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Complementation Analysis of *Agrobacterium tumefaciens* Ti Plasmid Mutations Affecting Oncogenicity

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A wide host range cosmid vector has been constructed by insertion of the λ *cos* site into the plasmid pRK2501. This cosmid, which is maintained in *Agrobacterium tumefaciens* and is compatible with the Ti plasmid, has been used to make a clone bank of the *A. tumefaciens* pTiA6 plasmid. Several pTiA6 cosmids have been used to complement Tn*5*-induced Ti plasmid mutations. Five avirulent mutations which map outside of the region of the plasmid maintained in plant tumors (T-DNA) could be complemented in a *trans* orientation. Two mutations which are located on a single *Hpa*I restriction fragment outside of the T-DNA, as well as three mutations which map within the T-DNA region, could not be complemented in a *trans* orientation in a REC$^-$ host.

Agrobacterium tumefaciens is the causative agent of crown gall disease, a neoplastic transformation of dicotyledonous plants. Oncogenic strains of *A. tumefaciens* contain large plasmids which are essential for tumorigenicity (26–28). A portion of the tumor-inducing (Ti) plasmid is transferred to the plant and is stably maintained in the tumor (T-DNA) (4, 25). In the plant cell, this T-DNA is actively transcribed into RNA (6, 9), at least some of which is polyadenylated (19).

The T-DNA, as well as other regions of the Ti plasmid, encodes functions necessary for tumorigenesis. Mutants altered in virulence as a result of Tn*5* (8), Tn*904* (22), and Tn*7* insertion (10) have been identified. These mutations mapped both inside the region of the T-DNA and in a large region of the plasmid flanking the T-DNA. Since the DNA outside of the T-DNA is not stably maintained in tumor tissue, the mutations in this region of the Ti plasmid may be involved in such functions as plant cell recognition and adsorption, plasmid transfer, and integration of T-DNA. However, the nature and number of plasmid loci involved in tumorigenesis remain unknown.

To gain insight into the roles of the various virulence loci, we have determined whether Tn*5*-induced Ti plasmid mutants can be complemented with wild-type loci *in trans*. This was accomplished by cloning portions of the Ti plasmid into a wide host range plasmid vector which is compatible with the Ti plasmid in *A. tumefaciens* and assaying the strain for virulence.

MATERIALS AND METHODS

Bacterial strains and culturing media. Bacterial strains containing Tn*5* insertions in Ti plasmids which were used are listed in Table 1. *A. tumefaciens* PC2594, a REC$^-$ derivative of LBA4011, was kindly provided by R. Schilperoort (15). All liquid culturing was done in L-broth (21). AB minimal medium (3) was solidified with 1.5% agar (Difco Laboratories). Octopine differential medium (BTB) was used for screening colonies utilizing octopine (12).

Construction of Ti plasmid bank. A wide host range cosmid vector was constructed in the following way. The plasmid pHC79 (11), containing bacteriophage λ cohesive ends (*cos*) on a 1.2-kilobase-pair (kbp) *Bgl*II fragment, was mixed in a 20:1 molar ratio with the plasmid pRK2501 (14), which contains a single *Bgl*II restriction site, and restricted with *Bgl*II. The DNA was ethanol precipitated, ligated (7), and transformed (5) into *Escherichia coli* HB101 selecting for kanamycin resistance (50 μg/ml). Size screening for pRK2501 containing the *cos* fragment was done by using the plasmid isolation procedure of Birnboim and Doly (1) followed by electrophoresis on vertical 0.8% agarose gels (20). One such plasmid, pHK17, was identified and used as a cosmid vector.

The Ti plasmid bank was constructed from pTiA6 as follows (see Fig. 1). pTiA6 was partially digested with *Eco*RI (Bethesda Research Laboratories), the fragments were fractionated on a 5 to 20% NaCl gradient, and fragments of approximately 25 kbp were ligated into the cosmid vector, pHK17. Ligated DNA was packaged and transduced into *E. coli* HB101 as described (2), selecting for resistance to kanamycin. Cosmids were mapped by comparison of restriction digest patterns with that of pTiA6 (24).

Transformation of *A. tumefaciens*. *A. tumefaciens* cells were transformed essentially as described by Holsters et al. (12). To construct strains for complementation, a recombination-deficient *A. tumefaciens* strain, PC2594, was first transformed with pTiA6::Tn*5* by selecting for kanamycin resistance (100 μg/ml). Ti plasmid-containing PC2594 was then transformed with the appropriate cosmid by selecting for tetracycline resistance (2 μg/ml).

TABLE 1. Strains containing Tn5 insertions in Ti plasmids

Strain	Plasmid
A1007	pTiA6NC *onc-3*::Tn5
A1010	pTiA6NC *onc-5*::Tn5
A1022	pTiB$_6$806 *onc-12*::Tn5
A1023	pTiB$_6$806 *onc-13*::Tn5
A1028	pTiB$_6$806 *onc-16*::Tn5
A1040	pTiB$_6$806 *onc-21*::Tn5
A1069	pTiB$_6$806 *onc-25*::Tn5
A1074	pTiB$_6$806 *onc-24*::Tn5
147[a]	pTiA6NC
169[a]	pTiA6NC
328[a]	pTiA6NC

[a] Strains derived from pTiA6 will be described in a separate manuscript. Strains 147 and 169 are similar in phenotype to A1070 (8), and strain 328 is similar in phenotype to A1022.

Complementation of virulence mutants. Merodiploid strains containing pTiA6::Tn5 and cosmids with those regions of the Ti plasmid corresponding to the site of Tn5 insertion were screened for virulence on *Kalanchoe daigremontiana* leaves and carrot root slices (*Daucus carota*) as previously reported (8).

Isolation of bacteria from tumors. Tumor slices were ground with a mortar and pestle and suspended in sterile distilled water. Bacteria were grown on AB minimal medium plates. Colonies which were capable of utilizing octopine were tested for virulence.

RESULTS

Construction of a cosmid vector and Ti plasmid bank. The use of cosmid vectors allows for the efficient cloning and transduction of large DNA inserts (11). To construct a cosmid bank of pTiA6 in a vector which is maintained in *A. tumefaciens* and is compatible with Ti plasmids, the cosmid vector pHK17 was constructed from the wide host range plasmid pRK2501 and the bacteriophage λ cohesive ends isolated from pHC79. The wide host range cosmid vector (Fig. 1) contains single restriction sites for *Eco*RI, *Xho*I, *Hin*dIII, *Sal*I and resistance to kanamycin and tetracycline and allows for the packaging of inserts from 19 to 35 kbp.

The construction of pHK17, as well as the construction of a pTiA6 cosmid clone bank, is outlined in Fig. 1. Partial *Eco*RI digests (average size of 25 kbp) of pTiA6 were ligated into the *Eco*RI site of pHK17. The ligated DNA was then packaged in vitro and transduced into *E. coli* HB101 (2). Cosmids containing portions of the entire pTiA6 plasmid were identified by restriction analysis. All of these cosmids are maintained in both *E. coli* and *A. tumefaciens* in the presence of either kanamycin or tetracycline but are rapidly lost in the absence of selective drug pressure.

Complementation of pTiA6::Tn5 mutants. A number of Tn5-induced Ti plasmid mutations have been previously demonstrated to result in avirulence (8). To further define the nature of these mutations, complementation analyses were done with wild-type DNA fragments isolated on cosmids. To eliminate the possibility of homologous recombination between mutated Ti plasmids and cosmids, these experiments were performed in the REC$^-$ host, PC2594 (15). Due to its *rec* genotype, PC2594 shows increased sensitivity to killing by UV light when compared with a *rec*$^+$ strain of *A. tumefaciens*. This UV sensitivity was tested before and after transformation with all Ti plasmids and cosmids. In addition, to definitively show that PC2594 is REC$^-$, its ability to promote homologous recombination was examined by the marker rescue system developed by Ruvkun and Ausubel (23). A Tn3 insertion in pHK121 could be marker exchanged onto pTiA6 at a frequency two orders of magnitude greater in a *rec*$^+$ host, A136, than in PC2594.

The pTiA6::Tn5 plasmids were first introduced into PC2594, selecting for kanamycin-resistant transformants. Cosmids containing the corresponding wild-type Ti plasmid DNA were transformed into these strains by selection for tetracycline resistance. Complementation by the cosmids was tested by virulence assays on both *Kalanchoe* leaves and carrot slices. Results of complementation of seven pTiA6::Tn5 plasmids by cosmids are summarized in Table 2 and Fig. 2. Five of the Ti plasmid mutations could be complemented, but two, A1023 and A1040, both of which map in *Hpa*I fragment 15, could not be complemented *in trans*.

None of the Ti plasmid mutations could be complemented when cells containing either the pTiA6::Tn5 plasmid or cosmid, but not both, were mixed together in the same wound. For complementation to occur, both the Ti plasmid and the cosmid containing the corresponding wild-type DNA must be in the same bacterium, indicating that any gene product which can complement a mutation does not diffuse out of the cell to complement a mutation in another bacterium.

Two mutants, A1069 and A1007, could be complemented by pHK121, but not by pHK120. Since pHK120 contains the wild-type DNA corresponding to the sites of Tn5 insertion for both of these mutants (Fig. 2), it seems likely that the promoter for the transcription of these regions lies outside of the DNA contained in pHK120.

It has been reported that the *Hpa*I fragment 7 region contains the genes coding for octopine catabolism (8, 16). To confirm that this region of the Ti plasmid does indeed code for the genes responsible for octopine catabolism, a cosmid containing *Hpa*I fragment 7, pHK111, was transformed into strain PC2594. The cosmid-

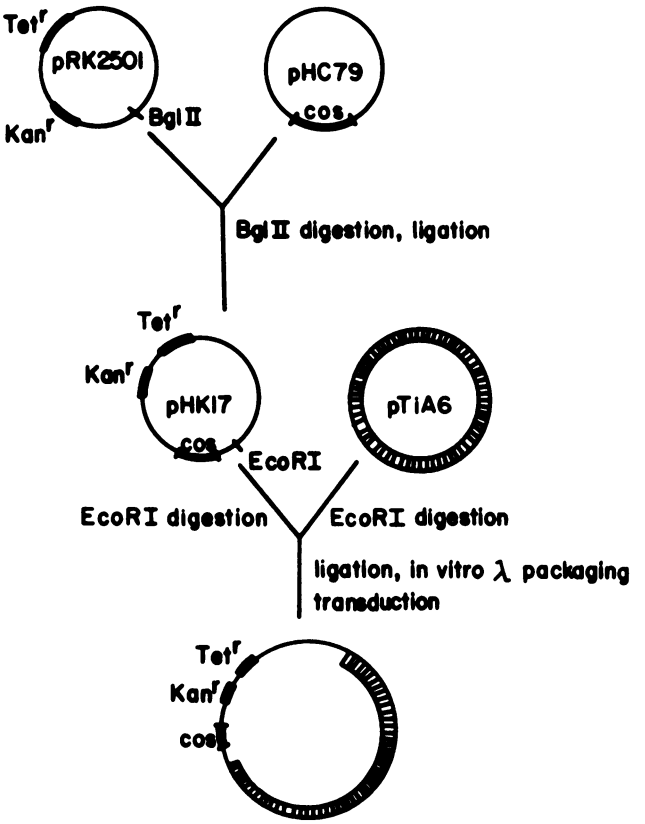

FIG. 1. Construction of a wide host range cosmid vector and pTiA6 cosmid bank. The *Bgl*II fragment containing the bacteriophage λ cohesive ends from pHC79 (11) was ligated into the single *Bgl*II site in pRK2501 (14), resulting in the cosmid vector, pHK17. A Ti plasmid bank was constructed by ligation of an *Eco*RI partial digest of pTiA6 into pHK17, in vitro packaging, and transduction into *E. coli* HB101.

containing strain tested positive on octopine differential medium (12), indicating that this cosmid does contain the genes responsible for octopine utilization.

The ability of cosmids to complement three different T-DNA mutations was examined in the REC$^-$ PC2594. None of these T-DNA mutants could be complemented by a cosmid containing the homologous regions of the T-DNA. This lack of complementation of mutations which are believed to affect genes expressed in plant cells suggests that cosmids cannot complement genes expressed in the plant. This may reflect the inability of the cosmid to enter or be maintained in the plant cell.

DISCUSSION

Transposon mutagenesis of Ti plasmids has demonstrated the existence of regions required for virulence both inside and outside of the T-DNA (8, 10, 22). The roles of these various loci in tumor induction or maintenance or both are not understood at present. We have attempted to complement *in trans* mutations in a number of these loci in an effort to elucidate the roles of these essential regions of the Ti plasmid.

The cosmid pHK17 allows for the cloning of large fragments of Ti plasmid DNA into a vector which replicates in *A. tumefaciens* and is compatible with the Ti plasmid. A bank of pTiA6 fragments covering the entire A6 plasmid has been cloned into this vector and has been used to complement a number of pTiA6::Tn*5* mutations. Five mutations, all of these outside of the T-DNA, could be complemented in a REC$^-$ bacterial host strain. This fact indicates that these areas of the Ti plasmid likely code for gene products which are responsible for tumorigenesis. Since the mixing together of bacteria which contain only cosmid and bacteria which contain only a mutant Ti plasmid failed to induce tumors, these gene products appear not to be transferable from one bacterial cell to another in a functional state.

There are several functions in the process of tumorigenesis in which these gene products

TABLE 2. Complementation of Ti plasmid mutations which affect oncogenicity[a]

Ti plasmid	Site of Tn5 insertion[b]	Complementing cosmid	Virulence of merodiploid
A1007	*Hpa*-9	pHK121	+
A1007	*Hpa*-9	pHK120	−
A1069	*Hpa*-16	pHK121	+
A1069	*Hpa*-16	pHK120	−
A1074	*Hpa*-12	pHK121	+
A1023	*Hpa*-15	pHK121	−
A1040	*Hpa*-15	pHK121	−
A1010	*Hpa*-6	pHK121	+
A1028	*Hpa*-6	pHK210	+
A1022	*Hpa*-14	pHK210	−
147	*Hpa*-13	pHK210	−
169	*Hpa*-13	pHK210	−
328	*Hpa*-3	pHK210	−

[a] Ti plasmids containing Tn5 insertions (8) and containing the corresponding wild-type DNA cosmids were transformed into *A. tumefaciens* and assayed for virulence on *Kalanchoe* and carrot slices as described in the text. (+) Complemented; (−) not complemented.

[b] These are restriction enzyme fragments.

might be involved. An early step in tumor formation in vivo is the attachment of bacteria to the plant cell (17). The Ti plasmid or a portion of it must then be transferred by an undefined mechanism to the plant cell. Once inside the plant cell, the T-DNA integrated into plant nuclear DNA. A mutation in any of the gene products involved in cell recognition, plasmid transfer, and integration would likely lead to avirulence. Avirulent mutants covering approximately 25 kbp of the Ti plasmid have been complemented, suggesting that several gene products are involved in the process of tumorigenesis. This does not seem unreasonable since at least 13 cistrons are required for formation of F pili and F plasmid transfer in *E. coli* (18). It is interesting to note that in the case of the F plasmid, one *cis* dominant locus, the transfer origin, has been identified. Two Ti plasmid mutations, both located in *Hpa*I fragment 15, cannot be complemented *in trans*. Although a number of explanations for this lack of complementation are possible, this region could correspond to a Ti plasmid transfer origin. It is also possible that the *Hpa*I-15 region contains a gene which is expressed only in plants, and the cosmid cannot be transferred to plants.

None of the Tn5 mutations localized to the T-DNA could be complemented in a *rec* host. This observation could be explained in several ways. For example, these T-DNA mutations may function only in a *cis* arrangement to other regions of the Ti plasmid, either in the bacterium or the plant cell. Alternatively, these mutations may

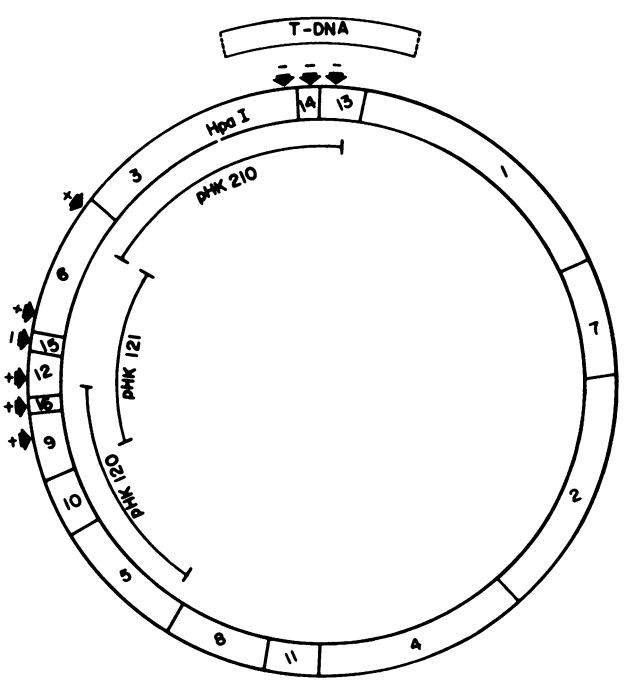

FIG. 2. Summary of complementation of Ti plasmid mutations which affect oncogenicity. Mutations were induced by insertion of Tn5 as described previously (8). Bars indicate the regions of wild-type pTiA6 plasmid included in the cosmids pHK120, pHK121, and pHK210. (+) Complemented; (−) not complemented.

affect genes which are expressed in the plant cell, but the cosmid, because it lacks the appropriate site(s), may not be transferred from the bacterium to the plant cell.

Complementation has been utilized to identify several loci whose gene products are necessary for Ti plasmid virulence as well as a locus which appears to act *in cis*. Work is currently underway to determine the number of gene products and their modes of action. Identification of these genes should lead to a better understanding of the mechanism of tumor induction.

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