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Construction and Application of R Prime Plasmids, Carrying Different Segments of an Octopine Ti Plasmid from *Agrobacterium tumefaciens*, for Complementation of *vir* Genes

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Several R prime plasmids have been obtained with high efficiency, by enclosing the R plasmid replicator, in an R::Ti cointegrate plasmid, between two copies of the transposon Tn1831, in the same orientation. These R primes carry different segments of an octopine Ti plasmid, and are compatible with Ti plasmids. They were used to study genetic complementation of Ti plasmid insertion mutants, outside the T-DNA region, which affected oncogenicity. Complementation was observed in both recombination-proficient and -deficient strains. The complementation *in trans* indicates that certain functions essential for tumor formation outside the T-DNA region are probably expressed in the bacterium. Therefore, the authors proposed to make a distinction between virulence (Vir) functions and oncogenic (Onc) functions of the octopine Ti plasmid of *Agrobacterium tumefaciens*. A large R prime was obtained, carrying the whole Ti plasmid, except a 7-Mdalton segment, containing the Ti plasmid replicator region. Strains harboring this plasmid induced normal tumors, showing that the replicator region of the octopine Ti plasmid is dispensible for tumor induction.

The plant tumor crown gall is caused by *Agrobacterium tumefaciens*. After wounding and subsequent infection, tumors can be induced on most dicotyledons. A large plasmid in *Agrobacterium* strains, the Ti plasmid, is essential for tumor formation (Van Larebeke *et al.*, 1974; Watson *et al.*, 1975; Zaenen *et al.*, 1974). Part of this plasmid, the T-DNA, is found to be stably integrated in the nuclear DNA of the tumor cell (Chilton *et al.*, 1977; Chilton *et al.*, 1980; Thomashow *et al.*, 1980; Wilmitzer *et al.*, 1980), and at least partly transcribed in RNA (Drummond *et al.*, 1977; Ledeboer, 1978; Gurley *et al.*, 1979; Willmitzer *et al.*, 1981). In addition, the Ti plasmid carries genes that specify the synthesis of tumor-specific compounds, so-called opines, in the tumor cell, and genes for the utilization of these compounds by the bacterium (Bomhoff *et al.*, 1976; Montoya *et al.*, 1977). It has been shown that the genetic information for the enzyme lysopinedehydrogenase (LpDH), required in the plant tumor cell for the synthesis of the opine octopine, is located on the

T-DNA (Schröder *et al.*, 1981). Thus, the prokaryote *Agrobacterium tumefaciens* represents a naturally occurring genetic engineering system for eukaryotic plants.

A physical and a gross genetic map of an octopine Ti plasmid has been constructed (Chilton *et al.*, 1978; Koekman *et al.*, 1979). It has been demonstrated that much of the right half of the Ti plasmid can be deleted, without altering oncogenicity. Even part of the T-DNA region can be deleted, but deleting the complete T-DNA region results in the loss of oncogenicity.

More recently, transposon mutagenesis was used to determine the location of functions on this Ti plasmid. Transposon insertions into the octopine Ti plasmid, close to the origin of replication, which is located in *HpaI* fragment 11 (Koekman *et al.*, 1980) have a strong effect on the oncogenicity of strains harboring these plasmids (Hooykaas, 1979b; Ooms *et al.*, 1981). Such strains only induce small tumors on tomato and kalanchoë. This implicates a possible role for the replicator region in tumorigenesis. Nonon-

cogenic mutants have been described with Tn904 (encoding resistance to streptomycin) and Tn5 (encoding resistance to kanamycin) insertions into the octopine Ti plasmid (Klapwijk *et al.*, 1980; Ooms *et al.*, 1980; Garfinkel *et al.*, 1980). These mutants have revealed a region on the left half of the Ti plasmid, outside the T-DNA region, which is essential for tumor induction. This region has never been found in plant tumor cells (Chilton *et al.*, 1977; Thomashow *et al.*, 1980b). Therefore, it is attractive to assume that this part of the Ti plasmid is expressed in the bacterium, and necessary, in whatever way, for tumorigenesis. If it is, in fact, expressed in the bacterium it should be possible to complement, *in trans*, an inactivated gene in that region, by the corresponding wild-type gene.

To perform such complementation studies, and to analyze, whether the replicator region of the Ti plasmid is essential for tumor induction, we cloned parts of the Ti plasmid on an Inc-P1 type R plasmid, which is compatible with Ti plasmids. To this end, we used a procedure by which it is possible to clone *in vivo* large segments of plasmid DNA on R plasmids (Hille and Schilperoort, 1981). It is based on the observation that deletions will occur between two directly repeated transposons, when both are temporarily present in one plasmid molecule. Data on the construction and the use of the R primes in complementation studies are described in the present paper.

MATERIALS AND METHODS

Bacteria. Bacterial strains are listed in Table 1. Bacteria were cultured at 29°C.

Media. Rich medium (TY) contained per liter: 5 g tryptone (Difco) and 3 g yeast extract. Bromothymol blue indicator medium was as described by Hooykaas *et al.* (1979a). Media were solidified with 1.8% (w/v) Bacto agar (Difco).

Conjugation conditions. Strains, grown overnight on TY medium, were diluted 10-fold in the same medium, and incubated for

a further 4 h. Donor and recipient bacteria were mixed 1:1; then 100 μ l was spotted on a nitrocellulose membrane filter (0.45- μ m pore size). Filters were incubated for 20 h on TY, after which the bacteria were resuspended in 2 ml 0.9% NaCl. Appropriate dilutions were plated on selective medium.

Selection of transconjugants. For selection, bacteria were plated on TY, to which the required antibiotics were added. In *Agrobacterium* the following final concentrations of antibiotics were used (μ g/ml): kanamycin, 50; carbenicillin, 100; spectinomycin, 250; streptomycin, 500; rifampicin, 20; gentamicin, 40.

Plasmid detection. This was essentially performed as described by Casse *et al.* (1979).

Plasmid isolation and restriction enzyme analysis. These were done as described by Koekman *et al.* (1980).

Tests for tumor induction. A sterile wooden toothpick was dipped in a colony of the strain to be tested, and then used to puncture the stem of tomato or sunflower. On kalanchoë stems, overnight cultures in TY were inoculated, 24 h after wounding, as described by Bomhoff *et al.* (1976).

RESULTS

Construction of R Prime Plasmids

To obtain R prime plasmids, we made use of a stable cointegrate plasmid, consisting of R772 and an octopine Ti plasmid. This cointegrate plasmid, pAL969, harbors all the genetic markers of R772 and of the Ti plasmid, and still confers oncogenicity to its host. The site of cointegration was determined on the physical map of the Ti plasmid (G. Ooms, personal communication, visualized in Fig. 1).

Previously, we demonstrated that deletions may occur when two directly repeated identical transposons are temporarily present in a plasmid molecule (Hille and Schilperoort, 1981). In the case of a cointegrate plasmid, carrying two functional replicators,

TABLE I
BACTERIAL STRAINS

Strain (LBA No.) ^a	Chromosomal markers	Plasmid(s)	Relevant plasmid markers						Source
			Sp	Sm	Km	Cb	Occ	Oncogenicity	
677	Rif, Nal	pTi	s	s	s	s	+	+	Hooykaas (1979b)
937	—	pTi, R772	s	s	r	s	+	+	Hooykaas (1979b)
949	Nal, Str	pAL116	r	r	r	s	+	—	Klapwijk (1979b)
973	Gen, Nov	pAL969	s	s	r	s	+	+	Hooykaas (1979b)
1509	Rif, Nal	pAL1509	r	r	s	s	+	+/-	Ooms <i>et al.</i> (1981)
1666	Rif, Nal	pAL1666	r	r	s	r	+	+	Ooms <i>et al.</i> (1981)
1802	Rif, Nal	pAL1802	r	r	r	s	+	+	This study
1822	Rif, Nal	pAL1822	r	r	r	s	+	+	This study
1823	Rif, Nal	pAL1823	r	r	r	s	+	+	This study
1824	Rif, Nal	pAL1824	r	r	r	s	+	+	This study
1825	Rif, Nal	pTi, pAL1820	r	r	r	r	+	+	This study
2228	Gen, Nov	—	s	s	s	s	—	—	Hooykaas (1979b)
4219	Rif	pAL237	s	r	s	s	+	—	Klapwijk <i>et al.</i> (1980)
4226	Rif	pAL243	s	r	s	s	+	—	Klapwijk <i>et al.</i> (1980)
4228 ^b	Rif	pAL245	s	r	s	s	+	—	Klapwijk <i>et al.</i> (1980)
4232	Rif	pAL248	s	r	s	s	+	—	Klapwijk <i>et al.</i> (1980)
4301	Rif, Rec	—	s	s	s	s	—	—	Klapwijk <i>et al.</i> (1979a)
4312	Rif, Rec	pTi	s	s	s	s	+	+	Klapwijk <i>et al.</i> (1979a)

Note. Abbreviations: Cb, carbenicillin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Gen, gentamicin; Nal, nalidixic acid; Nov, novobiocin; Rif, rifampycin; Str, streptomycin; Rec, recombination deficient; s, sensitive; r, resistant.

^a The 4000 numbers have an Ach5 background; all the others a C58 background. These two different genetic backgrounds each have a different restriction modification system.

^b This strain is weakly oncogenic on tomato and kalanchoë, but induces normal tumors on petunia.

it was shown that by enclosing one of the replicators between two copies of the transposon Tn1831 (carrying determinants for resistance against spectinomycin, streptomycin, and mercury chloride), *in vivo* cloning is possible. We have employed this approach to construct different R prime plasmids. First, a copy of Tn1831 was introduced at a fixed position into the coin-tegrate plasmid pAL969. Then, a second copy of Tn1831 was inserted into pAL969::Tn1831, at such a position that the R772 replicator was enclosed between the two transposons.

In order to obtain derivatives of pAL969, with Tn1831 inserted at a fixed position, strain LBA973, harboring pAL969, was crossed with different recipient strains carrying Ti::Tn1831 plasmids in which Tn1831 was inserted at different positions into

the Ti plasmid. Plasmids pAL969 and Ti::Tn1831 are incompatible and it was found that introduction of pAL969 into a strain with Ti::Tn1831 leads to the loss of Ti::Tn1831 (Hooykaas, 1979b). However, before loss of Ti::Tn1831, homologous recombination may occur between these plasmids. Therefore, after crossing, we selected for the presence of pAL969 in the recipient, and screened for recombinants that had gained the transposon Tn1831. The various plasmids, constructed in this way, are shown in Fig. 1. They are all the same as plasmid pAL969, but in addition contain a copy of Tn1831 at a fixed position.

Figure 2 gives a scheme for the construction of the R prime plasmids. Various donor strains carrying pAL969::Tn1831 were crossed with a recipient carrying a Ti::Tn1831 plasmid with Tn1831 inserted at

FIG. 1. Organization of cointegrate plasmids used in the experiments. The physical map for the restriction enzyme *Hpa*I is presented for the Ti part of this plasmid. The R part is represented by the open triangle (a physical map of this plasmid, R772, will be published separately). Plasmid pAL969 is the original plasmid; pAL1802, pAL1822, pAL1823, and pAL1824 carry, in addition, a Tn1831 insertion, the position of which is indicated on the map with a black arrow.

a different position compared to the cointegrate plasmid. The Ti::Tn1831 plasmid of the recipient also contained a carbenicillin resistance marker located close to the position of the Tn1831 insertion. It was assumed that, when recombination occurred and the Cb resistance marker was inserted into the plasmid of the donor, the chance of a recombination event, whereby Tn1831 and the Cb resistance marker remained linked, would be high. Figure 2c visualizes the hypothetical intermediate in which Tn1831 is present twice, enclosing the *ori* of the R plasmid. This intermediate is assumed to dissociate

into two circular DNA molecules, each carrying a functional replicator, one being an R prime, the other a deleted Ti plasmid. Table 2 shows the crosses that were performed to obtain R primes, and in Fig. 2d the structure of these R primes is visualized.

R plasmids, as well as the described cointegrate plasmids, are conjugative on a rich medium. This is in contrast to Ti plasmids, for which it is necessary to induce the *tra* genes with octopine in a minimal medium. Transconjugants, obtained in the crosses described in Table 2, were further crossed on rich medium with an *Agrobacterium* strain,

FIG. 2. Scheme for the construction of R prime plasmids. LBA1802 (pAL1802) was crossed on rich medium with LBA1666 (pAL1666). Under these conditions only the cointegrate plasmid (pAL1802) is transferable. Homologous recombination between the two plasmids can occur. Selection, after crossing, was for $Cb^r Km^r$. Transconjugants obtained have originated from the donor (Km^r) and from the recipient (Cb^r), and must thus be recombinant plasmids. It was assumed that if Cb^r was introduced into the cointegrate plasmid the chance that Cb^r and the $Tn1831$ insertion (in pAL1666) would remain linked in recombination, would be high. The hypothetical intermediate, a cointegrate plasmid with two copies of $Tn1831$, is visualized in (c). Recombination between two directly repeated copies of $Tn1831$, will subsequently dissociate the intermediate into two plasmids, each carrying a functional replicator. The R prime plasmid pAL1813 was purified by crossing on rich medium with LBA2228 (containing no Ti plasmid). Like pAL1802, the other cointegrate plasmids, described in Fig. 1, were used to obtain R primes. In (d) a number of R primes are shown, obtained by following this procedure. Black arrows show the position of a $Tn1831$ insertion. The small arrow indicates the position of the carbenicillin resistance marker. The stripes in the circle represent the R772 part of the (R772::Ti) cointegrate plasmid.

cured of its Ti plasmid (LBA2228). From this cross it could be seen whether the whole cointegrate or an R prime plasmid was transferred. These two possibilities can be distinguished because the R prime will not carry *occ* genes, for octopine catabolism, as can be seen for pAL1813, pAL1818, and pAL1819 in Fig. 2. In the case of the R prime pAL1820, a discrimination between both types of plasmids on Occ phenotype was

not possible, because also this R prime was expected to carry the corresponding genes. Here, the only way to determine whether an R prime was constructed indeed was to test whether the plasmid obtained was compatible with Ti plasmids. The problem then was that no genetic markers were available to determine whether both plasmids stably coexisted in one cell. However, the R prime and the Ti plasmid were supposed to have

TABLE 2
CROSSES AND TRANSCONJUGANTS OBTAINED

Donor		Recipient		No. of Cb ^r Km ^r transconjugants used as donor in a further cross with LBA2228 ^a	No. of strains carrying an <i>in vivo</i> -constructed, Ti ori-lacking, R prime plasmid	Collection No. of mutant Ti plasmid
Strain	Ti plasmid	Strain	Ti plasmid			
LBA1802	pAL1802	LBA1666	pAL1666	12	4	pAL1813
LBA1822	pAL1822	LBA1666	pAL1666	18	6	pAL1818
LBA1823	pAL1823	LBA1666	pAL1666	24	10	pAL1819
LBA1824	pAL1824	LBA1666	pAL1666	22	2	pAL1820

^a Cb^rKm^r transconjugants, obtained from the crosses described, were grown in rich medium and subsequently crossed with LBA2228 (Gen^r, cured of its Ti plasmid). From these transconjugants, the Occ phenotype was tested. For the detection of pAL1820, see the text.

different molecular weights, and therefore the problem could be solved biochemically. Transconjugants, expected to carry the R prime, were crossed with an *Agrobacterium* strain carrying a Ti plasmid (LBA677). According to the procedure of Casse (Casse *et al.*, 1979), plasmid DNA was isolated from several of the transconjugants obtained and analyzed on an agarose gel. In Fig. 3 an example of such a gel is shown. It reveals that the R prime pAL1820 is compatible with a Ti plasmid (like in LBA1825). Purified pAL1820 DNA was digested with the restriction endonuclease *Hpa*I and electrophoresed on an agarose gel (see Fig. 4). It has been shown that the origin of replication and incompatibility functions of the Ti plasmid are located on *Hpa*I fragment 11 (Koekman *et al.*, 1980). This fragment was absent in plasmid pAL1820, which unequivocally demonstrated that pAL1820 had lost the Ti plasmid replicator region.

Complementation Studies

The assumption that mutations in a region on the left half of the Ti plasmid, which in addition to T-DNA is essential for oncogenicity, might be complemented, was verified by using the constructed R prime plasmids (pAL1813 and pAL1818). These R primes did not confer oncogenicity to their host, as expected, because they do not contain the T-DNA region. Certain Ti::Tn904 plasmids

did neither, because Tn904 was inserted in the mentioned region (Ooms *et al.*, 1980). In Fig. 5 the positions of these Tn904 insertions are indicated. Crosses were performed to obtain strains carrying a Ti::Tn904 as well as an R prime plasmid, which made these bacteria diploid for genes located on the left half of the Ti plasmid. Table 3 shows results of plant tests of strains harboring the different combinations of plasmids. The IncP-1 type plasmid R772 itself did not influence oncogenicity, and neither did pAL116 (Klapwijk, 1979), an R prime plasmid containing the right part of the Ti plasmid (Fig. 5). Only when, respectively, pAL1813 or pAL1818 were present, in addition to the mutated Ti plasmid, did the strains become tumorigenic. This strongly suggests that complementation did occur.

Initially, these experiments were performed in *Rec*⁺ strains. To avoid problems due to the *Rec* system of the host, which could interfere with true complementation, the different plasmids were transferred, by conjugation, to a *Rec*⁻ *Agrobacterium* strain (LBA4301), and again tested for tumor induction (see Fig. 6). Results obtained were essentially the same as in the *Rec*⁺ situation.

The Replicator Region

We were able now to test the question, whether the replicator region of the Ti plasmid is required for tumor induction. The

FIG. 3. Agarose gel electrophoresis of native plasmid DNA.

Lane	Strain	Plasmids
1	LBA937	cryptic, pTi, R772
2	LBA1820	cryptic, pAL1820
3	LBA1825	cryptic, pAL1820, pTi

(a) Cryptic plasmid (± 300 Mdaltan); (b) pAL1820 (165 Mdaltan); (c) Ti plasmid (121 Mdaltan); (d) R772 (38 Mdaltan); (e) chromosomal DNA. It is obvious that plasmid pAL1820 is compatible with a Ti plasmid (lane 3), and that it contains additional DNA compared to R772 (lane 1).

largest R prime plasmid, pAL1820, only lacks a 7-Mdaltan fragment of the Ti plasmid containing the Ti replicator region (Fig. 5). A strain carrying pAL1820 was tested for tumor induction on kalanchoë, tomato, and sunflower, and in all cases was found to induce tumors of normal size and morphology. Different plant species were tested, since it was found earlier that the oncogenicity of Ti plasmid mutants varied for various species (Ooms *et al.*, 1981).

DISCUSSION

It was possible to clone large segments of the Ti plasmid on the wide host range plas-

mid R772. The *in vivo* cloning was very efficient, because we could directly select for recombinant plasmids in which the cointegrate plasmid of the donor had received the Cb^r marker of the Ti plasmid of the recipient.

The R prime plasmids obtained were compatible with Ti plasmids; pAL1813 and pAL1818 were used for genetic complemen-

FIG. 4. Fragmentation pattern of the R prime plasmid pAL1820. The plasmids pTi, pAL1820, and R772 were digested with *HpaI*, followed by agarose gel electrophoresis (respectively, lanes 1, 2, and 3). The Ti plasmid replicator is located on *HpaI* fragment 11 (Koekman *et al.*, 1980b); this fragment is absent in pAL1820.

FIG. 5. Map of Ti::Tn904 and R prime plasmids, used in complementation studies. The inner circles represent the physical map for the restriction endonucleases *HpaI* and *SmaI* of the octopine Ti plasmid (Chilton *et al.*, 1978). Arrows indicate the positions of Tn904 insertions in this plasmid, which cause loss of oncogenicity. The open triangle shows the position of R772 in the R prime plasmids pAL1813, pAL1818, pAL1819, and pAL1820, whereas the black triangle indicates the position of R702 in the R prime plasmid pAL116.

tation studies with Ti plasmids mutated in a region essential for tumor induction. Three Ti::Tn904 plasmids did not confer oncogenicity

to their host when tested on three different plants. The fourth Ti::Tn904 plasmid, pAL245, was somewhat different. Tumors

TABLE 3
COMPLEMENTATION STUDIES

Strain (LBA No.)	+R plasmid/ Ti plasmid	R772	pAL116	pAL1813	pAL1818
4301	—	—	—	—	—
4219	pAL237	—	—	+	+
4226	pAL243	—	—	+	+
4228	pAL245	—	—	—	+
4232	pAL248	—	—	+	+
4312	pTi	+	+	+	+

Note. The R plasmids were introduced, by conjugation, into strains containing a mutated or wild-type Ti plasmid. In the table the example of Rec⁺ strains is shown; the same combinations of plasmids were also tested in a Rec⁻ strain. Tumor induction was tested on tomato and kalanchoë.

+, Tumor induction; —, no tumor induction.

FIG. 6 Tumors induced on tomato stems. A Rec^- *Agrobacterium* strain, harboring the $\text{Ti}::\text{Tn904}$ plasmid pAL245, was tested for tumor induction. Then R772 and the R primes pAL1813, pAL1818, pAL1819, and pAL116 were introduced into this strain, and they were tested again (see also Table 3).

induced by LBA4228 (pAL245) were very small on tomato and kalanchoë, but normal on petunia. Strains carrying the R prime plasmids pAL1813 or pAL1818 were avirulent on all plants tested. This was expected because they do not carry the T-DNA region, but only parts of the region essential for tumor induction. Strains harboring the mentioned $\text{Ti}::\text{Tn904}$ plasmids as well as the R prime pAL1818 were in all cases oncogenic on all plants tested. The strain, carrying the combination of pAL245 and pAL1813, did not induce tumors on tomato and kalanchoë. This could be explained, however, because plasmid pAL245 contains two Tn904 insertions, both of which are not covered by pAL1813.

The possibility that strains carrying an R

prime and a mutated Ti plasmid become tumorigenic only when both plasmids have formed a cointegrate is rather unlikely. Small-scale plasmid DNA isolations, analyzed by agarose gel electrophoresis, did not show any cointegrate (results not shown), indicating that at most a small fraction of the bacteria may harbor such a cointegrate. Moreover, for wounds with a fixed size, tumor size is proportional to the amount of oncogenic bacteria used to induce the tumor (Schilperoort, 1969). If only a small amount of bacteria become oncogenic, one would expect rather small tumors. Furthermore, it is known that the presence of nononcogenic *Agrobacterium* inhibits tumor induction by the oncogenic bacteria (Lippincott and Lippincott, 1969; Schilperoort, 1969). So, if the

majority of the *Agrobacteria* were avirulent, tumor development would be negligible in our case. However, tumors of normal size were induced. The results were essentially the same in a recombination-proficient and in a recombination-deficient host. This showed that we were dealing with true complementation. In addition, it was demonstrated that an R prime plasmid, pAL116, carrying the right part of the Ti plasmid but not the region essential for tumor induction, could not complement such Ti::Tn904 plasmid mutations, showing that the complementation is region specific.

Garfinkel *et al.* (1980) described Ti::Tn5 mutants in the same region on the left part of the Ti plasmid, indicating that the region essential for tumor induction spans approximately 20 Mdaltan. Experiments are in progress to study genetic complementation of these Ti::Tn5 mutants using pAL1813 and pAL1818. Preliminary results show that these mutations can be complemented too.

The described 20-Mdaltan region of the octopine Ti plasmid, essential for tumor induction, was shown to be largely homologous to a region on nopaline Ti plasmids (Drummond and Chilton, 1978; Hepburn and Hindley, 1979), null type Ti plasmids (Drummond and Chilton, 1978), and to the virulence plasmid of *A. rhizogenes* (White and Nester, 1980). Parts of this region on the octopine Ti plasmid are also homologous to a *Rhizobium trifolii* plasmid, which carries *nif* genes (Prakash, 1981), and was found to be essential for root nodulation (Hooykaas *et al.*, 1981). Thus, this region seems to be essential for *Agrobacterium* to confer oncogenicity, and is conserved among plasmids of related bacteria. This all directs to some common function(s) for this region. Until now, this region has never been found in the plant tumor cell (Chilton *et al.*, 1977; Thomashow *et al.*, 1980b). This does not exclude the possibility, however, that it might have been present in the plant cell during the early stage of transformation, but is lost in later stages. Experiments are in progress to transform protoplasts with naked

Ti plasmid DNA, mutated in the described region. If transformation occurs, as with wild-type Ti plasmid DNA (F. Krens, personal communication), it would prove that this region does not have to function in the plant cell for tumor induction. In that case, the expression of this region in the bacterium would be essential in order to induce a tumor. We would like to suppose, that mutations in this region be referred to as virulence (Vir) mutations; this to distinguish them from oncogenic functions (Onc) on T-DNA, which is known to be expressed in the plant tumor cell (Ooms *et al.*, 1981; Willmitzer *et al.*, 1981). Current experiments are aimed at physically separating the T-DNA region and the Vir region on different compatible plasmids. This will give additional information on the role of the Vir region in tumor formation, and may result in interesting applications for genetic engineering in plants.

Since it was known that the right half of the Ti plasmid, and even part of the T-DNA region, could be deleted without loss of oncogenicity, the R prime plasmid pAL1819 was tested for tumor-inducing capacity. This R prime carries the intact Vir region and the leftmost part of the T-DNA region. Strains harboring this plasmid did not induce tumors on the different plant species tested (results not shown). Two possibilities could explain its nononcogenic character. First, Koekman *et al.* (1979) reported that part of the right end of the T-DNA region could be deleted, without losing oncogenicity completely. But in pAL1819 most of the T-DNA region is deleted and thereby essential functions for oncogenicity could have been lost. Second, the Ti plasmid replicator region might be necessary for tumor induction. In the R prime plasmid, pAL1819, this region has been deleted. To study the role of the replicator region in oncogenicity, an R prime was constructed, pAL1820, carrying almost the entire Ti plasmid, except for a 7-Mdaltan segment containing the Ti replicator region. It was found that this R prime confers normal oncogenicity to its host. Therefore, an essential role in tumor formation for the Ti

plasmid replicator region can be excluded. Moreover, our results imply that a different replicator at a different place in the Ti plasmid (deleted of its own replicator), does not alter normal oncogenicity. The T-DNA region, essential for oncogenicity, can now be defined somewhat more; by itself, the extreme left part of the T-DNA region (as in pAL1819) is apparently not sufficient. According to the gross genetic map, part of the right side of the T-DNA region can be deleted without complete loss of oncogenicity (Koekman *et al.*, 1979). This suggests that an approximately 3-Mdalton fragment in the T-DNA region next to the part present in plasmid pAL1819 has to be present for the bacterium to induce tumors.

Plasmid pAL1820 is stably present in *E. coli* (to be published separately). Sophisticated mutagenesis methods developed for *E. coli* are now exploitable for the R prime pAL1820, which confers oncogenicity to *Agrobacterium tumefaciens*. Experiments will be concentrated on the T-DNA region of pAL1820, to make it suitable as a vector for cloning foreign genes. These can subsequently be introduced into a plant genome.

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