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Genetics

Short direct repeats flank the T-DNA on a nopaline Ti plasmid

(transposable elements/site-specific recombination/plant tumors/transformation)

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ABSTRACT **Crown gall disease results from the insertion of a segment of the *Agrobacterium* Ti plasmid, called T-DNA, into host plant nuclear DNA. We have subjected to sequence analysis the border regions of pTi T37 (ends of T-DNA) and one left T-DNA/plant DNA border fragment isolated from BT37 tobacco teratoma by molecular cloning. These sequence studies, taken together with published sequence of a right T-DNA/plant DNA border fragment, allowed us to identify the positions of left and right borders at the DNA sequence level. Comparison of left and right border regions of the Ti plasmid revealed a "core" direct repeat of 13 of 14 bases (12 contiguous) precisely at the borders of T-DNA. An extended repeat of 21 of 25 bases overlaps this core repeat. T-DNA on the Ti plasmid exhibits no longer direct or inverted repeats in the border regions, based on Southern hybridization studies. The physical structure of T-DNA differs from that of known prokaryotic and eukaryotic transposable elements but bears a structural resemblance to the prophage of bacteriophage λ.**

Crown gall is a neoplastic disease of higher plants caused by oncogenic strains of *Agrobacterium tumefaciens* (1). Tumor cells are stably transformed in that they exhibit hormone autotrophy *in vitro* in the absence of the inciting bacterium (2). Axenic tumor cells synthesize one or more novel metabolites called opines (3–7) that are specific catabolic substrates for the inciting *Agrobacterium* strain (5, 7–9). The pathogen harbors large plasmids called Ti (tumor inducing) plasmids (10) that code for oncogenicity and specify which opines the tumor will synthesize as well as which opines the bacteria can catabolize (8, 9, 11, 12). A part of the Ti plasmid called T-DNA is stably maintained in the tumor cells (13–17) and is transcribed into polyadenylylated RNAs (14, 18–20). T-DNA transcripts are found on polysomes in the tumor cell (20) and can be translated into proteins *in vitro* (21). Insertion of transposons into the T-DNA region of the Ti plasmid can affect tumor morphology (22–24) or eliminate the synthesis of opines by the tumor (24, 25). T-DNA is in the nuclear fraction of the plant tumor cell (26, 27) covalently joined to host plant DNA (28–30). T-DNA insertion into plant DNA can be viewed as an example of genetic engineering that occurs in nature, for it diverts plant metabolites into opines useful only to the pathogen.

The mechanism by which T-DNA integrates into the plant genome is unknown. The T-DNA region of the Ti plasmid, when compared with T-DNA in the tumor cell by Southern hybridization, indicates that several octopine-type tumors contain two Ti plasmid segments (T_L and T_R) inserted into plant DNA independently (16). T_R has been found at 0–20 copies per tumor cell; T_L has been found at one to several copies, and its edges are variable in different tumor lines (16). A right border fragment of T_L joined to plant DNA, isolated by molecular cloning, hybridizes to a noncontiguous region of the Ti plasmid that maps in the center of T-DNA (29). In contrast to the complex T-DNA inserts found in the octopine tumors, T-DNA of the nopaline tumors has a more simple arrangement. It appears colinear with T-DNA of the Ti plasmid, with borders that appear fixed, within the resolution of the technique of Southern blot analysis. The host plant DNA sites at which it inserts appear to be variable (17). In addition, T-DNA in nopaline tumors includes "fusion" DNA fragments homologous to the left and right edges of T-DNA, presumed to derive from either tandem copies or circular forms of T-DNA (30).

If T-DNA is a discrete physical and genetic element, the DNA sequences at its edges might structurally define it. We have subjected to sequence analysis DNA from regions of the nopaline Ti plasmid pTi T37 that contain the left and right edges of T-DNA. The flanking Ti plasmid DNA exhibits short direct repeats that we propose to be border signals.

MATERIALS AND METHODS

Recombinant Plasmids. *Eco*RI fragments G and 29 (Fig. 1) were subcloned into pBR325 (31) from recombinant phage clone PC1 (28) and recombinant plasmid pBR322 (*Bam*HI-6) (28), respectively. *Hin*dIII fragment 23 (Fig. 1) was subcloned into pBR325 from *Eco*RI fragment 1 of pTi T37. *Eco*RI fragment 1 had previously been cloned into Charon 4A bacteriophage (32, 33).

M13 Cloning Vectors. M13mp7 vector (34) was obtained as *Eco*RI linear DNA from New England BioLabs. M13 phage derivative mWB2341 carries *lac* operon DNA that has been modified to contain one *Eco*RI site and one *Hin*dIII site adjacent to the commercially available primer (arrow on the vector in Fig. 2) for dideoxy sequence analysis (unpublished data). The *lac* DNA was cloned out of mWJ22 (two *Eco*RI sites, one *Hin*dIII site) (35) into the unique *Sau* 96 site of wild-type M13 DNA to obtain mWB2341. Details of construction of this and other vectors in this series will be published elsewhere.

Cloning of T-DNA Fragments into M13 Vectors. The restriction enzymes employed were from New England BioLabs. Sticky ends of restriction fragments were rendered blunt by treatment for 20 min at 37°C with the large fragment of *Escherichia coli* DNA polymerase I (36–38) (New England BioLabs) in 10 mM Tris·HCl, pH 7.9/10 mM $MgCl_2$/50 mM NaCl/10 mM 2-mercaptoethanol and 100 μM each of dATP, dCTP, dGTP, and dTTP at a DNA concentration of 200 μg/ml. All other enzymatic reactions were performed as recommended by the manufacturer and terminated by phenol extraction and ethanol precipitation of the DNA. T4 ligase, from cells infected by λ T4 lig (39), was a gift from M. Bittner. Ligation of sticky ends was carried out in 30 mM Tris·HCl, pH 7.9/4 mM $MgCl_2$/1 mM

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Abbreviation: bp, base pair(s).

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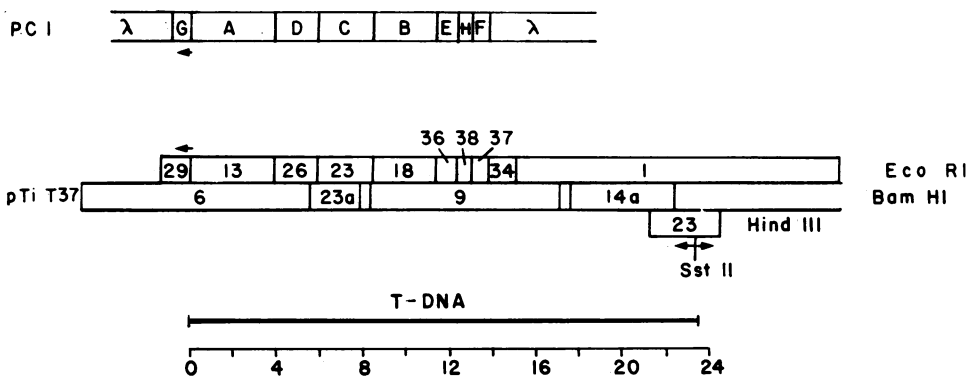

FIG. 1. Restriction maps of recombinant phage clone PC1 and the T-DNA region of pTi T37. PC1 is a recombinant phage containing T-DNA fragments from BT37 tumor tissue obtained as an *Eco*RI partial digest product (28); its *Eco*RI map is shown above. The map of the T-DNA region of pTi T37 shown (M. W. Bevan, personal communication) corrects details of a previous version (28). Phage inserts A, D, C, B, E, H, and F correspond to Ti plasmid *Eco*RI fragments 13, 26, 23, 18, 36, 38, and 37, respectively. Phage insert G is a border fragment containing T-DNA joined to tobacco DNA (28). The arrows indicate the direction of DNA sequence analysis of the fragments at the left and right edges of T-DNA. The scale indicates the size of T-DNA in kilobases.

EDTA/40 μM ATP/10 mM dithiothreitol at a DNA concentration of 16 μg/ml. Blunt-end ligations employed the same buffer with 1 mM ATP/1 mM hexamine cobalt chloride (British Drug House, Poole, England) (unpublished data) at a DNA concentration of 100 μg/ml. All ligations were performed at 4°C for 15 hr.

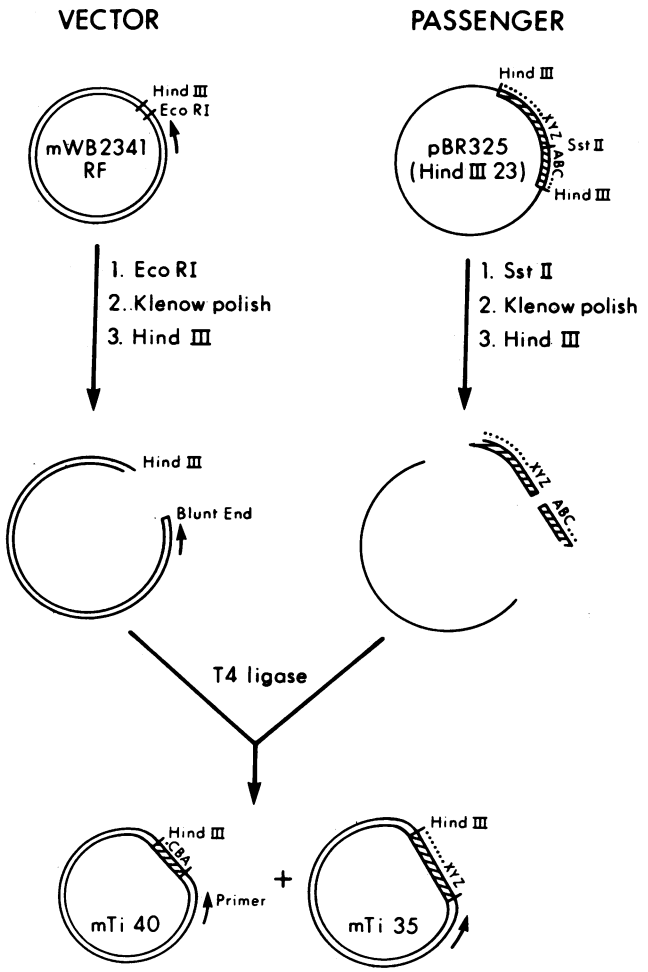

FIG. 2. Strategy for cloning *Hin*dIII fragment 23 subfragments into M13 phage vector. The replicative form (RF) of the M13 phage vector mWB2341 (double line) and the passenger DNA, consisting of *Hin*dIII/blunted *Sst* II subfragments of *Hin*dIII fragment 23, were ligated together as indicated. Recombinant phage clones contained the large (. . . XYZ) or small (ABC . . .) subfragment with the *Sst* II end adjacent to the primer site for DNA sequence determination (indicated by an arrow on the vector). mTi 40 and mTi 35 are not drawn to scale.

*Eco*RI fragments 29 and G were cloned into the *Eco*RI site of M13mp7 as described (34). The *Sst* II/*Hin*dIII subfragments of *Hin*dIII fragment 23 were subcloned out of pBR325 into mWB2341 replicative form by the strategy outlined in Fig. 2, which directs the blunted *Sst* II site adjacent to the *lac* primer (New England BioLabs) used for dideoxy sequence analysis. Phage clones containing the large and small (40) *Sst* II/*Hin*dIII subfragments were designated mTi 35 and mTi 40, respectively.

DNA Sequence Analysis. DNA sequence was obtained from recombinant M13 phage DNA by the method of Sanger *et al.* (41) as adapted by Barnes (40).

RESULTS

Left Boundary. To determine at the nucleotide sequence level the position of a left border of T-DNA in the nopaline crown gall tumor, we subjected to sequence analysis a cloned left T-DNA/plant DNA border fragment (fragment G) (28) and the corresponding region of pTi T37 (*Eco*RI fragment 29) (Fig. 1) leftward from their common *Eco*RI site. The sequence from fragment G perfectly matched the sequence from fragment 29 for the first 68 bases to the left of the *Eco*RI site and then diverged, presumably into plant DNA (Fig. 3A). Computer dot matrix analysis of 153 bases of fragment G sequence vs. fragment 29 sequence revealed no other direct or inverted repeats.

Computer analysis of the sequence of *Eco*RI fragment 29 revealed a Chi site, 5′-G-C-T-G-G-T-G-G-3′ (43), and four Chi-like octamer sequences, one differing from Chi by one base and the others by two bases (Fig. 3A). These all occur in the same orientation within a 34-base region that lies 12 bases outside the left T-DNA border. We found no such Chi-related elements in either orientation in the region of pTi T37 that contains the right border of T-DNA (see below).

Right Border. The BT37 tumor line contains several T-DNA copies; thus, the single right T-DNA/plant DNA border fragment that was subjected to sequence analysis by Zambryski *et al.* (30) may not derive from the insert whose left border our fragment G represents. Nevertheless, any right border fragment should serve to determine the location of the border, if T-DNA is a discrete element.

*Hin*dIII fragment 23, which contains the right border (17, 30), was subcloned as *Hin*dIII/*Sst* II fragments into M13 vector mWB2341 to yield mTi 35 and mTi 40 (Fig. 2). By Sanger (32) sequence analysis of mTi 35, 310 bases were determined to the left (M. W. Bevan, personal communication) of the *Sst* II site and from mTi 40, 270 bases to the right (Fig. 3B) (see arrows in

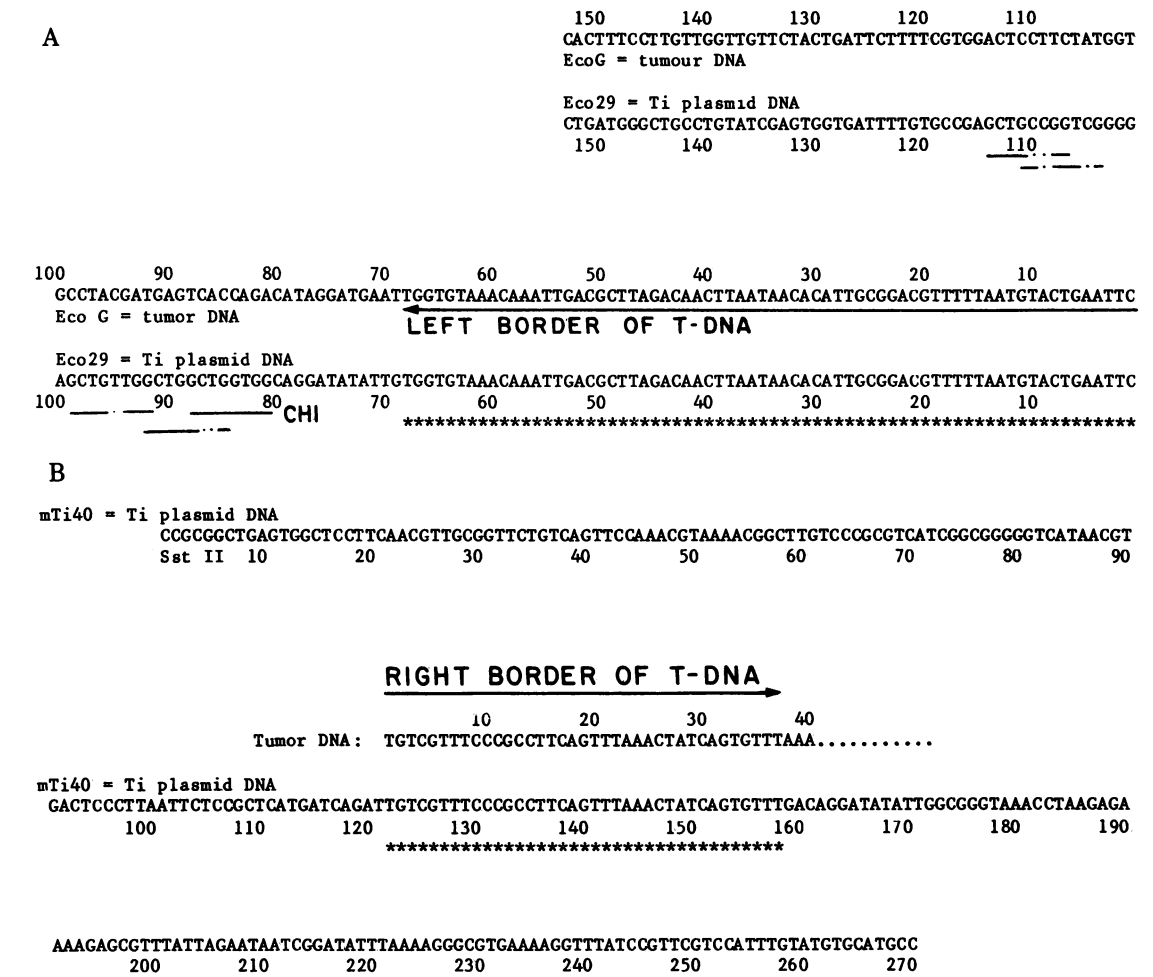

FIG. 3. DNA sequence of left and right border regions of pTi T37 vs. left and right border clones from BT37 tumor DNA. (*A*) Sequence of *Eco*RI fragments G (from tumor) and 29 (from Ti plasmid). Stars and arrow mark the 68 base pairs (bp) of matching sequence to the left of the *Eco*RI site (numbered 1–6). The figure presents the complement of the strands actually subjected to sequence analysis, so that the orientation of this sequence corresponds to the orientation of fragments G and 29 on the map in Fig. 1. A Chi site (G-C-T-G-G-T-G-G) (42) and four Chi-like octamers are underlined in the sequence of fragment 29. (*B*) Sequence of mTi 40 (from Ti plasmid) is compared with the published BT37 tumor border fragment sequence (30). Stars and arrow mark matching sequences that define the point of divergence into plant DNA 158 bp to the right of the *Sst* II site (numbered 1–6).

Fig. 1). Comparison with the partial sequence of the right border fragment of Zambryski *et al.* (30) (reproduced in Fig. 3*B*) shows the position of the right border to be 158 bases to the right of the *Sst* II site.

Direct Repeats Flank T-DNA on the Ti Plasmid. Comparison of Ti plasmid DNA sequence in the left and right border regions revealed that the first 14 bases outside T-DNA on the Ti plasmid constitute a direct repeat of 13 of 14 bases, 12 contiguous. This 14-mer forms a part of an extended repeat of 21 of 25 bases (Fig. 4). The Chi sequence near the left border overlaps the extended repeat by two bases. Computer dot matrix analysis revealed no other significant direct or inverted repeats in the 153 bases sequenced in *Eco*RI fragment 29 vs. 580 bases sequenced around the *Sst* II site in *Hin*dIII fragment 23.

Absence of Longer Repeats. To test whether there was a much longer repeated sequence at the borders of T-DNA in pTi

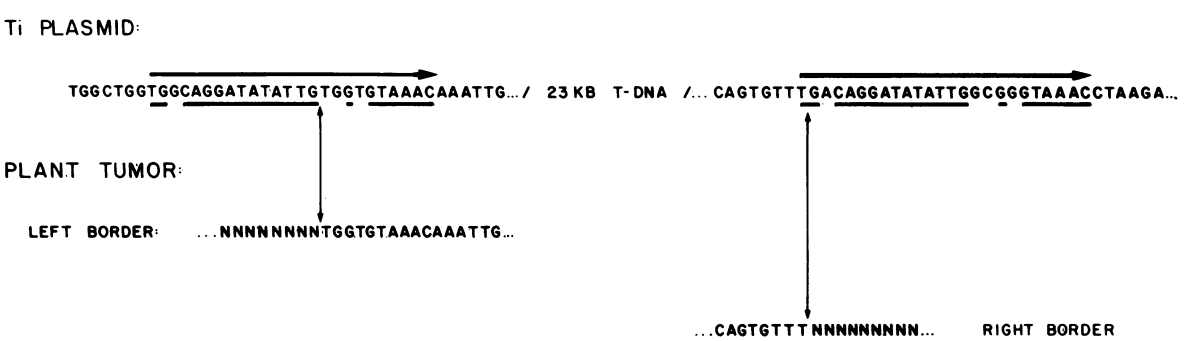

FIG. 4. DNA sequence of border repeats. Sequence data from the border regions (Fig. 3) are presented with the 25-bp direct repeats marked by horizontal arrows and bases within the repeat that match are marked by underlining. The border fragments from tumor DNA are abstracted, with NNNNN . . . representing presumed plant DNA to emphasize the fate of the 25-bp repeat upon T-DNA integration into plant DNA.

T37 that escaped detection by our limited sequence study, we looked for homology between regions of pTi T37 that contain the left and right borders of T-DNA by DNA hybridization. For this purpose, the recombinant clone of pBR325 containing *Hin*dIII fragment 23 was digested with *Hin*dIII, *Sst* II, and *Bam*HI. The three resulting subfragments (designated 1, 2, and 3 in Fig. 5) were excised and collected by electroelution. Similarly, *Eco*RI fragments 29 and G were isolated after digestion of the appropriate recombinant plasmids with *Eco*RI. Isolated fragments were subjected to gel electrophoresis and Southern blotting (42) and were hybridized to labeled *Eco*RI fragment 29 (Fig. 5) and labeled *Eco*RI fragment 13 (recombinant plasmid) (data not shown). These probes from the left border of T-DNA failed to hybridize to fragments 1, 2, and 3 from the right border region but hybridized strongly to homologous DNA. We conclude that there is no extended homology between the left and right border regions of pTi T37.

DISCUSSION

Conservation of 25-bp Repeat on Octopine Ti Plasmid. The data presented here suggest that the borders of T-DNA in nopaline-type Ti plasmid pTi T37 are determined by specific DNA sequences. T-DNA on the Ti plasmid is flanked by direct repeats of 13 of 14 bp (12 contiguous). Overlapping these are 25-bp imperfect repeats. The significance of these repeats is underscored by the finding of similar sequence at the left border of an octopine Ti plasmid (44):

Octopine Ti left repeat:

C ↓ **G-G-C-A-G-G-A-T-A-T-A-T-T-C** - A-A-T-T- **G-T-A-A-A-T**

Nopaline Ti left repeat:

T - G-G-C-A-G-G-A-T-A-T-A-T-T-G ↓ **T-G-G-T-G-T-A-A-A-C**

Nopaline Ti right repeat:

T ↓ **G-A-C-A-G-G-A-T-A-T-A-T-T-G** - G-C-G-G-**G-T-A-A-A-C**

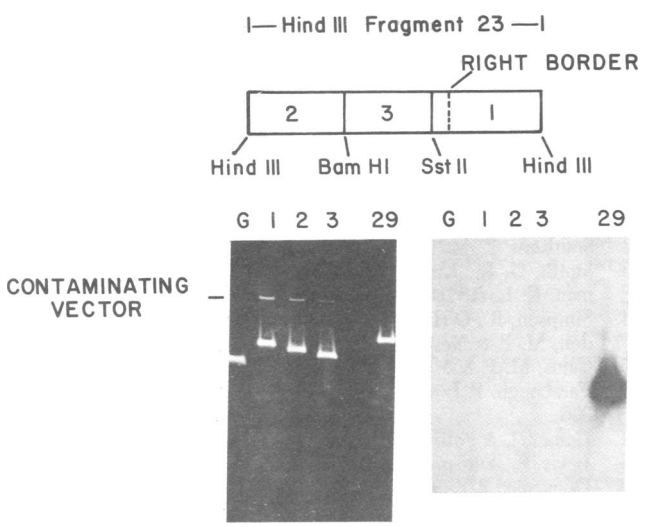

FIG. 5. Hybridization of labeled left border probe with Southern blots of right border region fragments. (*Upper*) Map of *Hin*dIII fragment 23. (*Lower Left*) Photograph of ethidium bromide-stained agarose gel containing 1 μg of the following DNA fragments, previously isolated from preparative agarose gels by electroelution: G (the left T-DNA/plant DNA border fragment); 1, 2, and 3, the subfragments of *Hin*dIII fragment 23; and 29, *Eco*RI fragment 29. (*Lower Right*) Autoradiogram of Southern blot made from the agarose gel after hybridization with *Eco*RI fragment 29. No homology is detected between left and right border regions. Similar results were obtained with *Eco*RI fragment 13 (data not shown). DNA fragments are numbered as in *Lower Left*.

Arrows in the above sequence indicate the ends of colinear T-DNA sequence found in border fragment clones isolated from plant tumors; bases shown in **boldface** are those conserved on both ends of nopaline T-DNA and their counterparts in the octopine Ti repeat. Arrows (points of discontinuity) occur at the left and right ends of the imperfect 14-mer G-N-C-A-G-G-A-T-A-T-A-T-T-N (N is any nucleoside), consistent with a site-specific recombination mechanism.

The DNA sequence surrounding the 25-bp repeat on the octopine Ti plasmid is unrelated to the context sequence of the nopaline Ti plasmid repeats, based on our dot matrix analysis (data not shown) of the sequence of the left border region of the octopine Ti plasmid (44). We infer that this repeat *per se* signals T-DNA borders and is of functional significance rather than structural significance because it is preserved on the octopine Ti plasmid whereas surrounding DNA is not.

T-DNA Structure Differs from That of Transposons. No known prokaryotic transposon or eukaryotic transposable element has border structures resembling those of T-DNA reported here. Prokaryotic transposons possess long direct or inverted repeats that stay joined to the transposon when it moves to a new location (45). Eukaryotic transposable elements characterized thus far have long direct repeats that appear to move with the element (46–49). Retrovirus proviruses structurally resemble the eukaryotic transposable elements, with direct repeats hundreds of bp in length (50). Thus, none of these elements affords a clear precedent for the structure and recombination pattern of T-DNA: short imperfect direct repeats that do not become inserted intact into the plant genome.

All of the known transposable elements generate short direct repeats of host DNA flanking each insert (45–50). These are diverse for a given element and appear to be a structural by-product of the transposition process (45). We have not determined whether the T-DNA insert in the plant genome is flanked by variable direct repeats of plant DNA (a study requiring isolation of both borders of a single T-DNA insert). T-DNA *on the Ti plasmid* (as defined by the border positions identified here) is flanked by imperfect 14-mers.

The closest parallel that we can find between T-DNA and a transposable element is a certain resemblance to the prophage of bacteriophage λ. Although present information is insufficient to allow any firm conclusion, the following parallels with λ exist: The λ prophage is flanked by functional 15-bp repeats, which are signals for site-specific recombination (51), resulting in excision of λ as a circle carrying one copy of the repeat. A similar model for T-DNA excision would predict the net preservation of one copy of the extended 25-bp repeat in each T-DNA insert, possibly split between the two borders where T-DNA adjoins plant DNA. Our data are consistent with such a model.

Further, a λ-like mechanism would predict that circular T-DNA would insert at plant DNA sites somewhat homologous to the 25-bp repeat. Consistent with this idea, we find homology between the presumed plant DNA component of border fragment G and the "missing" end of the 25-bp repeat. From the sequences of Fig. 3*A* we see the following matching bases (shown in **boldface**) on the plant DNA side of the border, 2 bp out of register:

Border fragment G:

A-G-A-C-A-T-**A-G-G-A-T**-G-A-**A-T** ↓ T-G-G-T-G-T-A-A-A-C

Left Ti plasmid repeat:

T-G-G-C-**A-G-G-A-T**-A-T-**A-T**-T-G- T-G-G-T-G-T-A-A-A-C

↑ Border

Insufficient data have been published for the right border fragment to allow similar analysis for it.

Chi Site. The role of the Chi element at the left end of T-

DNA on pTi T37 is not clear. Chi augments recombination in λ over a broad region; its greatest effect extends leftward as Chi is written here (52). Site-directed mutagenesis of Chi on the Ti plasmid will test whether it affects the process of T-DNA excision. We note that Chi is not transferred intact to the plant genome with T-DNA; thus, any effect it might exert would presumably be in the bacterium.

Genetic Analysis of Role of Repeats. The data presented here suggest that the border repeats define what will be T-DNA. Deletion of the right border repeat by Tn*1* has been found to attenuate but not abolish tumor induction (17, 24). The Tn*1* DNA in this case formed the border of T-DNA (17). This interesting result implies that alternative borders can substitute when a normal border is deleted. λ phage can utilize secondary attachment sites when the primary site is deleted, even when the homology is as low as 9 of 15, although the efficiency is diminished (53, 54).

The unusual nopaline Ti plasmid pTi AT181, which is closely related to pTi T37, has a large deletion at the left end of its T-DNA (compared to pTi T37); however, DNA sequence analysis has revealed that the left border repeat survives intact in this virulent strain (unpublished data).

Deletion of border repeats followed by their reintroduction at new positions can be achieved by site-directed insertions into the Ti plasmid (55–57). Such studies will rigorously test whether the border repeats signal the functional ends of T-DNA. The presence of the 25-bp direct repeats at the edges of T-DNA provides the first clue to the mechanism of illegitimate recombination between prokaryotic Ti plasmid DNA and the eukaryotic host plant genome.

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