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Genetics

T-DNA border sequences required for crown gall tumorigenesis

(*Agrobacterium tumefaciens*/transfer/integration/plant tumors)

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ABSTRACT **Similar 23-base-pair (bp) direct repeats occur at the ends of two adjacent but noncontiguous T-DNAs, T_L and T_R (left and right T-DNA), in the tumor-inducing plasmid pTiA6NC. Thus, three border repeats lie right and one lies left of T_L, which carries the genes needed for tumor maintenance. To determine whether T-DNA transfer and integration (subsequently called T-DNA transmission) require sequences in addition to the 23-bp border repeat, we constructed a deletion removing the three potential T_L right borders (the T_L right border and both T_R borders). Since this deletion severely attenuated virulence, we reintroduced restriction fragments containing the T_L right border repeat at a new location to the right of T_L and tested their ability to restore virulence. Fragments that carried the border repeat flanked by at least 67 bp of wild-type Ti plasmid sequences on the left and 1035 bp on the right restored virulence completely. Smaller fragments restored virulence significantly but not fully, even though the border repeat remained intact. Therefore, T-region sequences flanking the border repeat in the fully active fragments stimulated T-DNA integration. Fragments that restored virulence fully when inserted in the wild-type orientation stimulated virulence only slightly in the opposite orientation. Thus, the right border sequence promotes T-DNA transfer and integration best in one direction.**

Agrobacterium tumefaciens incites crown gall tumors on dicotyledonous plants when viable bacteria infect wounded plant tissue (1). Virulent *A. tumefaciens* strains contain a large [190 kilobases (kb)] tumor-inducing (Ti) plasmid that carries genes essential for tumorigenesis (2). During tumorigenesis a specific segment of the Ti plasmid, the T-DNA, integrates into plant nuclear DNA (1). Plant tumor cells express T-DNA genes responsible for tumorous growth, but T-DNA transmission into plant cells does not require T-DNA-encoded proteins and it can occur in the absence of tumor growth (3, 4). Mutations in virulence (*vir*) genes, located outside the T region, block T-DNA transmission into plant nuclear DNA (5). Presumably, some *vir* proteins play a direct role in T-DNA integration, but the mechanism of T-DNA integration remains unknown.

Specific sequences apparently signal T-DNA borders, because T-DNA ends usually occur at specific regions of the Ti plasmid (6–8). Similar 23-base-pair (bp) direct repeats lie at both ends of three different T regions, and T-DNA ends occur in or near these repeats in several different tumors (9–13). Although deletions that remove the left end of the T region do not affect virulence (14–16), deletions that remove the right end severely attenuate virulence (14–18). This implies that T-DNA transmission requires the right border repeat. Alternatively, these deletions might abolish virulence by removing other necessary sequences.

We conducted experiments to verify that T-DNA transmission requires the right border repeat and to determine whether this process requires additional sequences. We also tested the influence of orientation, location, and flanking sequences on border repeat function. To identify sequences that form a functional right T-DNA border, we introduced different restriction fragments, each containing a border repeat, into a Ti plasmid with the right borders deleted and tested their ability to restore virulence. These experiments defined an 80-bp region that contains a functional right T-DNA border and identified nonessential sequences outside the border repeat that stimulate T-DNA transmission. The right border repeat functioned normally when moved to a new location in the Ti plasmid only when it remained in its wild-type orientation.

MATERIALS AND METHODS

Bacterial Strains. All Ti plasmids, derived from the octopine-type plasmid pTiA6NC (19), reside in the *A. tumefaciens* A136 chromosomal background (20). We used *Escherichia coli* strains MM294 (*F⁻ pro⁻ hsdR⁻ endA⁻ supII*; ref. 21), JM103 (Δ *lac-pro thi⁻ rpsL⁻ supE endA⁻ sbcB15 hsdR4/F' traD36 proA⁺ proB⁺ lacI^Q lacZΔM15*; ref. 22), JM105 (Δ *pro-lac thi⁻ rpsL⁻ hsdR4 endA⁻ sbcB15/F' traD36 lacI^Q lacZΔM15 proA⁺ proB⁺*; ref. 23), and SF800 (*F⁻ polA1 thy⁻ gyrA⁻*; ref. 24). We transformed recombinant plasmids containing pBR322 (25) into MM294, pUC8 (26) into JM103 or JM105, and pRK290 (27) into SF800.

Media. We cultured *A. tumefaciens* on AB minimal agar or YEP broth (28) and *E. coli* on L agar or L broth (29). To select drug-resistant *E. coli*, we used tetracycline (10 μg/ml), kanamycin (25 μg/ml), or ampicillin (50 μg/ml). To select drug-resistant *A. tumefaciens*, we used gentamicin (50 μg/ml) and carbenicillin or kanamycin (100 μg/ml).

Transformation. We transformed *A. tumefaciens* (30) and *E. coli* (31) with plasmid DNA essentially as described.

Enzymes. We obtained DNA polymerases, ligase, and restriction endonucleases from New England Biolabs, Bethesda Research Laboratories, and International Biotechnologies (New Haven, CT). We used the enzymes according to the suppliers' instructions.

Right Border Deletion. To develop a test system for border sequence activity of restriction fragments, we constructed a right border deletion mutant (pWR113) of pTiA6NC. In the intact plasmid three potential right border repeats lie between *Eco*RI fragments 7 and 1 (Fig. 1; ref. 9); this region does not contain tumor morphology or virulence genes (6, 32). We constructed a broad host range plasmid, pWR45, with the rightmost 3761 bp of *Eco*RI fragment 7 adjacent to the leftmost 4.2 kb of *Eco*RI fragment 1 forming the desired deletion of *Eco*RI fragments 19a, 12, 20, and two smaller fragments (Fig. 1). We translocated this deletion to a pTiA6NC derivative by homologous recombination as described (33, 34). We confirmed the structure of pWR113 by restriction analysis using Southern blots (35).

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Abbreviations: Ti plasmid, tumor-inducing plasmid; kb, kilobase(s); bp, base pair(s).

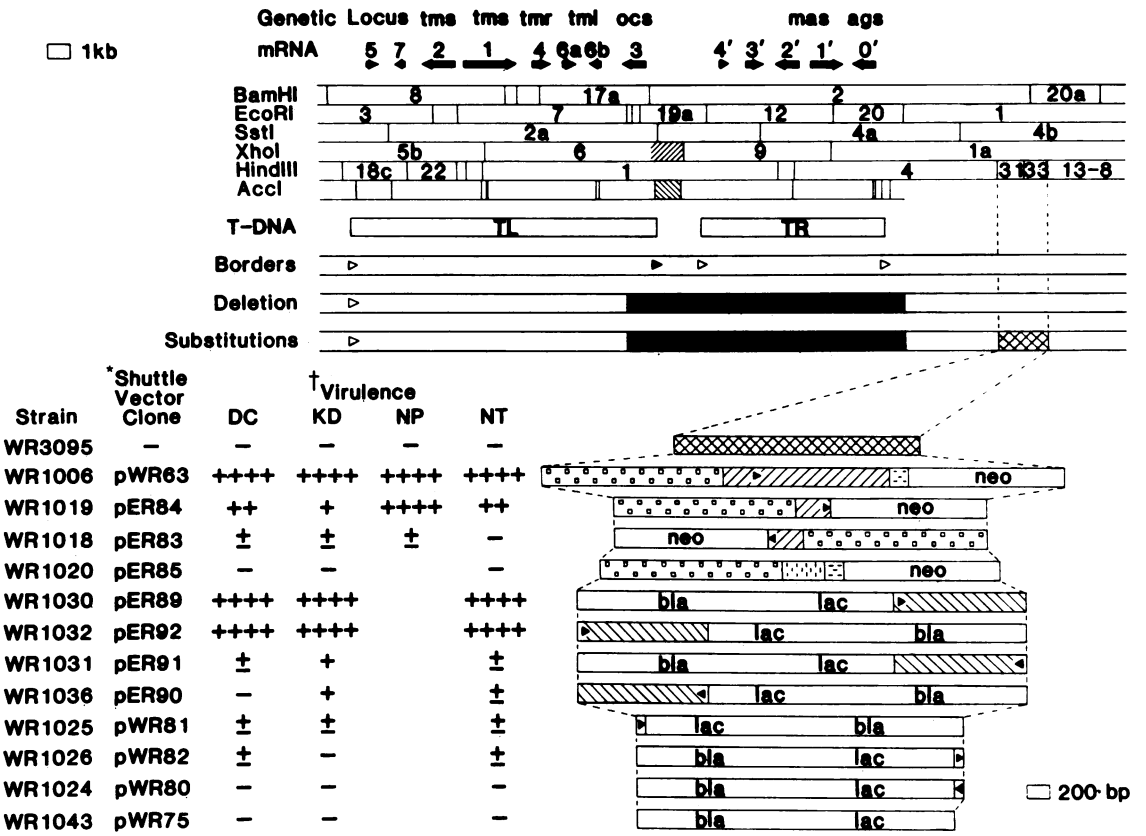

FIG. 1. T-DNA border mutants. *Shuttle vector clones represent pWR64-derivative plasmids used to translocate cloned border fragments into the right border deletion mutant Ti plasmid (pWR113).† We tested virulence on *D. carota* (DC), *K. daigremontiana* (KD), *N. plumbaginafolia* (NP), and *N. tabacum* (NT). Symbols are as follows: ++++, fully virulent; + to +++, parially virulent; ±, very weakly virulent; –, avirulent; □, pTiDNA; ■, deletion; ▨, site of substitutions; ▨, 1434-bp *Bam*HI/*Xho* I region containing the T_L right border repeat; ▨, 1568-bp *Hin*dIII/*Bam*HI fragment of Tn*5*; ▨, 166-bp *Sal* I/*Sma* I fragment of Tn*5*; neo, 1323-bp *Sma* I/*Hin*dIII fragment of Tn*5*; ▨, 372-bp *Sal* I/*Bam*HI fragment of Tn *5*; ▨, 1125-bp *Acc* I fragment containing the T_L right border repeat; lac bla, 2715-bp pUC8 plasmid; ▶, T_L right border repeat; ▷, other border repeats. *tms*, *tmr*, and *tml* symbolize *t*umor *m*orphology *s*hoot, *r*oot, and *l*arge. *ocs*, *ags*, and *mas* symbolize *oc*topine, *ag*ropine, and *ma*nnopine *s*ynthases. *neo* is neomycin phosphotransferase, *bla* is β-lactamase, and *lac* is β-galactosidase. Arrows indicate locations and directions of transcription.

Shuttle Vector. We constructed a shuttle vector (pWR64), which allowed us to translocate restriction fragments with border repeats into the right border deletion mutant Ti plasmid (pWR113). Our shuttle vector contains a derivative of *Eco*RI fragment 1 inserted at the *Eco*RI site of pRK290. We deleted *Hin*dIII fragments 31 and 33 by *Hin*dIII digestion and subsequent ligation; pTiA6NC lacks the *Hin*dIII site that separates fragments 13 and 8 in pTiAch5 (36). Thus, this mutant *Eco*RI fragment 1 contains a single *Hin*dIII site flanked by 4.2 kb of *Eco*RI fragment 1 to the left and 8.4 kb to the right. We cloned restriction fragments into the *Hin*dIII site on pWR64 and introduced them by homologous recombination 4.2 kb to the right of the T region in pWR113.

Border Sequence Clones. We isolated four different restriction fragments containing the left T-DNA (T_L) right border repeat. The largest fragment, a 1434-bp *Bam*HI/*Xho* I fragment (bases 13,774–15,208; ref. 9), contains 284 bp of Ti plasmid DNA to the left of the 23-bp border repeat (bases 14,059–14,081) and 1127 bp to the right (Fig. 1). We cloned this fragment into ColE1::Tn*5* at the *Bam*HI and *Sal* I sites of Tn*5*. The border repeat and *neo* (kanamycin resistance) lie between *Hin*dIII sites in the Tn*5* inverted repeats. We constructed plasmids with this *Hin*dIII fragment in the shuttle vector in its wild-type orientation (pWR63) and in pBR322 (pER81).

To remove Ti plasmid DNA lying right of the border repeat, we cleaved pER81 at the unique *Sst* I site (base 14,089) 8 bp to the right of the border repeat. Using T4 DNA polymerase, we removed the 4 base 3′ overhang generated by *Sst* I cleavage and created a blunt end (37). We cleaved the linear DNA with *Sma* I, producing a blunt-ended cut in the Tn*5* portion of pER81. We circularized the large blunt-ended fragment of pER81 with ligase to create pER82, a plasmid with a 311-bp *Bam*HI/*Sst* I border fragment. Sequence analysis (38) showed that the T_L right repeat remained intact. We inserted the border-containing *Hin*dIII fragment of pER82 into pWR64 in the wild-type (pER84) and inverted (pER83) orientations with respect to T_L.

We eliminated most of the Ti plasmid sequences lying left of the border repeat by testing a 1125-bp *Acc* I fragment (bases 13,991–15,116; Fig. 1); this fragment contains 67 bp of pTi DNA to the left of the repeat and 1035 bp to the right. We cleaved pER81 with *Acc* I, converted the 5′ overhang ends to blunt ends by using the large fragment of *E. coli* DNA polymerase I (37), and isolated the 1125-bp fragment by electroelution after agarose gel electrophoresis (37). We ligated the blunt-ended *Acc* I fragment into *Hin*cII-cleaved (blunt-ended) pUC8 in both orientations. We cleaved these plasmids with *Hin*dIII and inserted them into the *Hin*dIII site of the shuttle vector. We derived two types of shuttle vector inserts from each plasmid: those with the border repeat in its wild-type orientation relative to T_L (pER89 and pER92) and those with the opposite orientation (pER90 and pER91).

The smallest fragment tested, an 80-bp *Alu* I fragment (bases 14,007–14,087), also contained the intact border repeat with 51 bp of pTi DNA to the left of the repeat and 6 bp to the right (Fig. 1). We isolated the blunt-ended 80-bp *Alu* I fragment after polyacrylamide gel electrophoresis (37) and

ligated the fragment into *Sma* I-cleaved pUC8. We isolated clones containing the insert in both orientations. Sequence analysis proved that the *Alu* I fragment remained unaltered. Using the pUC8 *Hin*dIII site, we inserted each clone into the shuttle vector with the border repeat in the wild-type orientation (pWR81; pWR82), and we also inserted one clone in the inverted orientation (pWR80).

To ensure the vector portions of our constructs lacked border sequence activity, we inserted pUC8 (pWR75) and the *Hin*dIII fragment from Tn*5* (pER85) into the shuttle vector.

Translocations. We translocated each border fragment from the shuttle vector (pWR64) into *Eco*RI fragment 1 of the right border deletion mutant Ti plasmid (pWR113, in WR3095). We transformed WR3095 with a pWR64 derivative containing a border fragment and a selectable marker (kanamycin resistance for pWR63, pER83, pER84, and pER85; carbenicillin resistance for the pUC8 subclones) inserted into *Eco*RI fragment 1. Since the pWR64 derivatives and pWR113 shared homology in *Eco*RI fragment 1, recombination events between these plasmids on both sides of the border fragment–marker construct translocated the border fragment and marker into pWR113. WR3095 harbors pPH1JI (gentamicin resistance; ref. 34), a plasmid incompatible with the incoming pWR64; we identified recombinants as gentamicin-resistant transformants that inherited the incoming marker. From each recombinant we isolated total *A. tumefaciens* DNA (39) and confirmed the structure of each recombinant by Southern analysis (35) of *Bam*HI-digested DNA probed with isolated *Eco*RI fragment 1 DNA (labeled by nick-translation; ref. 37).

Containment. Containment levels used exceeded those required by the National Institutes of Health Guidelines for Recombinant DNA research.

Virulence Tests. We tested *A. tumefaciens* strains for virulence on leaves of *Kalanchoe daigremontiana* as described (40). We maintained axenic *Nicotiana pumbaginafolia* and *Nicotiana tabacum* var. Xanthi-nc plants in closed vessels on 50 ml of hormone-free Murashigi–Skoog medium (GIBCO) at 22°C–28°C with 12 hr of fluorescent light per day. We decapitated the plants with a sterile scalpel and smeared the wound with $\approx 5 \times 10^8$ *A. tumefaciens* cells taken from fresh AB minimal agar. We surface-sterilized fresh *Daucus carota* roots with 20% bleach for 20 min, rinsed with sterile water, sliced the root into 4- to 8-mm sections, placed the apical surface of each slice on water agar, and inoculated the basal surface with 25 μl of *A. tumefaciens* cells harvested from a logarithmic phase YEP broth culture and suspended in phosphate-buffered saline (41). We used from 2.5×10^3 to 2.5×10^7 cells per inoculation in 10-fold increments and compared slices that received the same-sized inoculum after 10–14 days. We tested each strain in at least four separate assays, and we tested at least two independent recombinants representing each construct.

RESULTS

Right Border Deletion. Wild-type pTiA6NC carries two adjacent but noncontiguous T regions: T_L, the left T-DNA, carries genes needed for tumor maintenance, but T_R, the right T-DNA, plays no apparent role in tumorigenesis (6, 32). Similar direct border repeats flank both T regions. Thus, three potential right border repeats could promote T_L transmission into plant DNA. To test the importance of these right border repeats for T_L transmission, we deleted all three potential right border repeats of pTiA6NC to form pWR113 (strain WR3095; Fig. 1). This 13-kb deletion removed the T_L right border sequence and all of T_R, but it did not affect tumor morphology or virulence genes. This deletion abolished virulence on *K. daigremontiana* leaves and stems of *N. plumbaginafolia* and *N. tabacum*; we observed rare tumors on *K. daigremontiana* stems and *D. carota* root discs. Thus, efficient T-DNA transmission requires either a right border repeat, other sequences affected by the deletion, or both.

Border Restoration. To determine whether the right border deletion abolished virulence solely by removing border repeats, we introduced restriction fragments containing the T_L right border 4.2 kb to the right of the T region in the right border deletion mutant Ti plasmid (pWR113; Fig. 1). By inserting into pWR113, in the wild-type orientation, border-containing fragments of 1434 bp (*Bam*HI/*Xho* I; WR1006) or 1125 bp (*Acc* I; WR1030 and WR1032; Fig. 1), we fully restored virulence (Fig. 2). Tumors induced by these strains appeared normal in size and morphology. Derivatives of pWR113 that received vector DNA without border sequences (WR1020; WR1043) remained avirulent. Thus, the 1125-bp *Acc* I fragment contained all the information required to fully restore virulence to the large (13 kb) right border deletion mutant, and efficient T-DNA transmission required no more than 67 bp of the Ti plasmid DNA left of the border repeat. Also, the border repeat remained completely functional when placed 4.2 kb to the right of its normal location.

To determine the role in T-DNA transmission of sequences lying right of the border repeat, we inserted a 311-bp border-containing *Bam*HI/*Sst* I fragment into pWR113 (Fig. 1; WR1019). This restriction fragment contains 4 bp of Ti plasmid sequences right of the repeat. This strain exhibited wild-type virulence on stems of *N. plumbaginafolia*, reduced virulence on carrot root discs and *N. tabacum*, and substantially less virulence on *K. daigremontiana* leaves than strains containing the larger border fragments (Fig. 2). Although T-DNA transmission required no more than 4 bp of the Ti plasmid sequences right of the border repeat, changing the sequences lying right of the border repeat influenced T-DNA integration efficiency. The 1124 bp removed from the fully active 1435-bp *Bam*HI/*Xho* I border fragment (in WR1006) to form the partially active 311-bp *Bam*HI/*Sst* I border fragment (in WR1019) may normally stimulate T-DNA transmission. Alternatively, the Tn*5* sequences brought next to the border repeat in cloning the 311-bp *Bam*HI/*Sst* I fragment may partially inhibit border repeat function.

To determine if flanking Tn*5* sequences inhibit border repeat activity, we constructed pWR113 derivatives (WR1025 and WR1026) containing an 80-bp border-containing *Alu* I fragment flanked by pUC8 (Fig. 1). This fragment contains 51 bp of T-region DNA to the left of the border repeat and 6 bp to the right. We cloned the *Alu* I fragment into the *Sma* I site of pUC8 in both possible orientations and constructed pWR113 derivatives with each clone. In WR1026, 28 bp of pUC8 DNA flanked the right end of the border fragment and the remaining 2.7 kb of pUC8 flanked the left end; WR1025 carried the flanking sequences reversed. These strains showed reduced virulence even though they contained an intact border repeat. WR1019, WR1025, and WR1026 each flanked the right end of the border repeat with completely different sequences, but all three exhibited reduced virulence. Thus, foreign sequences inserted next to the border repeat probably did not inhibit its function.

To test whether the orientation of the right border sequence relative to the T-DNA affects its function, we inserted border-containing fragments into pWR113 inverted relative to T_L. The resulting strains contained the inverted 1125-bp *Acc* I (WR1031 and WR1036), 311-bp *Bam*HI/*Sst* I (WR1018), and 80-bp *Alu* I (WR1024) border-containing fragments (Fig. 1). These strains produced a very weak response on *K. daigremontiana* leaves and carrot discs (Fig. 2) and occasionally induced small growths on stems of *N. plumbaginafolia* and *N. tabacum*. Even in the improper orientation, the larger fragments produced greater responses than the smaller fragments. Thus, the ability of the border repeat to promote

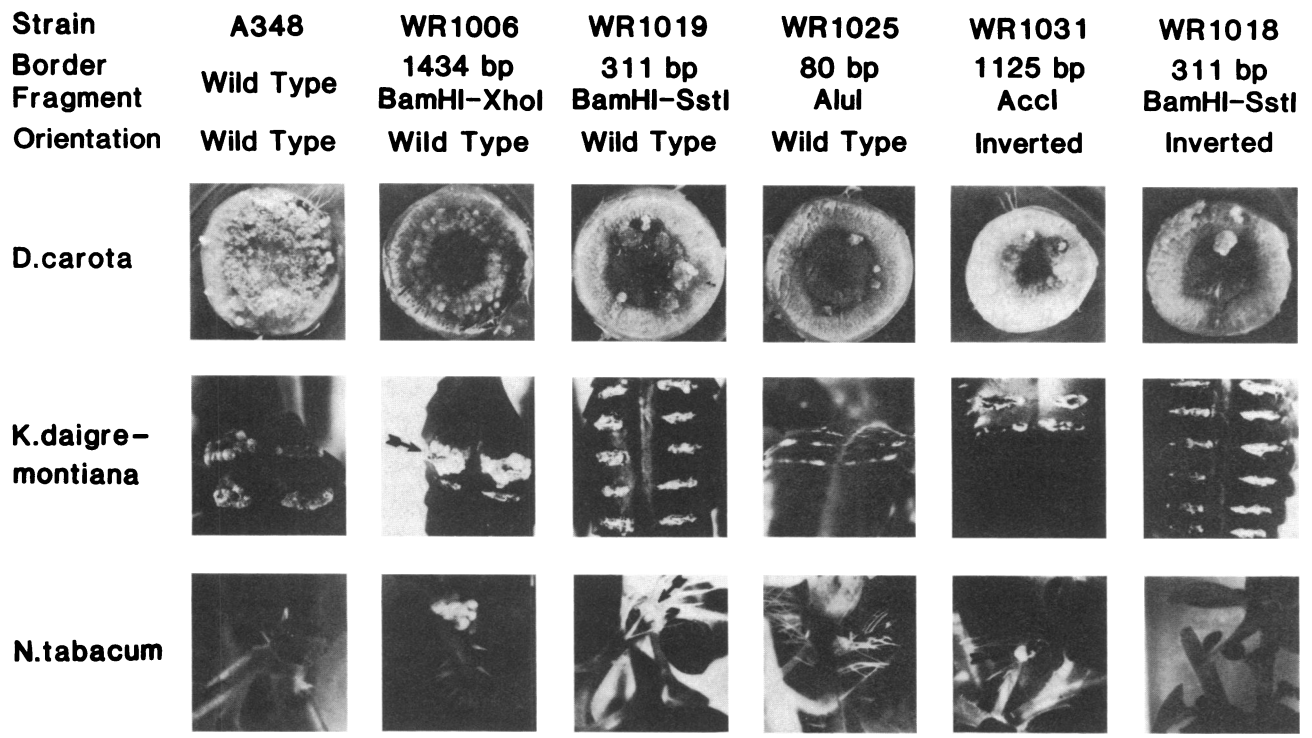

FIG. 2. Virulence tests. *N. tabacum* tumors were photographed 2 weeks (A348, WR1006), 4 weeks (WR1019), or 5 weeks (WR1025, WR1031, WR1018) after inoculation. We inoculated the *K. daigremontiana* leaf in the second column with WR1006 (top) and WR3095 (bottom, no response), and we photographed all *Kalanchoe* tumors after 4 weeks. We photographed the *D. carota* tumors 2 weeks (A348, WR1006, WR1019) or 4 weeks (WR1018, WR1025, WR1031) after inoculation.

T-DNA transmission depended greatly on its orientation relative to the T-DNA.

DISCUSSION

A deletion removing all three potential right T-DNA borders of pTiA6NC abolished virulence completely, even though the tumor morphology and virulence genes remained intact. Restriction fragments containing the T_L right border repeat restored virulence to this right border deletion mutant when inserted to the right of the T-DNA in the wild-type orientation. An 80-bp *Alu* I fragment with the 23-bp T_L right border repeat flanked by 51 bp of Ti DNA to the left and 6 bp to the right contained a functional right T-DNA border. Therefore, T-DNA transmission required a right border repeat. Others have recently reached similar conclusions for the nopaline-type Ti plasmid pTiC58 (42).

The exact position of the introduced border sequences did not affect virulence, but their orientation did. Border sequences introduced 4.2 kb to the right of T_L functioned normally provided they remained in the wild-type orientation. The same border fragments introduced in the opposite orientation exhibited only very slight activity. This activity depended on T-region sequences, because larger border fragments produced a greater response than smaller fragments, and vector DNA alone produced no response. Thus, the right border repeat functioned directionally and may resemble the chi sequence in RecA–RecBC-mediated recombination (43) or the origin of transfer (*oriT*) in bacterial plasmid conjugation (44). Both chi and *oriT* act unidirectionally and serve as substrates for endonucleolytic cleavage and recombination. In addition, *oriT* acts as an origin of DNA replication. Border sequences may function similarly to promote T-DNA transfer and integration into plant nuclear DNA.

Our experiments tested the importance of flanking sequences on border repeat efficiency. The 1125-bp *Acc* I fragment remained fully active even though it carries only 67 bp of the sequences normally lying to the left of the repeat. In contrast, sequences to the right of the T_L right border repeat, while not essential for T-DNA transmission, stimulated this process significantly. Several features of this region may explain its ability to stimulate T-DNA transmission. It contains two open reading frames preceded by Shine–Dalgarno ribosome binding sites (9). Thus, this region could encode proteins that promote T-DNA transmission. This region also contains a number of sequences potentially able to fold into secondary structures (9). Such structures could help proteins recognize, bind, or cleave the adjacent border repeat. Alternatively, the primary sequence may stimulate T-DNA transfer and integration, although the first 25 bp flanking the right ends of the six sequenced border repeats do not share significant homology. Binding sites for enzymes that act at the border repeat may occur well outside the repeat, or, if a promoter occurs upstream from the open reading frames, transcription might read through the border repeat and increase its accessibility to presumptive T-DNA integration enzymes. Mutagenesis of this region will allow us to distinguish between these possibilities.

Our results differ from those of Wang *et al.* (42) on one point: in the nopaline-type plasmid pTiC58, the border repeat alone restored virulence fully, but in the octopine-type plasmid pTiA6NC, we observed only partial activity with border fragments that lacked sequences normally lying to the right of the repeat. These experiments differed in several ways: (*i*) the Ti plasmids and chromosomal backgrounds, (*ii*) the sizes of the deletions, (*iii*) the extents of the deletions beyond the ends of the repeats, (*iv*) the sites where borders were reintroduced, (*v*) the sequences of the border repeats, and (*vi*) the sequences flanking the border repeats. Since the first five differences did not prevent large border fragments from fully restoring virulence in both systems, we suspect sequences flanking the border repeats may have caused the differences observed by using the small border fragments.

Alternatively, the nopaline-type right T-DNA border repeat differs at two positions (bases 3 and 19) from the sequences conserved in the other five known border repeats, and these may allow the nopaline-type right border to function more effectively than other borders in the absence of normal flanking sequences. We will distinguish between these possibilities by testing the nopaline-type right border in our assay and by testing octopine-type borders in a mutant nopaline-type Ti plasmid lacking the normal right border.

We have recently synthesized an oligonucleotide containing only the T_L right border repeat and tested it for activity. This repeat restored virulence partially. Thus, T-DNA transmission required only the border repeat, but flanking sequences influenced border efficiency.

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