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Genetics

The right border region of pTiT37 T-DNA is intrinsically more active than the left border region in promoting T-DNA transformation

(crown gall/Ti plasmid/binary vectors/mini-T plasmid/T-DNA excision)

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ABSTRACT **Deletions of border regions of T-DNA on *Agrobacterium* Ti plasmid or mini-T plasmid have shown the right border region of pTiT37 T-DNA to be more active than the left border region in promoting T-DNA transformation. In this study we examine the possibility that the apparent difference in activity of left and right border regions may be due to position or orientation differences between the left and right borders with respect to the transferred *onc* genes. We have constructed eight similar single-border mini-T plasmids that contain a left border segment or a right border segment of pTiT37 T-DNA at various positions with either orientation with respect to the *onc* genes. We assayed the plant tumor-inducing activity of these mini-T plasmids in *Agrobacterium tumefaciens* LBA4404 containing the virulence helper plasmid pAL4404. Regardless of the position and orientation of the border-containing segment in the mini-T plasmid, mini-T plasmids with the right border segment were highly virulent, whereas those with the left border segment were only weakly so. These results indicate that the difference in transformational activity between the left and right border regions is intrinsic and not an effect of position or orientation with respect to the *onc* genes. The pattern of the mini-T plasmid sequences integrated into the plant genome suggests that T-DNA transformation involves the directional transfer of a linear intermediate bounded by the border repeats.**

Agrobacterium tumefaciens is the causative agent of crown gall disease of dicotyledonous plants. It induces tumor formation on the host plant by a gene transfer mechanism. Large plasmids called Ti (tumor-inducing) plasmids in the bacterium carry genetic determinants for tumor induction (1). During the transformation process, a specific segment of the plasmid, called the T-DNA (transferred DNA), is transferred to the plant cells and incorporated into their nuclear genomes (2, 3) and occasionally into their chloroplast genomes (4). The T-DNA encodes three known oncogenicity (*onc*) functions involved in phytohormone biosynthesis (5–9). The expression of these *onc* genes in the transformed plant cells results in tumor formation. The T-DNA also encodes genes involved in the biosynthesis of novel metabolites called opines (10). For recent reviews of the molecular biology of crown gall disease see refs. 11 and 12.

Two regions of the Ti plasmid are essential for T-DNA transformation process. One is the virulence (*vir*) region located counterclockwise from the T-DNA on the Ti plasmid map (13, 14). Mutations in the *vir* region can result in the loss of T-DNA transformation activity. Transcriptional activities within the *vir* region are induced when *Agrobacterium* is incubated with plant cells or extracts (15). This induction effect suggests that *vir* functions are involved in T-DNA transfer from the bacterium to the plant cell. The other region essential to T-DNA transformation is the right border region of the T-DNA. Ti plasmids with the T-DNA right border region deleted are avirulent (16, 17). Interestingly, deletion of the left border region, which has structural similarity with the right border region (see below) of T-DNA, has no effect on virulence (17).

Imperfect direct repeats [25 base pairs (bp) in length] flank the T-DNA on the Ti plasmid (18). These repeats appear to precisely define the ends of the T-DNA (18, 19). The 25-bp repeat is an essential element of the right border region; removal of parts or the whole of the 25-bp repeat abolishes the activity of the right border region to promote T-DNA transformation (20, 21).

We have investigated the requirement for T-DNA border regions in T-DNA transformation by using a binary transformation system (22, 23). In the binary system, the T-region (the region containing the T-DNA) is located on an independent replicon, which we have termed a mini-T plasmid (24), separate from that containing the *vir* region. The high transformational activity of binary systems indicates that the *vir* functions can act in *trans* to promote T-DNA transfer and integration (22–24). In a recent study with mini-T plasmids constructed from cosmid clones of the T-region of nopaline Ti plasmid pTiT37, we showed that mini-T plasmids must possess at least one T-DNA border region in order to transform the host plant (24). Surprisingly, in contrast to Ti plasmids, we found that mini-T plasmids without a right border region but containing a left border region were transformationally active, albeit significantly weaker than those containing both border regions or only the right border region. Because the left-border-only mini-T plasmids of this earlier study contained the left border region in its natural position with respect to the *onc* genes, it was possible that their weaker transformational activity might be due to unfavorable positioning of the left border region with respect to the transferred *onc* genes. In this study we examine this possibility by comparing the virulence of a series of very similar mini-T plasmids that contained either a single right border segment or a single left border segment at different positions and orientations with respect to the *onc* genes. The results demonstrate that the right border region of pTiT37 T-DNA is intrinsically more active than the left border region in promoting T-DNA transformation, independent of position or orientation.

In this study we also examined the structure of the T-DNA transmitted by two single-border mini-T plasmids. The patterns of the T-DNA are consistent with a T-DNA transformation process that involves the directional transfer of a linear intermediate.

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Abbreviations: kb, kilobase(s); bp, base pair(s).

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MATERIALS AND METHODS

Bacterial Strains and Media. *Escherichia coli* strain χ1891 (F⁻, *thr-16*, *tsx-63*, *purE41*, *supE42*, λ⁻, Δ*trpE63*, *his-53*, *gyrA23*, *srl-2*, Δ*thyA57*, *tte*, *mtlA9*, *polA12*, *cycB2*, *cycA1*) was kindly provided by R. Curtiss III. Other bacterial strains and plasmids used in this study were described previously (24). The constructions of C1–C8 mini-T plasmids are described in *Results*.

E. coli χ1891 and derivatives were grown on LT2 agar or LT2 broth [LB medium (25) supplemented with thymidine at 40 μg/ml and tryptophan at 20 μg/ml] at 30°C except during and after selection for cointegrate mini-T plasmids. Other bacterial strains were grown on LB agar or LB broth. To select for drug-resistant *E. coli*, we used tetracycline (15 μg/ml), carbenicillin (100 μg/ml), kanamycin (50 μg/ml), or nalidixic acid (50 μg/ml). To select for drug-resistant *Agrobacterium tumefaciens* LBA4404, we used tetracycline (5 μg/ml) and carbenicillin (10 μg/ml).

Four border-containing pRK325 shuttle vectors were constructed by inserting *Bam*HI fragment 3a or *Hin*dIII fragment 10 of Ti plasmid pTiT37 into the unique *Bam*HI or *Hin*dIII site of pRK325. Clones containing the insert in both orientations were isolated; these were pRKB14 and pRKB24, containing the *Bam*HI fragment 3a, and pRKH4 and pRKH27, containing the *Hin*dIII fragment 10.

Bacterial Conjugations and the Formation of Cointegrate Mini-T Plasmids. The pOT16 plasmid was transferred from *E. coli* LE392 to *E. coli* χ1891 containing pRK325 or pRK325-derived plasmids by using the triparental mating procedure (26). Twenty microliters of each fresh overnight culture of LE392(pRK2013), LE392(pOT16), and χ1891 containing a pRK325-derived plasmid were plated on a 60-mm LT2 agar plate. The mixture was grown for 12–24 hr at 30°C. χ1891 transconjugants containing pOT16- and pRK325-derived plasmids were selected at 30°C on LT2 plates containing carbenicillin, tetracycline, and nalidixic acid. Purified χ1891 transconjugants were streaked out on LT2 plates containing tetracycline and carbenicillin and were grown at 42°C to select for isolates containing mini-T plasmids formed by cointegration of pOT16 with pRK325-derived plasmids. Plasmid DNA was prepared from χ1891 containing cointegrate plasmids by using the alkali lysis procedure and was digested with *Eco*RI restriction endonuclease to reveal diagnostic fusion fragments indicative of cointegrate formation. Cointegrate mini-T plasmids were transferred from χ1891 to *A. tumefaciens* LBA4404 by the triparental mating procedure. χ1891 containing cointegrate mini-T plasmid was grown in LT2 broth at 37°C; 20 μl of overnight culture was plated on LT2 agar with 20 μl of overnight culture of LE392(pRK2013) and 40 μl of a 2-day-old culture of LBA4404 in AB minimal medium. The mating mixture was grown for 2 days at 30°C. LBA4404 containing a mini-T plasmid was selected from the mating mixture by plating on AB minimal plates containing carbenicillin at 10 μg/ml and tetracycline at 5 μg/ml.

Preparation and Analysis of DNA. The isolation and analysis of DNA were described previously (24).

Virulence Assays. We tested *A. tumefaciens* strains for virulence on leaves of *Kalanchoe daigremontiana* and carrot disks as described previously (24).

RESULTS

Construction of a Borderless Mini-T Plasmid (pOTS16). We formed borderless pOTS16 mini-T plasmid by cointegrating pOT16 plasmid (24) containing borderless T-DNA oncogenes with the wide host range shuttle vector pRK325 (24, 27). For this purpose, pOT16 (24), a pHC79 clone containing the *onc* genes of pTiT37, was transferred from *E. coli* LE392 into *E. coli* χ1891 containing pRK325 by using the triparental mating procedure (26). χ1891 transconjugants were isolated by drug selection at 30°C.

To select for cointegration of pOT16 with the shuttle vector, we exploited the fact that χ1891 has a temperature-sensitive DNA polymerase I and is therefore unable to replicate pOT16, a pMB1 replicon, at restrictive temperature (42°C). When χ1891 transconjugants are subjected to selection for carbenicillin resistance (a pOT16 marker) at 42°C, maintenance of carbenicillin resistance is predominantly due to cointegration between pOT16 and the pRK325 shuttle vector, whose replication is independent of DNA polymerase I.

The cointegration can occur in two ways because pRK325 possesses homology to two separate regions of pBR322, the progenitor of pHC79 (28). The two regions of homology are contained within the 1.4-kilobase (kb) *Eco*RI–*Bam*HI fragment of pBR325 (29) present in pRK325. One region is a 482-bp segment from the C-terminal part of the tetracycline resistance gene of pHC79 (30); the other region is the 375-bp *Hin*dIII–*Bam*HI fragment common to both pHC79 and the 1.4-kb *Eco*RI–*Bam*HI fragment of pBR325 (28–30). In the 1.4-kb *Eco*RI–*Bam*HI fragment, the 482-bp segment is adjacent to the 375-bp *Hin*dIII–*Bam*HI segment and is inverted relative to the 375-bp *Hin*dIII–*Bam*HI fragment (30). Because cointegration between pOT16 and pRK325 can occur through either region of homology, it gives rise to two different mini-T plasmids (Fig. 1). Cointegration via the 482-bp segment (Fig. 1, plasmids a and b) produces "type a" pOTS16 mini-T plasmid (Fig. 1, plasmid d), whereas cointegration via the 375-bp *Hin*dIII–*Bam*HI fragment (Fig. 1, plasmids b and c) produces "type b" pOTS16 mini-T plasmid (Fig. 1, plasmid e).

Addition of T-DNA Border Regions to pOTS16. We showed previously that the pOTS16 mini-T plasmid that contains the *onc* genes of pTiT37 but no T-DNA border regions is avirulent in a binary transformation system (24). To examine the position and orientation effects of border regions, T-DNA left and right border regions were added in two positions and two orientations to the shuttle vector into which pOT16 was to cointegrate. Border-region-containing fragments were inserted into pRK325 at either the unique *Bam*HI or the unique *Hin*dIII restriction site (Fig. 1, plasmids a and c). These border-region-containing shuttle vectors were cointegrated with pOT16 to form mini-T plasmids containing T-DNA border regions. To examine the effect of the right border region, the 14-kb *Bam*HI fragment 3a of Ti plasmid pTiT37 was inserted into the *Bam*HI site of pRK325. The right border of pTiT37 T-DNA is located in this fragment at approximately 1 kb from its left end (18, 31). To examine the effect of the left border region, the *Hin*dIII fragment 10 of pTiT37 was inserted into the *Hin*dIII site of pRK325. The *Hin*dIII fragment 10 is approximately 7 kb long and contains the left border approximately in its center (18, 31). Each border fragment was inserted in both possible orientations into pRK325, to examine the effect of the border region orientation on T-DNA transformation efficiency.

The mechanics of cointegrate formation between pOT16 and each of the border-region-containing pRK325 derivatives are the same as those for the formation of pOTS16; each cointegration can give rise to the a and b types of mini-T plasmid as already noted for pOTS16 (see Fig. 1). The final locations of the inserted border fragment are different for a and b types of mini-T plasmid as shown in Fig. 2, because the *Hin*dIII and *Bam*HI restriction endonuclease sites into which they are cloned have different fates in the two cointegration pathways (see underlined *Hin*dIII and *Bam*HI sites in Fig. 1, plasmids d and e). The two types of mini-T plasmid were distinguished from each other by *Eco*RI restriction enzyme digestion, which yields type-specific diagnostic fusion fragments.

In total, we constructed eight different derivatives of pOTS16 mini-T plasmids containing a single T-DNA border

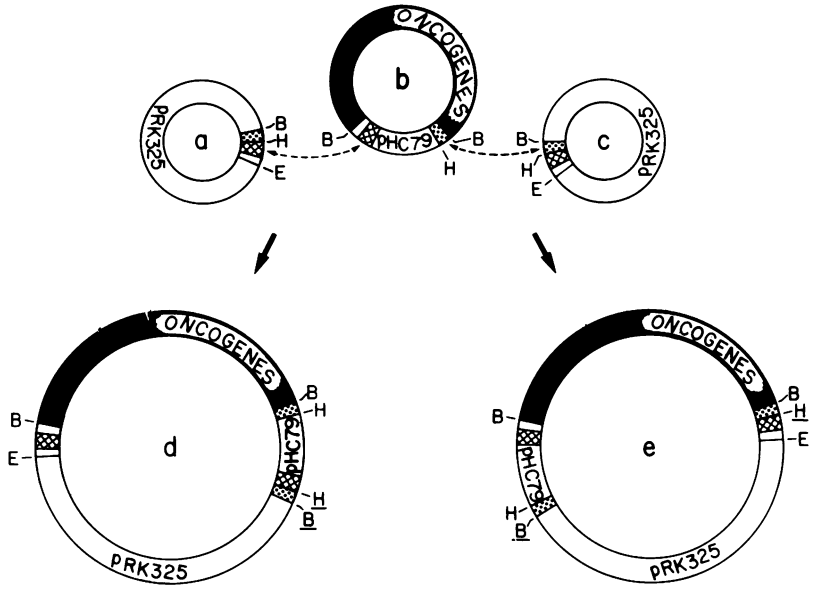

FIG. 1. Mini-T plasmid formation. The pOTS16 mini-T plasmids were formed by cointegration between pOT16 and the shuttle vector pRK325. The c form of pRK325 is inverted with respect to the a form. Cointegration between pOT16 (b) and pRK325 (a) via the 482-bp common segment (the cross-hatched segment) gives rise to "type a" pOTS16 mini-T plasmid (d). Cointegration between pOT16 (b) and pRK325 (c) via the 375-bp segment (the stippled segment) gives rise to "type b" pOTS16 mini-T plasmid (e). Each cointegration occurs by a single crossover event between the common segments indicated by the broken line with arrowheads. See text for explanation of the two pathways of cointegration. The locations of the unique *Bam*HI (B) and *Hin*dIII (H) restriction sites of pRK325 in the cointegrate mini-T plasmids are underlined (d and e). E, *Eco*RI site.

region (Fig. 2) by this approach in χ1891 (two borders × two orientations × two cointegrate types). After verification of their structures with restriction enzyme digest analysis (data not shown), the mini-T plasmids were transferred by triparental mating into *A. tumefaciens* LBA4404 to assay their virulence.

Effects of T-DNA Border Region Position and Orientation on Transformation Activities of Mini-T Plasmids. The virulence of *A. tumefaciens* LBA4404 containing each of the eight single-border pOTS16 derivatives was assayed by the carrot disk and kalanchoe leaf assays. The results are summarized in Fig. 2.

The right border region in pOTS16-C2 mimics closely the normal position and orientation, as found in pTiT37, of the T-DNA right border region with respect to the T-DNA *onc* genes (Fig. 2, construct 2). Construct 2 is highly virulent (score of ++++ in the virulence assay, Fig. 2). Neither inverting the orientation of the right border fragment with respect to the *onc* genes (Fig. 2, constructs 1 and 5) nor placing it farther from the *onc* genes (Fig. 2, constructs 1, 5, and 6) on the pOTS16 mini-T plasmid significantly altered the virulence from that of construct 2.

In pTiT37 the DNA segment containing the left border region of T-DNA is to the left of the *onc* genes. We showed previously that mini-T plasmids with the left border region in its normal position and orientation and without a right border region were weakly active (24). Placing the T-DNA left border region at the position and orientation normally assumed by the right border region (Fig. 2, constructs 4 and 8) or inverting its orientation at this new position (Fig. 2, constructs 3 and 7) did not result in more virulent mini-T plasmids.

Analysis of T-DNA in Mini-T Plasmid-Transformed Plant Cells. Carrot tumor tissues induced by pOTS16-C2 or pOTS16-C6 mini-T plasmids were analyzed to determine what part of the mini-T plasmid was transferred and integrated into the plant genome. We prepared DNA from uncloned tumor tissue, digested it with restriction endonuclease *Eco*RI, and prepared Southern blots of the separated fragments. The blots were probed with radiolabeled pOTS16-C2 plasmid DNA. Positive control lanes of *Eco*RI-digested pOTS16-C2 and -C6 plasmid DNA were included on the blots. A negative control of *Eco*RI-digested normal carrot DNA exhibited three strong bands of hybridization due to homologies with the pRK325 sequences in the mini-T plasmid (G.C.J., unpublished observations). These bands were located at positions that presented little interference with the analysis of the mini-T plasmid sequences in the tumor cells

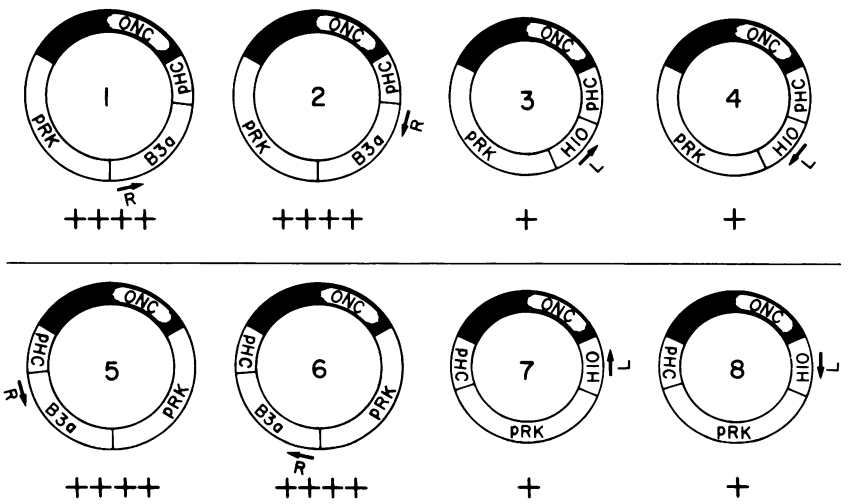

FIG. 2. Border additions to pOTS16 and their effect on virulence. C1 and C5 mini-T plasmids (1 and 5, respectively) are formed by cointegration between pOT16 and pRKB14; C2 and C6 mini-T plasmids (2 and 6, respectively), by cointegration between pOT16 and pRKB24; C3 and C7 mini-T plasmids (3 and 7, respectively), by cointegration between pOT16 and pRKH27; C4 and C8 mini-T plasmids (4 and 8, respectively), by cointegration between pOT16 and pRKH4. C1, C2, C3, and C4 mini-T plasmids are formed by the type a pathway; C5, C6, C7, and C8 mini-T plasmids, by the type b pathway (see Fig. 1). The locations and orientations of the left (L) and right (R) border repeats are indicated by the arrows. The left end of the repeat is at the tail of the arrow and the right end of the repeat is at the head of the arrow. The virulence of these mini-T plasmids in *A. tumefaciens* LBA4404 was assayed on carrot disks and kalanchoe leaves, and the results are shown below each mini-T plasmid: ++++, fully virulent; +, weakly virulent (see ref. 24 for explanation of virulence scoring).

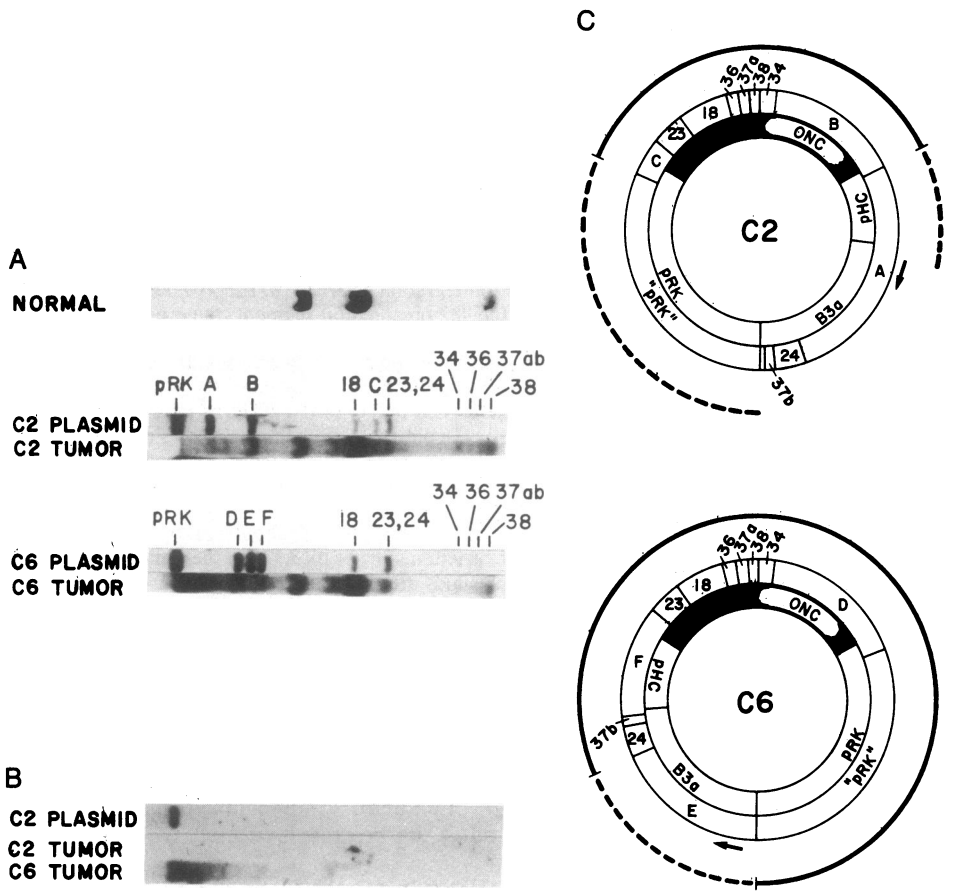

FIG. 3. Southern hybridization analysis of T-DNAs of C2 and C6 carrot tumors. The isolated tumor DNA was digested with *Eco*RI, subjected to agarose electrophoresis, and transferred to nitrocellulose as previously described (24). (*A*) Results of hybridization with radiolabeled C2 mini-T plasmid DNA. The identities of the bands are noted in the margin above each plasmid lane (see *C* for the *Eco*RI restriction maps of C2 and C6 mini-T plasmids). The C2 and C6 plasmid lanes show 0.2-copy-per-diploid-genome reconstructions. The Southern blot was regenerated by washing in boiling 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ and hybridized with radiolabeled pMO25 plasmid DNA (*B*). (*C*) Structures (inner ring) and *Eco*RI restriction map (outer ring) of C2 and C6 mini-T plasmids. The arrows show the locations and orientations of the right border repeats. The outer line indicates the extent of T-DNA of each mini-T plasmid. The solid line represents the DNA segments found intact in the carrot tumors. The broken line indicates the presumed locations of one end of the T-DNAs.

(Fig. 3*A*). C2 tumor DNA contains as internal fragments all *Eco*RI fragments mapping in or near the *onc* genes (Fig. 3*A*) as summarized by the solid perimeter line in Fig. 3*C*. C6 tumor DNA contains the same fragments, plus substantially more vector DNA fragments (Fig. 3*A*), as summarized by the solid perimeter line in Fig. 3*C*.

To confirm that C6 tumor DNA but not C2 tumor DNA contains intact pRK325 DNA, the probe was washed off of the blot shown in Fig. 3*A* and the blot was reprobed with radiolabeled pMO25 plasmid DNA. The pMO25 plasmid is a deletion derivative of pRK325 that does not exhibit hybridization with normal carrot DNA (G.C.J., unpublished observations). C6 tumor DNA exhibited strong hybridization at the position of pRK325 DNA (Fig. 3*B*), whereas C2 tumor DNA failed to do so. The solid line for integrated DNA of C6 therefore extends through the pRK325 region (Fig. 3*C*). The broken lines in Fig. 3*C* indicate the locations of the presumed ends of the integrated plasmid sequences.

The above results indicate that not all of the single-border mini-T plasmids is necessarily transferred and integrated into the host plant genome and that the left end of the border repeat (the tail end of the arrows in the figures) appears to define one end of the DNA transmitted. This is especially clear for pOTS16-C2, whose tumor DNA does not contain *Eco*RI fragment 24 or pRK325 as internal fragments. In the case of pOTS16-C6, the border repeat is approximately halfway around the circular DNA molecule from the *onc* genes and more of the plasmid appears to be transmitted.

DISCUSSION

Intrinsic Difference Between Left and Right Border Regions in Promoting T-DNA Transformation. It is clear from the results presented here that independent of position or orientation, the left border region of Ti plasmid pTiT37 is intrinsically much less active than the right border region in promoting T-DNA transformation from mini-T plasmids. This is a surprise finding, given that the 25-bp repeat at the left border is nearly identical (21 out of 25 bp) (18) to that at the right border. The large difference in activity between the right and left border regions may be due to these small differences between their border repeats, or it could reside in neighboring sequences included on the long restriction fragments employed here as "border fragments." Consistent with the latter possibility, results from border region resection experiments indicate that sequences a short distance to the right of the right border repeat are essential to high efficiency of transformation (ref. 32 and unpublished observations).

T-DNA Transferred and Integrated from Single-Border Mini-T Plasmids. Here and in our earlier study (24), we have analyzed the structure of T-DNA transmitted to the plant genome by single-border mini-T plasmids. Two features are characteristic of the T-DNA transferred and integrated by single-border mini-T plasmids. First, in general, only a part of the single-border mini-T plasmid is transmitted to the plant cell as T-DNA. Of the four single-border mini-T plasmids examined (two here and two in ref. 24), three cases show partial transmission of the mini-T plasmid; and only in one case—that of C6 mini-T plasmid—does it appear that the entirety of the mini-T plasmid may have been transmitted. Second, sequences mapping to the left of the right border repeat (i.e., sequences behind the arrows in the figures) up to and including the *onc* genes always form a part of the T-DNA, whereas sequences beyond the *onc* genes do not necessarily do so.

These integration patterns are consistent with a T-DNA transformation process that involves the linearization of single-border mini-T plasmids at the border repeat and the polar transfer or integration, starting from the left end of the border repeat and proceeding leftward, of the linearized mini-T plasmid into the plant genome.

Generality of a Linear Intermediate in T-DNA Transformation. Studies of T-DNA transformation from Ti plasmid are

also consistent with the involvement of a linear intermediate that is formed by a cleavage at the right border repeat and directionally transferred or integrated into the plant genome. Evidence in support of this model is as follows: (*i*) Deletion of the left border region has no noticeable effect on virulence (17). (*ii*) Deletion of the right border region renders Ti plasmids avirulent (16, 17). (*iii*) Inversion of the right border fragment also renders Ti plasmids essentially avirulent (21, 32).

By homology to the right repeat, we suppose that the left border repeat is also cleaved during the T-DNA transformation process. This cleavage may serve to delimit the other end of the linear intermediate or to terminate the polar transfer or integration event initiated at the right border repeat. The intrinsic weak activity of the left border region demonstrated here predicts that sequences located to the left of the left border repeats are also transmitted to the plant cell but at a very low frequency during T-DNA transformation. The fact that these sequences are not normally detected in transformed tissue (3, 33) is consistent with the proposed rarity of these events and the null selective value of these sequences in the plant cell.

We presume that virulence of the mini-T plasmid is insensitive to border orientation because of its substantially smaller size. Border inversion in mini-T plasmids of this study increases the polar transfer distance from the repeat to oncogenes by no more than 20 kb; in Ti plasmids, a difference of 150 to 200 kb is created by right border inversion.

Recently, Koukolíková-Nicola *et al.* (34) have proposed that T-DNA transformation proceeds through a circular T-DNA intermediate formed by a recombination of the left and right border repeats. Their evidence for a circular intermediate is based on two types of rescue cloning of T-DNA from *Agrobacterium* after "induction" by exposure to plant cell exudates. As we have argued elsewhere (24), the circular intermediate hypothesis is inconsistent with a number of experimental observations, and the rescue cloning approach used to isolate the intermediate would not distinguish a circular structure from a linear structure with cohesive ends. Cleavage of the border repeats in the form of a staggered cut could generate a nicked circle by base pairing of cohesive ends of a linear T-DNA excision product. Independent experiments of Z. Koukolíková-Nicola and B. Hohn (personal communication) indicate that denaturation blocks, and renaturation restores, clonability of the T-DNA intermediate, consistent with a linear structure with cohesive ends.

Implications for Design of T-DNA Transformation Vectors. An implication of the proposed polar transfer mechanism for the design of T-DNA transformation vectors is that the location of the insert DNA in the vector may be important for the effective transmission of the insert DNA to the plant cell. Given that the transfer or integration process is initiated at the left end of the right border repeat and proceeds leftward, the best location for the insert DNA would be between the origin of transfer/integration—i.e., left end of the right border repeat—and the marker genes used to select for transformants. This location would establish a tight transmission linkage between insert DNA and the selectable marker gene. This consideration is particularly important for large insert DNA, whose complete transmission is more likely to be disrupted by size-related breakage events than that of smaller insert DNA.

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