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trans-Acting Virulence Functions of the Octopine Ti Plasmid from *Agrobacterium tumefaciens*

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All Ti plasmid-encoded virulence functions that were studied act in *trans*. An octopine Ti plasmid-specific *vir* operon, called *vir*-O, located on an *Eco*RI restriction fragment has been characterized. Sequences with promoter activity in *Escherichia coli* were identified for a second *vir* operon, called *vir*-C, which was located close to the position of *vir*-O.

A large plasmid, called the Ti plasmid, which is present in oncogenic *Agrobacterium tumefaciens* strains, is essential for crown gall tumor induction (18, 19, 20). Part of this plasmid, the T region, is transferred to and integrated in the plant genome during tumorigenesis (2, 17). Physically distinct from the T region, a second region on the Ti plasmid which is essential for tumor induction has been identified (3, 6, 16). Several mutations in this region are complementable in *trans*, suggesting that this region has to function inside the bacterium for virulence (7, 8, 13). Therefore, this segment of the Ti plasmid is called Vir (virulence) region.

In a previous study, we demonstrated complementation of virulence functions for some Ti::Tn*904* mutations (7). These results have now been extended by using 23 different Ti::Tn*5* and 2 different Ti::Tn*1831* plasmids, in which the transposons were inserted at various positions within the Vir region (Fig. 1). *Agrobacterium* strains, carrying these mutated Ti plasmids, were used in complementation studies with R-prime plasmids, which are compatible with Ti plasmids, and carry different segments of the Vir region of the wild-type octopine Ti plasmid (Fig. 1). In all cases, complementation was observed, demonstrating that all these functions act in *trans*.

This observation is in contradiction with results obtained by Klee et al. (13), who showed that two mutations in the Vir region (within *Hpa*I fragment 15) could not be complemented in *trans* by cosmid clones. The same *A. tumefaciens* mutants were used in this study, and were shown to be complementable in *trans* by R-prime plasmids. The reason for this contradiction is as yet not understood, but might be due to differences in size of cloned Vir region fragments. Recently, it has been shown that Vir region and T region of an octopine Ti plasmid can physically be disconnected (10), which excludes the possibility that these virulence functions act in *cis* with regard to the T region.

For several reasons, we have concentrated on *Eco*RI fragment 4, a relatively small section of the Vir region. First, heteroduplex mapping between the octopine Ti plasmid pTiAch5 and the nopaline Ti plasmid pTiC58 revealed an area that is not homologous between the plasmids in this fragment (reference 5 and Fig. 1). Second, mutations in the homologous part cause the bacterium to be avirulent. And third, some mutations in the nonhomologous part also cause avirulence of the bacterium. These data suggest the existence of octopine Ti plasmid-specific virulence functions, which for *Eco*RI fragment 4 might be distinguished between general and octopine-specific virulence functions.

We used three different Ti::Tn*5* plasmids for cloning *Eco*RI fragment 4 and for subsequent complementation studies. Two of the Tn*5* insertions (in pAL1583 and pAL1586) are located in the homologous region, and one (in pAL1597) is positioned in the nonhomologous region (Fig. 1). The different *Eco*RI-4::Tn*5* fragments were cloned on the IncP plasmid pRK290 (4), and the IncQ plasmid pKT247 (1). The broad-host-range vector plasmids pRK290 and pKT247 themselves did not have an effect on the virulence of *A. tumefaciens* (9). One set of mutations (in pAL1597, pAL1541, and pAL245) could only completely be restored to virulence if complementation was performed with IncP plasmids. With IncQ plasmids, however, a partial recovery of virulence (small tumors) was found for the same set of mutations. In one case, an IncQ plasmid carrying *Eco*RI fragment 4 even reduced the normal virulence of an *A. tumefaciens* strain. This might indicate that a higher copy number of plasmids, carrying *vir* genes, influences virulence. The virulence of none of the other avirulent mutants tested was restored completely with IncP *Eco*RI fragment 4 *vir* clones. Complementation was ineffective for LBA-1583(pAL1583), whereas only small tumors were formed with LBA1586(pAL1586). From these results, we conclude that a new *vir* operon, which is called *vir*-O, is present on *Eco*RI fragment 4. This operon, mutated in pAL1597, pAL1541, and pAL245, was fully restored to virulence by plasmids carrying *Eco*RI fragment 4, which harbors Tn*5* insertions surrounding *vir*-O. Moreover, a second operon, referred to as *vir*-C, may also be present on *Eco*RI fragment 4, since the mutation in LBA1586(pAL1586) could be partially restored (small tumors occurred) by the other *vir*::Tn*5* mutations cloned on pRK290.

Genetic data indicated that a *vir*-O operon could be present on a 3-kilobase segment within *Pst*I-O, which is part of EcoRI fragment 4 (between the positions of the transposon insertions in pAL1636 and pAL1586 [Fig. 1]). This fragment contains the intact *vir*-O operon indeed, since it restores virulence of *A. tumefaciens vir*-O mutants, but not of a *vir*-C mutant. Because of the positions of different Tn*5* insertions, the right-hand end of the *vir*-O operon could be placed in a 300-base-pair segment, and the left-hand end could be placed in an 1,100-base-pair *Hin*dIII-*Pst*I segment. The size of the *vir*-O operon is therefore estimated to be in the range of 800 to 2500 base pairs (Fig. 2).

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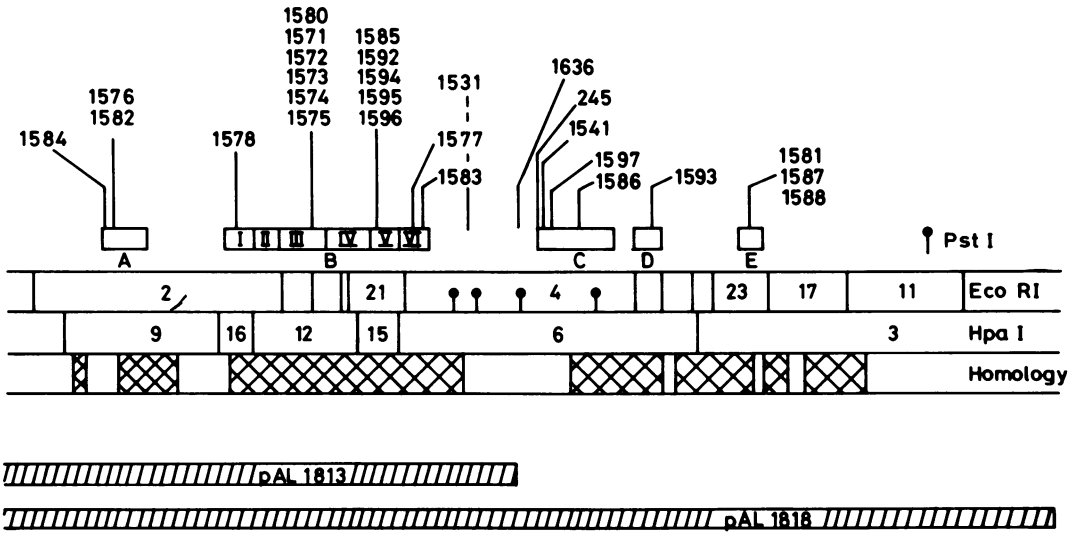

FIG. 1. Location of *vir* mutations. The position of the different transposon insertions is indicated on a physical map of the Vir region for the restriction enzymes *Eco*RI and *Hpa*I. Only in *Eco*RI fragment 4, also *Pst*I sites are shown. The numbers above the insertion positions correspond to pAL numbered Ti plasmids. All insertions cause *Agrobacterium* to be avirulent on tomato, except for LBA1636(pAL1636) which is normally tumorigenic. Homology between octopine and nopaline Ti plasmids has been indicated by double-hatched boxes. In dashed lines the R-prime plasmids pAL1813 and pAL1818, used in the complementation studies, are indicated. Blocks refer to different Vir transcription units (12, 14).

From the present study, an indication has been found for both existence and position of an octopine-specific *vir* operon, the function of which is still unknown. Mutations in this operon cause a host-range effect on *Agrobacterium* spp. (on tomato, no tumor formation; on petunia, normal tumor formation); i.e., the *vir*-O product is not an absolute requirement for virulence in all plant species. Moreover, it has been shown that in the presence of a nopaline Ti plasmid, the octopine T region is transferred to plant cells (11). Thus, *vir* products, and in this case the *vir*-O product, obviously can be replaced by a product encoded by the nopaline Ti plasmid.

The *vir*-C mutation, present in pAL1586 (causing avirulence on all plant species tested), is located in *Pst*I-O; however, it was located in a homologous region between octopine and nopaline Ti plasmids. This *Pst*I fragment, cloned on an IncP plasmid, did not complement the mutation in *vir*-C, indicating that the *vir*-C operon cannot be present on this fragment completely. We cloned *Pst*I-O in the *Eco*RI site of the *gal*K promoter probe plasmid pKO-1 (15), and in

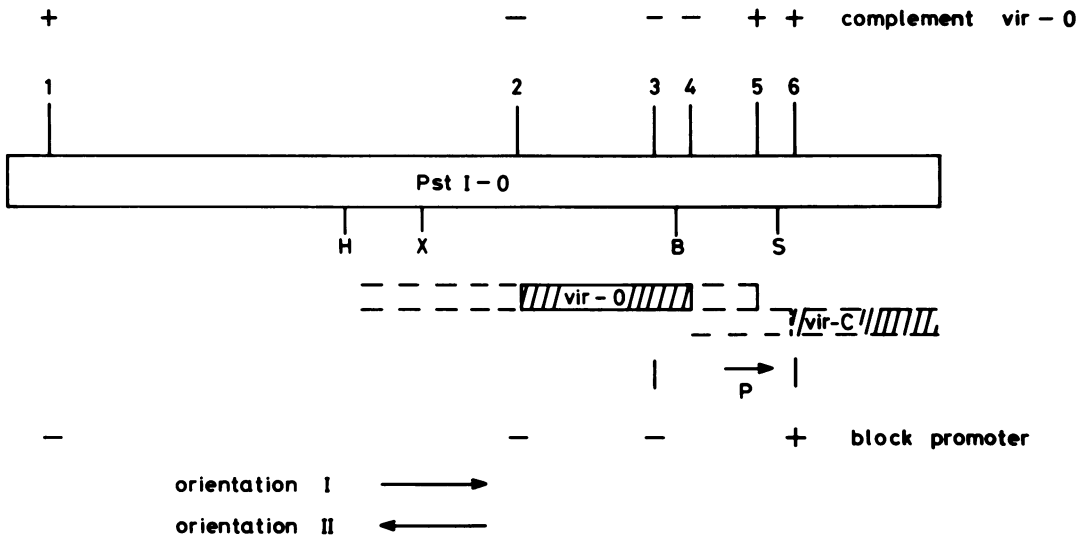

FIG. 2. Localization of *vir*-O and a putative promoter of *vir*-C. The 3.5-kilobase *Pst*I fragment O, cloned in the *Eco*RI site of IncP plasmid pRK252, was used for complementation of *Agrobacterium vir*-O mutants. The numbers 1 to 6 refer to the positions of different Tn*5* insertions into this fragment. Indications + or − above the numbers show whether complementation of *vir*-O mutations occurs. Thus genetically *vir*-O must be positioned between Tn*5* insertions 1 and 5. The numbers 1, 2, 3, and 6 were recloned as *Eco*RI fragments in both orientations on the *gal*K promoter probe plasmid pKO-1, and tested for promoter activity in *E. coli*. −, No blocking; +, blocking of promoter activity in orientation I by Tn*5*. H, *Hin*dIII; X, *Xho*I; B, *Bam*HI; S, *Sma*I.

E. coli significant promoter activity was detected only for the fragment in one orientation (pKO-1; 1 unit of *gal*K activity; pKO-1 harboring *Pst*I-O in orientation I, 30 units, and in orientation II, 1 unit). To map the position of the promoter, we recloned a number of mutated *Pst*I-O::Tn*5* fragments on plasmid pKO-1. In this way, the promoter was localized to a 500-base-pair segment, somewhere at the right-hand end of the *vir*-O operon, which is at the position of divergence of homology between the octopine and the nopaline Ti plasmid (Fig. 2). The direction of this promoter activity is towards the homologous region. This promoter has been identified by its activity in *E. coli*, but no evidence is available yet that this promoter is also functional in *A. tumefaciens*.

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