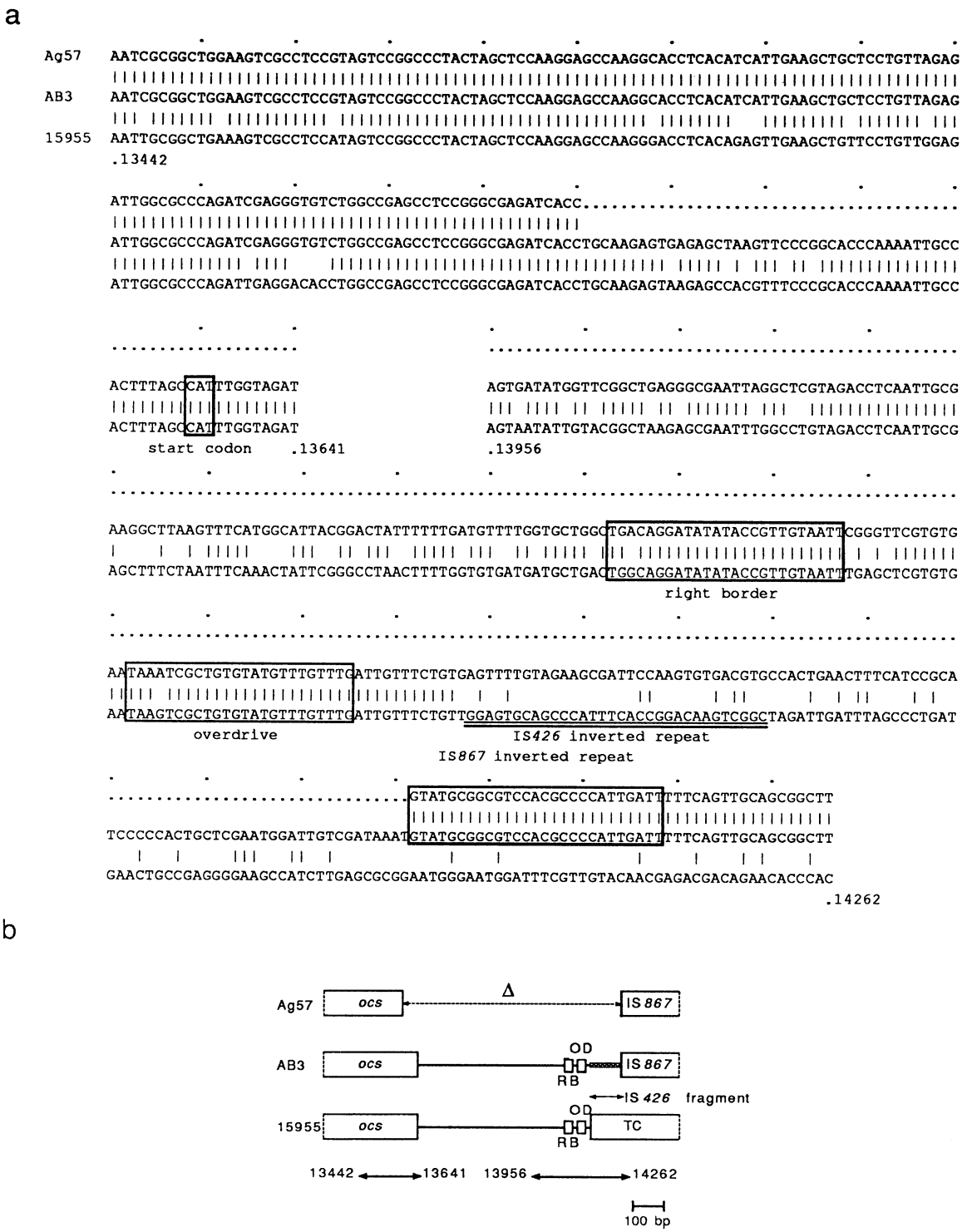

Fig. 3. A, Sequences of the right TA border regions of pTiAg57, pTiAB3, and pTi15995. The upper line shows the pTiAg57 sequence. The dotted line indicates the deleted region. The middle line shows the pTiAB3 sequence. The lower line indicates the pTi15955 sequence, with numbers indicating the published coordinates (Barker *et al.* 1983). The most important features are boxed. **B**, Summary of TL right border region structures of pTiAg15955 and TA right border region structures of pTiAB3 and pTiAg57. Coordinates are from Barker *et al.* (1983). RB, right border repeat; OD, *overdrive*; and dotted heavy line, AB3-specific sequence.

ment to the left and a 2.9-kb *Hin*dIII-*Bam*HI fragment to the right. Recombination between the left and right fragments of pPM3118 with the corresponding regions in pTiAg57 yielded a pTiAg57 plasmid in which the 2.9- and 0.3-kb *Hin*dIII fragments were deleted (for the extent of the deletion, see Fig. 1A). The resulting strain, C58C9-(pPM3119), was less virulent on *N. rustica* than the control strain C58C9(pTiAg57) (Fig. 5A, B), showing that the TA-region of pTiAg57 is required for full oncogenicity. In a second experiment, the role of the Ag57 TA-region was confirmed in a different way by testing the tumor-inducing activity of a pTiAg57 TB-*iaa* gene deletion mutant. This mutant was constructed by double recombination of pTiAg57 with the intermediate vector pPM4004, which carries a fragment of the pTiTm4 TB-region, in which a small internal deletion had been made to inactivate the TB-*iaaH* and TB-*iaaM* genes (Huss *et al.* 1990). The restriction map of this pTiTm4 fragment is identical with the map of the corresponding pTiAg57 fragment (Huss *et al.* 1989; this study). The pTiAg57 TB-*iaa* deletion mutant C58C9(pPM3197) still induced small callus growths on *N. rustica*, indicating that the TA-region with its gene 6b was transferred (Fig. 5C). The effect of C58C9(pPM3197) was compared with the effect of pTiAg57 gene 6b. To this end, the 2.5-kb *Eco*RI pTiAg57 TA fragment (Fig. 1A) was cloned into pGP100, yielding pPM40, which was inserted into pPM6000. pTiAg57 gene 6b induced the same small outgrowths on *N. rustica* (Fig. 5C) as pTiAB3 gene 6b (Fig. 2A). The pTiAg57 gene 6b-induced calli are also very similar to those induced by the pTiAg57 TB-*iaa* deletion mutant (Fig. 5C), supporting the assumption that the residual effect of the TB-*iaa* deletion mutant results from transfer of gene 6b of the TA-region.

Finally, on *N. rustica* the oncogenicity of the pTiAg57 TA deletion mutant could be restored to the wild-type level by coinfection with the pTiAg57 TB-*iaa* mutant or with pTiAg57 gene 6b (results not shown), again indicating that gene 6b is transferred.

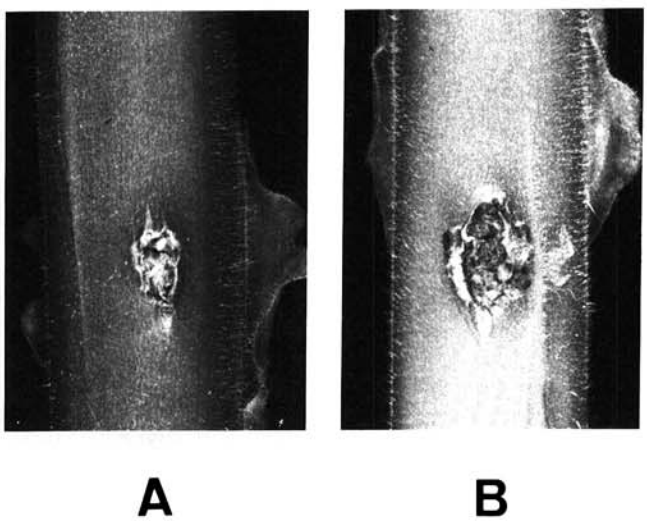

Fig. 4. Restoration of oncogenicity of C58C9(pTiAg57) on *Nicotiana tabacum* stems by addition of an AB3 right TA border sequence to the pTiAg57 TA-region. **A**, C58C9(pTiAg57). **B**, C58C9(pTiAg57::pPM3125), carrying the AB3 right TA border. Stems are shown 2 months after infection.

DISCUSSION

Sequence analysis of the biotype III octopine/cucumopine pTiAB3 TA-region shows that this T-region has left and right border repeats and an *overdrive* sequence which are very similar to the biotype I octopine pTi15955 TL border sequences. This suggests that the TA- and TL-regions are derived from the same ancestor T-region and are transferred by very similar T-region transfer systems. The *overdrive* sequence is required for efficient T-strand production (Van Haaren *et al.* 1988; Toro *et al.* 1988) and interacts most probably with the VirC1 protein. Yanofsky *et al.* (1985b) noted that the AB3-related strain Ag162 does not have a functional *virC* locus. Further studies should establish the role of the *overdrive* sequence in pTiAB3 and its possible

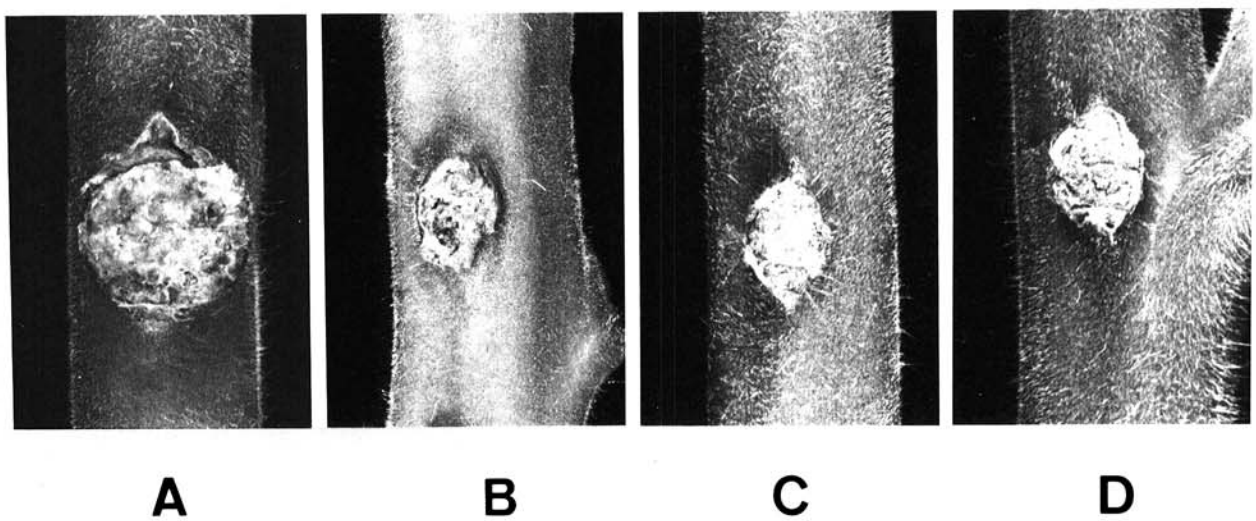

Fig. 5. Oncogenicity of C58C9(pTiAg57) and different mutants on *Nicotiana rustica*. **A**, C58C9(pTiAg57). **B**, pTiAg57 TA deletion mutant C58C9(pPM3119). **C**, pTiAg57 TB-*iaa* deletion mutant C58C9(pPM3197). **D**, GV3101(pPM6000::pPM40), carrying gene 6b of pTiAg57. Stems are shown 2 months after infection.

interaction with Vir proteins. The AB3 and 15955 sequences diverge 12 bp to the right of the *overdrive* sequence. In pTi15955 this position marks the start of the inverted repeat of the IS*426* insertion sequence fragment, which is part of a composite transposon in the 1.9-kb TC-region between the TL- and TR-regions (De Meirsman *et al.* 1987). In AB3, the *overdrive* is followed, at a distance of 96 bp, by the inverted repeat of the 2.7-kb IS*867* element (unrelated to IS*426*). Thus, in both octopine Ti plasmids, insertion sequences are found close to the right border of the T-regions. Studies of other border regions may establish whether this is a coincidence or whether T-region borders constitute "hot spots" for IS elements. The TA-region of the limited host range strain Ag57 resembles the TA-region of AB3, but a deletion has removed the right border sequences of the TA-region. The left end of the deletion is situated in the octopine synthase gene, the right end at the start of the left inverted repeat of the TA-associated IS*867* element. This suggests that the deletion resulted from recombination between two IS*867* copies, the one found to the right of the right TA border and another one inserted into the *ocs* gene. The TA-associated IS*867* element of pTiAg57 is somewhat smaller than the IS*867* copies from the pTiTm4 and pTiAB3 TA-regions and those of the pTiTm4, pTiAB3, and pTiAg57 TB-regions (Bonnard *et al.* 1989b; Paulus *et al.* 1989b), possibly due to the proposed recombination event. The right border deletion occurred very recently, as shown by the fact that the common pTiAB3 and pTiAg57 fragments which were sequenced (225 nucleotides in the right TA border region, this study) and an additional 1,156 nucleotides in the TA *ipt* region (F. Paulus, unpublished) are identical. In biotype I Ti plasmids, the right border repeat and the *overdrive* sequence are essential (Wang *et al.* 1984, 1987; Peralta and Ream 1985; Peralta *et al.* 1986; Toro *et al.* 1988). Since the analysis of limited host range Ti mutants (Yanofsky *et al.* 1985a; our results) indicated an important role for the TA-region in tumor induction, we expected that the lack of a right TA border region in pTiAg57 would affect oncogenicity.

Tumor induction by AB3 results from the combined activity of gene 6b of the TA-region and the *iaa* genes of the TB-region. These results confirm and extend studies with pTiAg162 (Yanofsky *et al.* 1985a) showing that the TA- and TB-regions have oncogenic properties and act synergistically. The precise mechanism by which gene 6b itself induces plant cell growth and enhances the effect of *iaa* genes is unknown. It also remains to be established whether gene 6b induces indefinite, tumorous growth or whether the growth of gene 6b-induced tissues is self-limiting. Preliminary experiments with gene 6b-induced calli indicate that these tissues do not grow in a hormone-independent way.

pTiAB3 induces large tumors on *N. tabacum*; pTiAg57 does not. Introduction of the right TA border of pTiAB3 into pTiAg57 restores tumor induction. Thus, the right TA border deletion of pTiAg57 strongly reduces transfer of TA-located gene 6b into *N. tabacum* cells.

N. rustica and *V. vinifera* respond to pTiAg57 by the formation of large tumors. This may be explained if (contrary to pTiAB3) pTiAg57 would not need its gene 6b for tumor induction on these plants. However, several lines of evidence show that the pTiAg57 TA-region is essential for tumor induction on *N. rustica* and must, therefore, be transferred even in the absence of normal right border sequences (border repeat and *overdrive*).

First, a pTiAg57 TA deletion mutant induces smaller tumors on *N. rustica* than the wild-type Ti plasmid and can be complemented by a strain carrying pTiAg57 gene 6b. Second, a pTiAg57 TB-*iaa* deletion mutant induces small, 6b-like outgrowths on *N. rustica* and is able to complement, by mixed infection, the TA deletion mutant.

On *N. rustica* and *V. vinifera*, the TA-region of pTiAg57 may be transferred by another, cryptic border sequence. Pseudoborders have been described in the biotype I octopine T-region, but they were very inefficient (Van Haaren *et al.* 1988). A deletion of the right border of the nopaline Ti plasmid pTiT37 did not reduce tumorigenicity on *Linum usitatissimum* L. In the latter case it was proposed that a pseudoborder in the 3′ untranslated region of the *ipt* locus was used (Hepburn and White 1985). This region is part of the gene 6a deletion characteristic for octopine/cucumopine strains like AB3 and Ag57. In Ag57, a pseudoborder could be situated to the left of gene 6b, between the right of gene 6b and the deleted area, or to the right of the deleted area. In the case of the biotype I octopine strain B6S3, a TL right border deletion mutant was found to be virulent, possibly due to the use of the right TR border, or to the use of a pseudoborder situated to the left of the deleted region (Leemans *et al.* 1981). Studies on pTiA6 T-DNA intermediates have shown the formation of large, 25-kb T-strands that extended from the right border of the TR-region to the left border of the TL-region (Stachel *et al.* 1987; Veluthambi *et al.* 1988). If, in the case of pTiAg57, one of the TB-region borders is used, the T-DNA would be at least 35 kb long (Yanofsky *et al.* 1985a; F. Paulus, unpublished results). The observed differences between *N. tabacum* and *N. rustica* could then be due to differences in stability of the T-DNA intermediates in different plant species. In this respect it is interesting to note that octopine *virF* mutants are nearly avirulent on *N. tabacum*, but normally virulent on *N. rustica* (Hooykaas *et al.* 1984), an observation which might be explained by a lesser sensitivity of *N. rustica* cells to inefficient T-DNA transfer. Yanofsky *et al.* (1985a, working with Ag162) and Buchholz and Thomashow (1984a, working with Ag63) did not find DNA from the region between the TA- and TB-regions in *V. vinifera*, *N. glauca*, and *N. rustica* tumors. Further experiments should establish how the TA-region of pTiAg57 is transferred, for example by studying the formation of T-DNA intermediates in induced bacteria or by Southern analysis of Ag57-induced tumors. Our studies show that *Agrobacterium* host range may not only be determined by virulence genes and oncogenes, but also by T-region border structure. The natural deletion of a border sequence and its *overdrive* without apparent consequences for T-DNA transfer and tumor induction on *N. rustica* and *V. vinifera* shows the extraordinary flexibility of the Ti plasmid system.

ACKNOWLEDGMENTS

We thank P. Hooykaas for providing LBA649, C. Panagopoulos for Ag57, and E. Szegedi for AB3. B. Tinland and B. Huss were supported

by a doctoral grant from the French Ministry of Research and Technology, Paris, and A. Herrmann was supported by a grant from the European Community, Brussels.

LITERATURE CITED

Barker, R. F., Idler, K. B., Thompson, D. V., and Kemp, J. D. 1983. Nucleotide sequence of the T-region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Mol. Biol. 2:335-350.

Bonnard, G., Tinland, B., Paulus, F., Szegedi, E., and Otten, L. 1989a. Nucleotide sequence, evolutionary origin and biological role of a rearranged cytokinin gene isolated from a wide host range biotype III *Agrobacterium* strain. Mol. Gen. Genet. 216:428-438.

Bonnard, G., Vincent, F., and Otten, L. 1989b. Sequence and distribution of IS*866*, a novel T-region-associated insertion sequence from *Agrobacterium tumefaciens*. Plasmid 22:70-81.

Buchholz, W. G., and Thomashow, M. F. 1984a. Comparison of T-DNA oncogene complements of *Agrobacterium tumefaciens* tumor-inducing plasmids with limited and wide host ranges. J. Bacteriol. 160:319-326.

Buchholz, W. G., and Thomashow, M. F. 1984b. Host range encoded by *Agrobacterium tumefaciens* tumor-inducing plasmid pTiAg63 can be expanded by modification of its T-DNA oncogene complement. J. Bacteriol. 160:327-332.

De Meirsman, C., Desair, J., Vanderleyden, J., Van Gol, J., and Jen, C. G. 1987. Similarities between nucleotide sequences of insertion elements of *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* in relation to *Agrobacterium tumefaciens* TC-DNA. Nucleic Acids Res. 15:10591.

Devereux, J., Haeberli, P., and Marquess, P. 1987. The program manual for the sequence analysis software package of the Genetics Computer Group. Nucleic Acids Res. 12:387-395.

Dhaese, P., De Greve, H., Decraemer, H., Schell, J., and van Montagu, M. 1979. Rapid mapping of transposon insertion and deletion mutants in the large Ti-plasmids of *Agrobacterium tumefaciens*. Nucleic Acids Res. 7:1837-1849.

Hepburn, A. G., and White, J. 1985. The effect of right terminal repeat deletion on the oncogenicity of the T-region of pTiT37. Plant Mol. Biol. 5:3-11.

Hoekema, A., de Pater, B. S., Fellinger, A. J., Hooykaas, P. J. J., and Schilperoort, R. A. 1984. The limited host range of an *Agrobacterium tumefaciens* strain extended by a cytokinin gene from a wide host range T-region. EMBO J. 3:3043-3047.

Hooykaas, P. J. J., and Schilperoort, R. A. 1984. The molecular genetics of crown gall. Adv. Genet. 22:209-283.

Hooykaas, P. J. J., Hofker, M., den Dulk-Ras, H., and Schilperoort, R. A. 1984. A comparison of virulence determinants in an octopine Ti-plasmid, a nopaline Ti-plasmid and an Ri-plasmid by complementation analysis of *Agrobacterium tumefaciens* mutants. Plasmid 11:195-205.

Hooykaas, P. J. J., den Dulk-Ras, H., and Schilperoort, R. A. 1988. The *Agrobacterium tumefaciens* T-DNA gene 6b is an *onc* gene. Plant Mol. Biol. 11:791-794.

Huss, B., Bonnard, G., and Otten, L. 1989. Isolation and functional analysis of a set of auxin genes with low root-inducing activity from an *Agrobacterium tumefaciens* biotype III strain. Plant Mol. Biol. 12:271-283.

Huss, B., Tinland, B., Paulus, F., Walter, B., and Otten, L. 1990. Functional analysis of a complex oncogene arrangement in biotype III *Agrobacterium tumefaciens* strains. Plant Mol. Biol. 14:173-186.

Knauf, V. C., Panagopoulos, C. G., and Nester, E. W. 1982. Genetic factors controlling the host range of *Agrobacterium tumefaciens*. Phytopathology 72:1545-1549.

Knauf, V. C., Yanofsky, M., Montoya, A., and Nester, E. W. 1984. Physical and functional map of an *Agrobacterium tumefaciens* plasmid that confers a narrow host range. J. Bacteriol. 160:564-568.

Leemans, J., Shaw, C., Deblaere, R., De Greve, H., Hernalsteens, J.-P., Maes, M., Van Montagu, M., and Schell, J. 1981. Site-specific mutagenesis of *Agrobacterium* Ti plasmids and transfer of genes to plant cells. J. Mol. Appl. Genet. 1:149-164.

Leemans, J., Hernalsteens, J.-P., Deblaere, R., De Greve, H., Thia-toong, L., van Montagu, M., and Schell, J. 1983. Genetic analysis of T-DNA and regeneration of transformed plants. Pages 322-330 in: Molecular Genetics of the Bacteria-Plant Interaction. A. Pühler, ed. Springer, Berlin.

Nester, E. W., Gordon, M. P., Amasino, R. M., and Yanofsky, M. F. 1984. Crown gall: A molecular and physiological analysis. Annu. Rev. Plant Physiol. 35:387-413.

Otten, L., Piotrowiak, G., Hooykaas, P., Dubois, M., Szegedi, E., and Schell, J. 1985. Identification of an *Agrobacterium tumefaciens* pTiB6S3 *vir* region fragment that enhances the virulence of pTiC58. Mol. Gen. Genet. 199:189-193.

Panagopoulos, C. G., and Psallidas, P. G. 1973. Characteristics of Greek isolates of *Agrobacterium tumefaciens*. J. Appl. Bacteriol. 36:233-240.

Paulus, F., Huss, B., Bonnard, G., Ridé, M., Szegedi, E., Tempé, J., Petit, A., and Otten, L. 1989a. Molecular systematics of biotype III Ti plasmids of *Agrobacterium tumefaciens*. Mol. Plant-Microbe Interact. 2:64-74.

Paulus, F., Ridé, M., and Otten, L. 1989b. Distribution of two *Agrobacterium tumefaciens* insertion elements in natural isolates: Evidence for stable association between Ti plasmids and their bacterial hosts. Mol. Gen. Genet. 219:145-152.

Peralta, E. G., and Ream, L. W. 1985. T-DNA border sequences required for crown gall tumorigenesis. Proc. Natl. Acad. Sci. USA 82:5112-5116.

Peralta, E. G., Hellmiss, R., and Ream, W. 1986. *Overdrive*, a T-DNA transmission enhancer on the *A. tumefaciens* tumour-inducing plasmid. EMBO J. 5:1137-1142.

Rao, R. N., and Rogers, S. G. 1979. Plasmid pKC7: A vector containing ten restriction endonuclease sites suitable for cloning DNA segments. Gene 7:79-82.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Stachel, S., Timmerman, B., and Zambryski, P. 1987. Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: Requirement for 5′ *virD* gene products. EMBO J. 6:857-863.

Szegedi, E. 1985. Host range and specific L(+)tartrate utilization of biotype 3 of *Agrobacterium tumefaciens*. Acta Phytopathol. Acad. Sci. Hung. 20:17-22.

Thomashow, M. F., Panagopoulus, C. G., Gordon, M. P., and Nester, E. W. 1980. Host range of *Agrobacterium tumefaciens* is determined by the Ti plasmid. Nature (London) 283:794-796.

Thomashow, M. F., Knauf, V. C., and Nester, E. W. 1981. Relationship between the limited and wide host range octopine-type Ti plasmids of *Agrobacterium tumefaciens*. J. Bacteriol. 146:484-493.

Tinland, B., Huss, B., Paulus, F., Bonnard, G., and Otten, L. 1989. *Agrobacterium tumefaciens 6b* genes are strain-specific and affect the activity of auxin as well as cytokinin genes. Mol. Gen. Genet. 219:217-224.

Toro, N., Datta, A., Yanofsky, M. F., and Nester, E. W. 1988. Role of the *overdrive* sequence in T-DNA border cleavage in *Agrobacterium*. Proc. Natl. Acad. Sci. USA 85:8558-8562.

van Haaren, M. J. J., Sedee, N. J. A., Krul, M., Schilperoort, R. A., and Hooykaas, P. J. J. 1988. Function of heterologous and pseudo border repeats in T region transfer via the octopine virulence system of *Agrobacterium tumefaciens*. Plant Mol. Biol. 11:773-781.

Van Haute, E., Joos, H., Maes, M., Warren, E., van Montagu, M., and Schell, J. 1983. Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: A novel strategy for the reversed genetics of Ti plasmids of *Agrobacterium tumefaciens*. EMBO J. 2:411-417.

Veluthambi, L., Ream, W., and Gelvin, S. B. 1988. Virulence genes, borders and *overdrive* generate single-stranded T-DNA molecules from the A6 Ti plasmid of *Agrobacterium tumefaciens*. J. Bacteriol. 170:1523-1532.

Wang, K., Herrera-Estrella, L., van Montagu, M., and Zambryski, P. 1984. Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. Cell 38:455-462.

Wang, K., Genetello, C., van Montagu, M., and Zambryski, P. 1987. Sequence context of the T-DNA border repeat element determines its relative activity during T-DNA transfer to plant cells. Mol. Gen. Genet. 210:338-346.

Yanofsky, M., Montoya, A., Knauf, V., Lowe, B., Gordon, M. P., and

Nester, E. W. 1985a. Limited-host-range plasmid of *Agrobacterium tumefaciens*: Molecular and genetic analysis of transferred DNA. J. Bacteriol. 163:341-348.

Yanofsky, M., Lowe, B., Montoya, A., Rubin, R., Krul, W., Gordon, M. P., and Nester, E. W. 1985b. Molecular and genetic analysis of factors controlling host range in *Agrobacterium tumefaciens*. Mol. Gen. Genet. 201:237-246.

Zambryski, P., Tempé, J., and Schell, J. 1989. Transfer and function of T-DNA genes from *Agrobacterium* Ti and Ri plasmids in plants. Cell 56:193-201.