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Site-Directed Mutagenesis in *Escherichia coli* of a Stable R772::Ti Cointegrate Plasmid from *Agrobacterium tumefaciens*

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The host range of an octopine Ti plasmid is limited to *Rhizobiaceae*. This has been extended also to *Escherichia coli* in the form of a stable cointegrate with the wide-host-range plasmid R772. Its structure was studied by constructing a physical map of R772 and of the R772::pTiB6 cointegrate. An insertion sequence present in R772, called IS*70*, turned out to be involved in cointegrate formation. We found one intact copy of IS*70* and a small segment of IS*70*, respectively, at the junctions of R772 and Ti DNA. The absence of a complete second copy of IS*70* is a likely explanation for the stability of the cointegrate plasmid. A procedure for site-directed mutagenesis of this cointegrate plasmid in *E. coli* is described. The effect of mutations in the Ti plasmid part can be studied subsequently by transferring the cointegrate into *Agrobacterium tumefaciens*. The advantage of this procedure for Ti plasmids over other methods used at present is discussed.

The Ti plasmid of *Agrobacterium tumefaciens* is essential for the capacity of this bacterium to induce tumors on dicotyledonous plants (27, 29, 33). Upon tumor induction part of this plasmid, the T-region, is integrated into the plant genome, where it is transcribed into polyadenylic acid-containing mRNAs, some of which have been found to be capped and translated into proteins (2, 3, 7, 10, 25, 26, 30–32; A. M. Ledeboer, thesis, University of Leiden, Leiden, The Netherlands, 1978). Strains of *Agrobacterium* harboring a Ti plasmid lacking the T-region are no longer able to induce tumors (17). Physically distinct from the T-region, a second segment on the Ti plasmid has been identified that is essential to bacterial oncogenicity (8, 21). This segment has never been found in the plant tumor cell. Because mutations in this segment were shown to be complementable in trans, this area is called the *vir* (virulence) region (11, 12, 15).

To get a more detailed insight into the organization and function of Ti plasmid genes, procedures have been reported for site-directed mutagenesis of Ti plasmids (9, 18, 19, 24). In all of these cases the final step of Ti plasmid mutagenesis is performed in *Agrobacterium* itself, because the host range of Ti plasmids is limited to *Rhizobiaceae*. In short, the following steps are made. A cloned fragment of the Ti plasmid is mutagenized in *Escherichia coli*, e.g., by insertion of a transposon. Then, the mutagenized fragment is subcloned on a wide-host-range vector and transferred into a Ti plasmid-containing *Agrobacterium* strain. Here, by homologous recombination via double crossing over, the insert is placed in the Ti plasmid. The wide-host-range plasmid is subsequently cured with an incompatible plasmid, or the Ti plasmid is transferred conjugatively to another *Agrobacterium* recipient. Transconjugants are tested for proper placement of the mutation into the Ti plasmid. Because these procedures are rather laborious, it would be advantageous if the site-directed mutagenesis of the Ti plasmid itself could directly be performed in *E. coli*. To this end, the Ti plasmid requires a replicator that functions in *E. coli*.

Cointegrate plasmids have been isolated in our group between the wide-host-range IncP1 type plasmid R772 and an octopine Ti plasmid (pTiB6). When such cointegrates are transferred from *A. tumefaciens* into *E. coli*, they usually dissociate and the Ti part of the cointegrate is lost (6, 14). Here, we describe a particular R772::Ti cointegrate plasmid that appeared to be perfectly stable in both *A. tumefaciens* and *E. coli*. Moreover, *Agrobacterium* strains carrying this cointegrate can normally induce tumors on several plant species. The structure of this cointegrate was studied by constructing a physical map of the R772::Ti plasmid. Furthermore, it is shown that this cointegrated Ti plasmid can be used for site-directed mutagenesis of Ti plasmid regions within *E. coli*. The procedure has been applied to the T-region of this octopine Ti plas-

TABLE 1. Bacterial strains and plasmids used in this work

Strain/plasmid	Restriction fragment	Vector	Relevant phenotype	Source
E. coli strain				
KMBL1164			Pro$^-$ Thi$^-$	P. van de Putte
KMBL1001				P. van de Putte
A. tumefaciens strain				
LBA937			Rif^r Nal^r (R772, pTiB6)[a]	P. J. J. Hooykaas et al. (14)
LBA973			Gen^r Nov^r (pAL969)	P. J. J. Hooykaas
LBA1831			Rif^r Nal^r (pAL1831)	This work
Plasmid				
R772			Km^r	R. Hedges
pAL969			Km^r	P. J. J. Hooykaas
pAL1831			Km^r Ap^r Cm^r	This work
pRAL3501			Tc^r Cm^s	This work
pRL220			Tc^r Cm^r Ap^r Sm^r	J. Hille and R. A. Schilperoort (13)
pRL234			Tc^r Ap^r Cm^r	This work
Clones derived from R772				
Plasmid				
pRL231	*Hin*dIII-4	pTR262	Tc^r	This work
pRL232	*Hin*dIII-3	pTR262	Tc^r	This work
pRL233	*Hin*dIII-3 + 4	pTR262	Tc^r Km^r	This work
pRL236	*Eco*RI-1 + 2		Km^r IncP	This work
pRL237	*Eco*RI-2	pBR322	Ap^r Tc^r Km^r	This work
pRL238	*Hin*dIII-1 + 2	pTR262	Tc^r IncP	This work
pRL247	*Pst*I-6	pBR325	Cm^r Tc^r	This work
pRL248	*Pst*I-3	pBR325	Cm^r Tc^r	This work
pRL246	pBR322::IS*70*		Ap^r Tc^r	This work
pRL239			Km^r Ap^r Cm^r IncP	This work

mid. The mutated Ti plasmid was subsequently transferred to *A. tumefaciens* to study the phenotype of the mutation.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this work are listed in Table 1.

Conjugation conditions. Conjugations were performed as previously reported (11).

Plasmid isolation. Plasmid DNA was isolated from *E. coli* as described by Birnboim and Doly (1) and from *A. tumefaciens* as described by Koekman et al. (16).

Restriction endonuclease digestion, agarose gel electrophoresis, Southern blotting, and DNA-DNA filter hybridization. Restriction endonuclease digestion, agarose gel electrophoresis, Southern blotting, and DNA-DNA filter hybridization were carried out as described by Prakash et al. (22).

Ligation conditions. Ligation of restriction fragments was carried out in 6.5 mM $MgCl_2$–60 mM Tris-hydrochloride (pH 7.6)–10 mM dithiothreitol–0.4 mM ATP for 20 h at 14°C. The DNA concentration in the ligation mixture was usually about 100 μg/ml, with a fivefold excess of the DNA to be cloned compared with vector DNA. After ligation, the mixture was directly used for transformation.

Transformation. *E. coli* cells were transformed by the $CaCl_2$ method of Cohen et al. (5).

RESULTS

From an experiment in which an octopine Ti plasmid (pTiB6) was mobilized with the wide-host-range IncP1 type plasmid R772, a particular R772::Ti cointegrate was obtained (pAL969 [14]). Strains harboring this plasmid show 100% cotransfer of R772 and Ti plasmid markers when used as a donor in further crosses. To understand the stability of this cointegrate, its physical map was determined. First, a map of the IncP1 type plasmid R772 was made. Subsequently, a transposable element was identified and isolated from R772 and a physical map for plasmid pAL969 was made, in which copies of the transposable element were indicated.

Physical map of R772. Ten restriction endonucleases were tested on R772 DNA. Three of them, *Bgl*II, *Kpn*I, and *Xba*I, did not cut this plasmid, and one, *Bam*HI, gave a single cut. The remaining enzymes, *Hpa*I, *Sal*I, *Sma*I, *Hin*dIII, *Eco*RI, and *Pst*I, gave more than one cut (Fig. 1). When the sizes of all fragments were taken together, the size of R772 DNA turned out to be 40.5 megadaltons (Md). Double digestions and Southern blot hybridizations were performed to establish the order of the fragments.

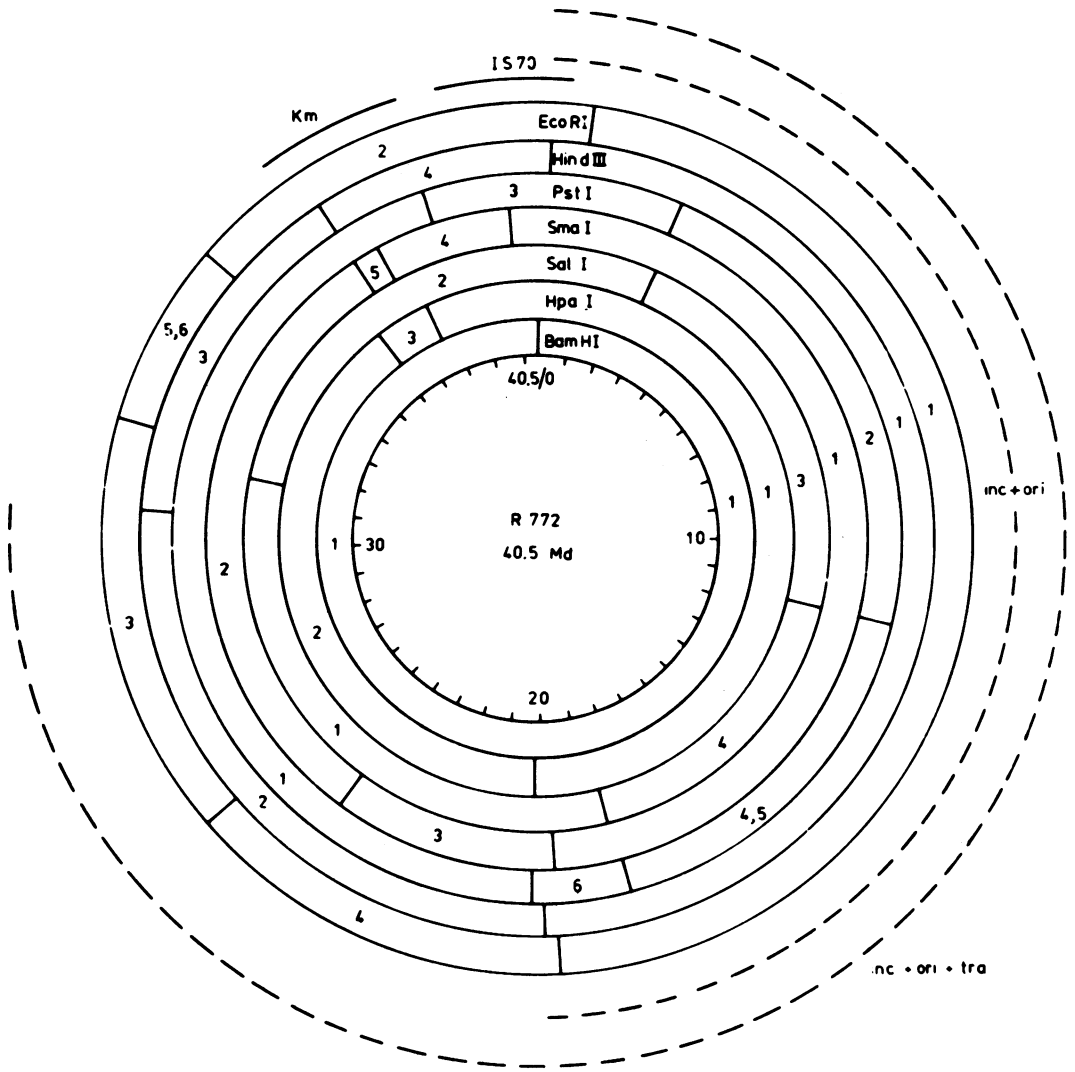

FIG. 1. Physical map of R772. The unique *Bam*HI site is taken as zero point. Size of restriction endonuclease fragments of R772 (in megadaltons): *Hpa*I, 23.0, 16.1, and 1.4; *Sal*I, 13.0, 11.6, 8.9, and 7.0; *Sma*I 20.4, 12.5, 4.4, 2.7, and 0.5; *Hin*dIII, 19.8, 10.7, 6.0, and 4.0; *Eco*RI, 19.1, 6.4, 6.4, 5.8, 1.8, and 1.0; *Pst*I, 18.3, 9.0, 4.7, 4.4, 2.4, and 1.7. Genetic traits are further described in the text. Abbreviations: inc, incompatibility functions of R772; ori, origin of replication of R772; tra, functions required for conjugal self-transfer of R772.

Because many restriction endonuclease sites appeared to be clustered in such a way that the alignment of sites could not be established, we decided to clone certain R772 restriction fragments on multicopy plasmids (see Table 1). By using these clones in restriction endonuclease analysis, sites could be mapped very accurately. In Fig. 1 the circular map of R772, with all known restriction endonuclease sites, is shown.

The cloning experiments also gave some information about certain genetic traits. From the *Hin*dIII cloning experiment on pTR262, it appeared that neither fragment 4 nor fragment 3 carried an intact Km^r locus (the sole antibiotic resistance marker of R772). But when cloned together, as one segment in which the original orientation was maintained, kanamycin resistance was expressed. To prove this, we cloned *Eco*RI fragments of R772 on pBR322. *Eco*RI fragment 2 turned out to carry the Km^r locus (see Fig. 1). The *Eco*RI cloning also gave rise to a plasmid that only carried the Km^r determinant and no pBR322 markers. This plasmid, pRL236, was shown to consist of R772 fragments 1 and 2,

which indicates that these fragments contain all information required for autonomous replication. Since plasmid pRL236 appeared to be transfer deficient, *tra* functions should locate in the area containing *Eco*RI fragment 3, 4, 5, or 6. Clone pRL238, consisting of pTR262 and *Hin*dIII fragments 1 and 2, was found to be tra^+ and to be able to replicate in *Agrobacterium*. The pTR262 replicator does not function in *Agrobacterium*, and therefore *Hin*dIII fragments 1 and 2 of R772 must carry all the information required for self-transfer and autonomous replication. These data together indicate that replication and incompatibility functions of R772 are located within *Hin*dIII fragment 1 (see Fig. 1).

Stable R772::Ti cointegrate plasmid. It has been shown that mobilized Ti plasmids of *A. tumefaciens* carry an insertion sequence or transposon, originating from the mobilizing plasmid (14). If it is assumed that mobilization is mediated by cointegrate formation due to transposition, then two directly repeated copies of a transposable element are expected to be present in an unstable intermediate. Instability is the result of homologous recombination between the two copies of the transposable element, which will lead to dissociation of the cointegrate into its constituents. Studies on position and structure of copies of the transposable element will enable us to understand the stability of a cointegrate plasmid.

Recently it was observed that a pBR322 derivative, when mobilized with R772, had acquired a transposable element (P. Hooykaas and G. Moolenaar, manuscript in preparation). In the same way we have isolated this transposable element from R772 on pBR322. The plasmid concerned was called pRL246 and analyzed in detail. The transposable element did not carry the sole antibiotic marker of R772 (Km^r); thus, it was designated as an insertion sequence (named IS*70*). Homoduplex analysis of plasmid pRL246 (not shown) revealed that IS*70* carries short inverted repeats at its ends (approximately 50 base pairs in length). In Fig. 2 results of the restriction endonuclease mapping of the 2.5-Md IS*70* on pBR322 are presented. With this map IS*70* was precisely positioned on the map of R772 (see Fig. 1).

For the R772::Ti cointegrate plasmid pAL969, the site of cointegration between R772 and the Ti plasmid was determined by using six different restriction endonucleases (data not shown). From the results it could be deduced that on R772 the site of cointegration was in a 1.5-Md fragment (the overlapping part of *Sma*I fragment 4 and *Pst*I fragment 3; see Fig. 1), and on the Ti plasmid the site was in a 0.8-Md fragment (*Eco*RI fragment 16 and *Hin*dIII fragment 28a).

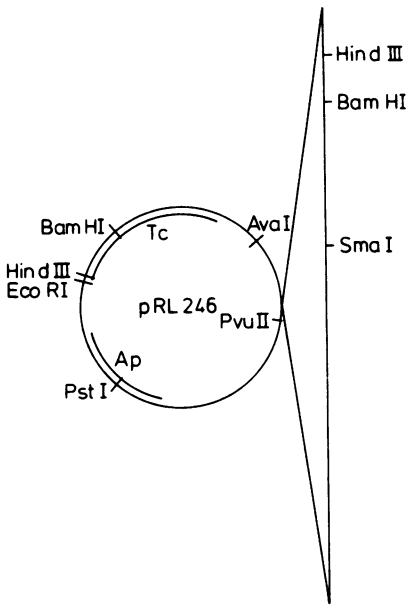

FIG. 2. Physical structure of pBR322::IS*70* (pRL246) is vizualized. The 50-base pair inverted repeats of IS*70*, at its ends, are not indicated.

The 1.5-Md fragment on R772 where cointegration had occurred carries part of the insertion sequence IS*70*. This suggests that cointegration indeed occurred via IS*70*.

If the cointegrate plasmid pAL969 was cut with an enzyme that does not cut in IS*70*, one would expect to find two fragments showing sequence homology with IS*70*, namely, the two R772::Ti fusion fragments. However, when an enzyme was used that cuts once in IS*70*, four such fragments would be expected.

Plasmid pBR322::IS*70* (pRL246) was labeled in vitro and hybridized to blots containing pAL969 DNA, separately digested with different enzymes. In the case of an enzyme that does not cut in IS*70* (*Eco*RI, *Hpa*I), two different bands were detected on the autoradiograms, as expected. However, with an enzyme that cuts once in IS*70* (*Sma*I), three bands were detected (Fig. 3). In a control experiment pRL246 was hybridized to a blot containing Ti plasmid DNA. No hybridization was observed to this blot, indicating that neither pBR322 nor IS*70* contains sequences homologous to the Ti plasmid. Several conclusions can be drawn from these results. First, it proves that cointegration between R772 and the Ti plasmid did occur through IS*70*, since it appears to be present twice. Second, it can be envisaged that there is only one complete copy of IS*70*, whereas the other is a remnant of IS*70*. Both are located at the R772::Ti fusion fragments. The size of the deleted IS*70* can be

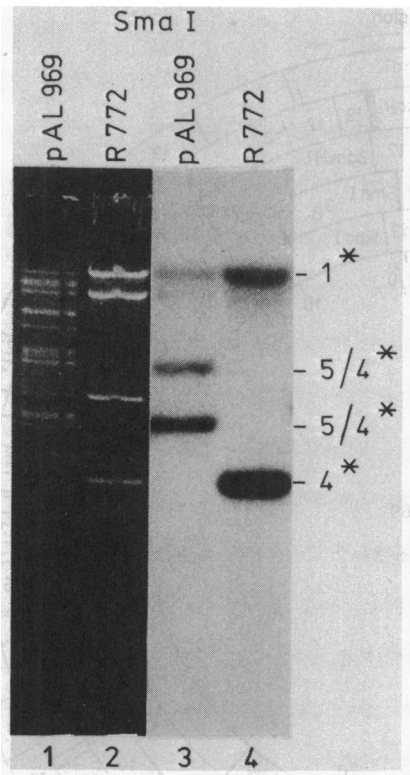

FIG. 3. Restriction endonuclease digestion of plasmids pAL969 and R772. In lanes 1 and 2, respectively, pAL969 and R772 were digested with *Sma*I and run on a 0.8% agarose gel. Plasmid pRL246, consisting of pBR322 and IS*70* (see Fig. 2), was hybridized to a blot of this gel. Since IS*70* is cut once with *Sma*I, two bands were found to hybridize in the case of R772 (lane 4). In the case of plasmid pAL969, three bands appeared (lane 3), indicating the presence of an extra part of IS*70* in pAL969. The relatively low intensity of the highest bands (lanes 3 and 4) is due to bad transfer of high-molecular-weight DNA during blotting. Fragments derived from R772 are indicated by a star, and R772::Ti fusion fragments are given two numbers, the Ti number and the R772 number.

estimated to be, at most, 0.5 Md. Third, when the size of fusion fragments of the cointegrate are summed, a small deletion of, at most, 0.5 Md must have occurred in the pTi DNA, although this deletion obviously has not eliminated any of the previously identified restriction sites. With these results a physical map of pAL969 was constructed, and IS*70*, the deleted IS*70*, and the Km^r locus were located on it (Fig. 4). The remarkable stability of pAL969 can now be explained by assuming that the size of the incomplete second IS*70* copy is not sufficient to allow efficient homologous recombination, which otherwise would lead to dissociation of the cointegrate.

Site-directed mutagenesis. A model experiment was designed to study the use of the stable R772::Ti cointegrate (pAL969) for site-directed mutagenesis in *E. coli*. Because of the relatively small size of R772 (40.5 Md), alterations in the restriction patterns of a mutated R772 plasmid would be easier to interpret than those for the cointegrate plasmid (with a size of 162 Md). Therefore, a site-directed mutation was first introduced into R772, and after it had been characterized it was introduced into plasmid pAL969.

*Hin*dIII fragment 3 of R772 (with a size of 6 Md) was cloned on the insertional activation vector pTR262. This fragment does not carry functions essential for self-transfer or autonomous replication of R772 (see Fig. 1). The resulting plasmid, pRL232, has three *Eco*RI sites, all located within the cloned fragment. The vector part (pTR262) has no *Eco*RI sites. The two small internal *Eco*RI fragments of pRL232 (1.8 and 1.0 Md) were eliminated and replaced by an *Eco*RI fragment of plasmid pRL220 (13), carrying Ap and Cm resistance determinants (with a total size of 5.8 Md). In this way a plasmid was obtained, pRL234, that carried Ap, Cm, and Tc resistance loci (Fig. 5). At both sides of the Cb Cm^r fragment, a segment with homology to R772 and approximately 1.5 Md in size existed. Now R772 and pRL234 were brought together in one bacterial cell (KMBL1164) to force the transfer of the Cb Cm segment into R772 via homologous recombination. This strain was then used as a donor in a cross with KMBL1001. Transfer of the IncP1 plasmid R772 was high; about 1% of the recipients received this plasmid. In earlier experiments it was found that R772 mobilizes pBR322 with a frequency of 10^{-5} per transferred R772 (via IS*70* from R772). Thus, via IS*70*, we also could expect R772 to mobilize pRL234 with a frequency of approximately 10^{-5}. We actually found a transfer value for the Ap determinant of 10^{-2} per transferred R772, which is much higher than we would expect for mobilization through a transposition event. This suggests that the homology between pRL234 and R772 is responsible for the high transfer frequency. For further analysis, 22 Ap^r colonies were picked with a toothpick; 14 were Ap^r Cm^r Km^r Tc^r and 8 turned out to be Ap^r Cm^r Km^r Tc^s. The set of 14 colonies expressed all markers of R772 as well as pRL234, which indicated that the complete pRL234 was mobilized (mobilization frequency, 6.5×10^{-3} per transferred R772). In contrast, the set of eight colonies carried the R772 marker (Km^r) and the Ap^r Cm^r markers from the mutagenized *Hin*dIII fragment 3 of R772, but not the marker of the vector plasmid (Tc^r). These eight colonies, therefore, were likely candidates for having received the site-directed mutation (fre-

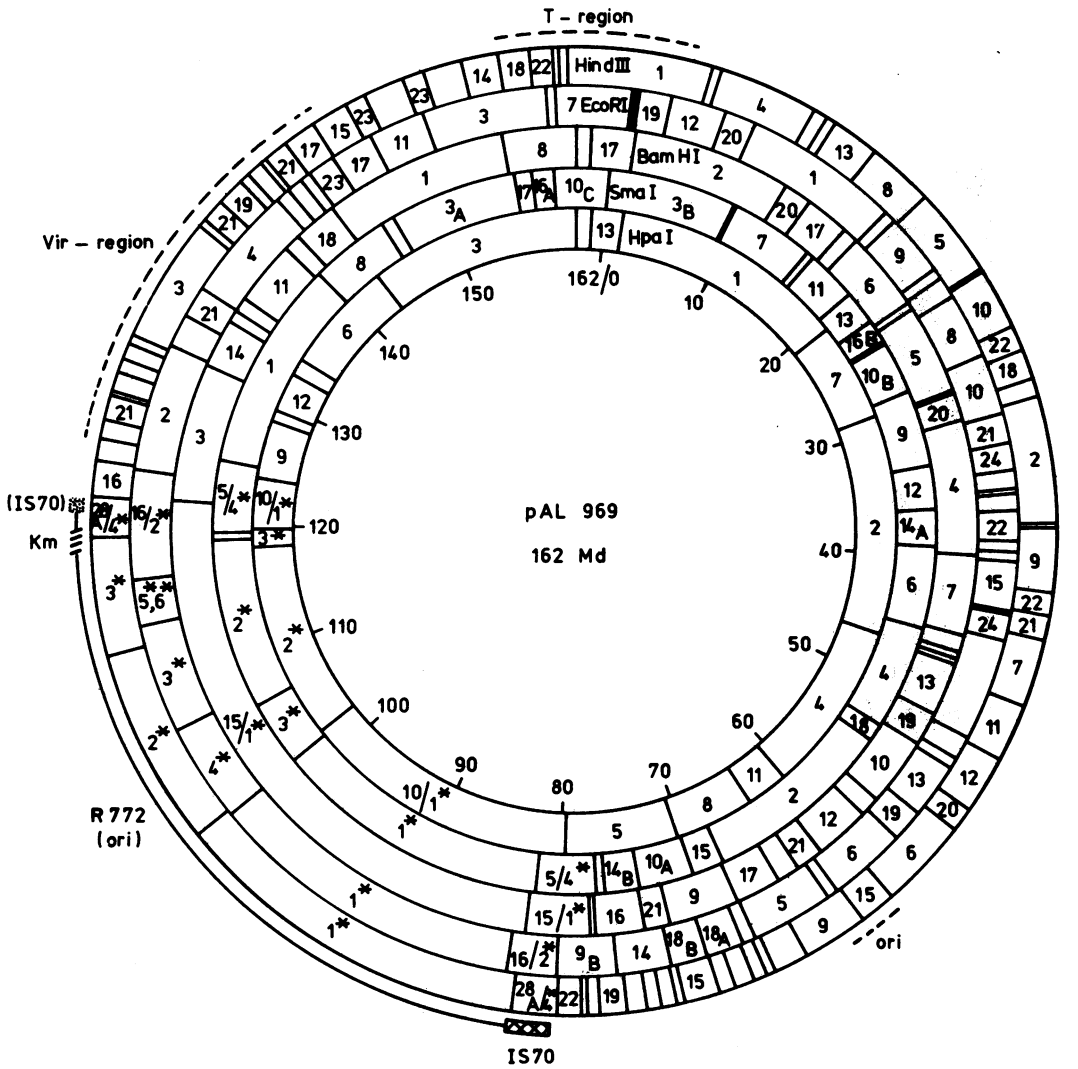

FIG. 4. Map of the R772::Ti cointegrate plasmid pAL969. On the map, the R772 fragments are indicated with a star. The R772::Ti fusion fragments have been given two numbers, the R772 number and the Ti number. The map of the Ti part is shown as described by De Vos et al. (20).

quency of replacement, 3.5×10^{-3} per transferred R772). From three independent colonies, plasmid DNA was isolated and analyzed on agarose gels: only one plasmid of the same size was observed for all three. Also, the fragment pattern after digestion with *Hin*dIII was identical for these plasmids (see Fig. 5 for an example). *Hin*dIII fragment 3 of R772 had disappeared, and two new fragments appeared. These two new fragments were identical to the mutagenized *Hin*dIII fragment 3 of R772 in pRL234. In the R772 derivative, pRL239, no band of the vector plasmid (pTR262) is seen. This conclusively shows that R772 had been site-directedly mutagenized. Similarly, the same mutation has been introduced into the cointegrate plasmid pAL969. Results obtained were essentially the same as observed for R772, and when such a mutated R772::Ti plasmid was transferred to *A. tumefaciens*, normal tumor induction was obtained. This was as expected, since the mutation was not located at the Ti part of the cointegrate plasmid.

To test the feasibility of the described procedure for site-directed mutagenesis to the T-region of the Ti plasmid, a T-region fragment (*Eco*RI fragment 7) was cloned on vector pACYC184. The resulting plasmid, pRAL3501,

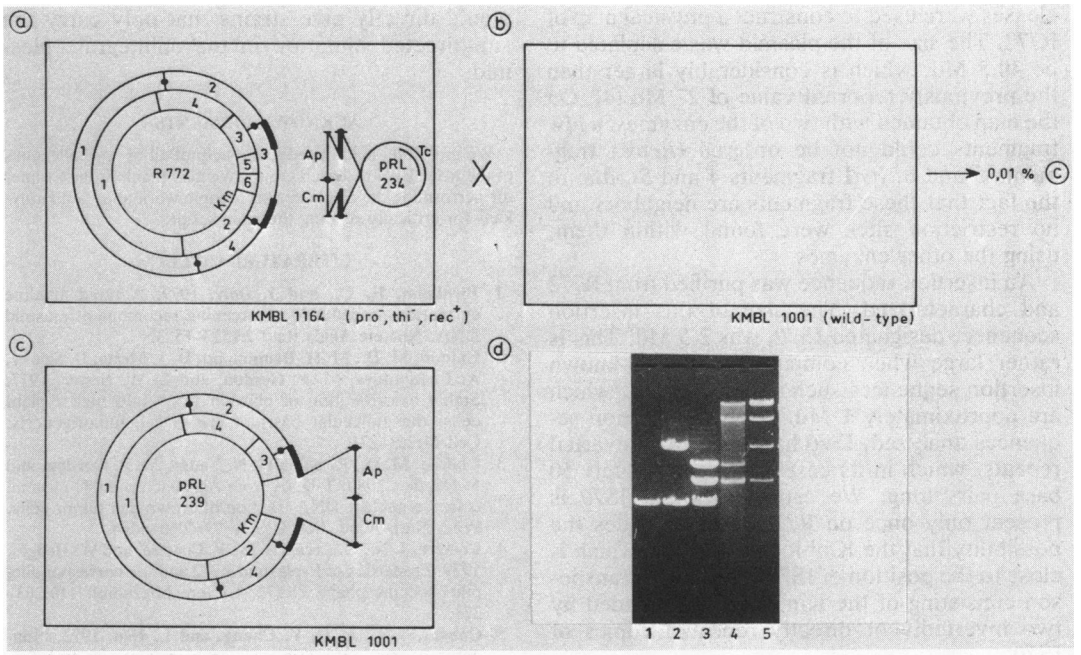

FIG. 5. Model experiment for site-directed mutagenesis of R772. In one *E. coli* cell, KMBL1164 (*pro*$^-$ *thi*$^-$ *rec*$^+$), R772, and pRL234 were brought together (a). Sequence homology between both plasmids is indicated by a thick line. *Eco*RI sites are visualized by stripes, and *Hin*dIII sites by a stripe with a black dot. This strain was used then as a donor in a cross with KMBL1001 (*E. coli* wild type) (b). One percent of the recipients received the Km resistance marker of R772, and of these, approximately 1% were also Ap resistant. In a frequency of one of three, the genetic data indicated that the Ap and Cm markers might have been recombined into R772 via a double crossover (c). From one such strain, plasmid pRL239 was isolated and the restriction enzyme fingerprint was analyzed on a 0.8% agarose gel (d). All digestions were performed with restriction endonuclease *Hin*dIII. In lane 1 the insertional activation vector pTR262 is shown, which was used to clone *Hin*dIII fragment 3 of R772 (pRL232, lane 2). Plasmid pRL232 was used for substitution of its two small *Eco*RI fragments (5 and 6 in R772) for a 5.8-Md *Eco*RI fragment, carrying Ap^r and Cm^r, from pRL220, resulting in plasmid pRL234 (lane 3). The Ap^r Cm^r segment in pRL234 was subsequently introduced into R772, resulting in plasmid pRL239 (lane 4). The original plasmid R772 is shown in lane 5.

contains two *Pst*I sites, separated by 0.5 Md, within the T-region fragment (vector pACYC184 has no *Pst*I site). The 0.5-Md *Pst*I fragment was substituted for a 2.7-Md fragment carrying a Cm determinant, derived from plasmid pRL220. At both sides of the segment containing the Cm determinant there existed now, respectively, 2.5- and 1.8-Md sequence homology to the T-region. This mutation was subsequently introduced into the cointegrate plasmid pAL969 as described before. With a frequency of one of three Cm^r transconjugants, the mutation was found to be introduced into pAL969. One mutated pAL969, called pAL1831, was isolated and digested with several restriction endonucleases, and the fragment patterns were studied by agarose gel electrophoresis. This confirmed that the 0.5-Md *Pst*I fragment of pAL969 was substituted for a 2.7-Md *Pst*I fragment in pAL1831 (data not shown). When this *Pst*I substitution in pAL1831 is compared with the genetic map of the T-region (9, 20), the mutation is located in the locus which causes a rooting tumor morphology. Compared with the transcription map of the T-region (32), transcript 4 has been mutated. Plasmid pAL1831 was transferred from *E. coli* to *A. tumefaciens*, and one of the transconjugants was tested for tumor-inducing capacity on various plant species. In contrast to tumors induced by wild-type octopine strains, on tobacco the small tumors induced by agrobacteria carrying pAL1831 developed roots. Root formation from tumors on kalanchoë stems was also more abundant than normal. On tomatoes only small tumors were formed. These observations are all in full agreement with the known phenotype of such mutations.

DISCUSSION

Physical map of R772 and of the R772::Ti cointegrate plasmid. Seven restriction endonu-

cleases were used to construct a physical map of R772. The size of the plasmid was calculated to be 40.5 Md, which is considerably larger than the previously reported value of 27 Md (4). On the map obtained with two of the enzymes, a few fragments could not be ordered (*Eco*RI fragments 5 and 6; *Pst*I fragments 4 and 5), due to the fact that these fragments are neighbors and no restriction sites were found within them, using the other enzymes.

An insertion sequence was purified from R772 and characterized. The size of this insertion sequence, designated IS*70*, was 2.5 Md. This is rather large when compared with well-known insertion sequences such as IS*2* and IS*4*, which are approximately 1 Md. Like all insertion sequences analyzed, IS*70* has at its ends inverted repeats, which in its case are approximately 50 base pairs long. We established that IS*70* is present only once on R772. This excludes the possibility that the Km^r locus on R772, which is close to the position of IS*70*, is part of a transposon consisting of the Km^r locus surrounded by two invertedly or directly repeated copies of IS*70*.

Evidence is presented that IS*70* is involved in the cointegrate formation between R772 and the Ti plasmid. The presence of only one complete and one largely deleted IS*70* copy in pAL969 might explain the stability of this R772::Ti cointegrate.

Site-directed mutagenesis. We have shown that in an efficient way Ti plasmids can be site-directedly mutated in *E. coli*. The method is based on using a stable cointegrate between a Ti plasmid and the wide-host-range plasmid R772. The cointegrate plasmid can be transferred reciprocally between *A. tumefaciens* and *E. coli*. The advantages of using *E. coli* as a host seem obvious. *E. coli* has a much faster growth rate, a lot of mutants are available, and special cloning vehicles such as cosmids and insertional activation vectors have been developed. Another advantage is that subcloning of a mutagenized fragment on a wide-host-range vector is no longer required as it was in previously described procedures. The necessity of using a wide-host-range plasmid raises difficulties in size and available restriction sites of these vectors. We have demonstrated site-directed mutagenesis of an R772::Ti cointegrate by conjugative transfer of this plasmid and testing for replacement of a selectable marker. The procedure probably can be simplified even further by the use of *polA*(Ts) strains. In such strains, ColE1-derived plasmids can replicate at 32°C, but not at 42°C, although other plasmids, such as the described cointegrate plasmid pAL969, are stably maintained at both temperatures. Therefore, simply selecting for, e.g., an antibiotic resistance marker at 42°C would directly give strains that only carry the site-directed mutation in the cointegrate plasmid.

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