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Overdrive, a T-DNA transmission enhancer on the *A. tumefaciens* tumour-inducing plasmid

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During crown gall tumorigenesis a specific segment of the *Agrobacterium tumefaciens* tumour-inducing (Ti) plasmid, the T-DNA, integrates into plant nuclear DNA. Similar 23-bp direct repeats at each end of the T region signal T-DNA borders, and T-DNA transmission (transfer and integration) requires the right-hand direct repeat. A chemically synthesized right border repeat in its wild-type orientation promotes T-DNA transmission at a low frequency; Ti plasmid sequences which normally flank the right repeat greatly stimulate the process. To identify flanking sequences required for full right border activity, we tested the activity of a border repeat surrounded by different amounts of normal flanking sequences. Efficient T-DNA transmission required a conserved sequence (5′ TAAPuTPy-CTGTPuT-TGTTTGTTTG 3′) which lies to the right of the two known right border repeats. In either orientation, a synthetic oligonucleotide containing this conserved sequence greatly stimulated the activity of a right border repeat, and a deletion removing 15 bp from the right end of this sequence destroyed its stimulatory effect. Thus, wild-type T-DNA transmission required both the 23-bp right border repeat and a conserved flanking sequence which we call *overdrive*.

Key words: Agrobacterium tumefaciens/crown gall/plant tumours/recombination enhancer/T-DNA borders

Introduction

Agrobacterium tumefaciens incites crown gall tumours on many dicotyledonous plants when viable bacteria infect wounded plant tissue (Bevan and Chilton, 1982; Nester *et al.*, 1984). Virulent strains contain a 190-kb tumour-inducing (Ti) plasmid that carries genes essential for tumorigenesis (Van Larebeke *et al.*, 1974; Garfinkel *et al.*, 1981). During tumorigenesis a specific segment of the Ti plasmid, the T-DNA, integrates into plant nuclear DNA (Thomashow *et al.*, 1980; DeBeuckeleer *et al.*, 1981; Lemmers *et al.*, 1980). Plant tumour cells express T-DNA genes responsible for tumorous growth, but T-DNA transmission does not require tumorigenesis or T-DNA-encoded proteins (Leemans *et al.*, 1982; Ream *et al.*, 1983). Similar 23-bp direct repeats lie at both ends of four different T regions (Barker *et al.*, 1983; Yadav *et al.*, 1982; Slightom *et al.*, 1986), and these repeats signal the T-DNA borders since T-DNA ends occur in or near these repeats in several different tumours (Simpson *et al.*, 1982; Zambryski *et al.*, 1982; Holsters *et al.*, 1983; Slightom *et al.*, 1985). Deletions that remove the T region right border severely attenuate virulence on most plants even though the tumour maintenance genes remain intact (Ooms *et al.*,1982; Holsters *et al.*, 1980; Joos *et al.*, 1983; Shaw *et al.*, 1984; Wang *et al.*, 1984; Peralta and Ream, 1985a, b; Hepburn and White, 1985). Such deletion mutants provide an assay for right border function: we can reintroduce T-DNA borders to the right of the T region and measure their ability to restore virulence. T-DNA transmission requires only a right border repeat in *cis* (Wang *et al.*, 1984; Peralta and Ream, 1985a, b), but Ti plasmid sequences that lie to the right of the right border repeat greatly stimulate its function (Peralta and Ream, 1985a, b); Jen and Chilton, in preparation).

In this study we identified a specific sequence flanking a right border repeat that stimulated T-DNA transmission. This flanking sequence, which we call *overdrive*, lies to the right of the TL right border repeat in the octopine-type plasmid pTiA6NC. Related sequences occur to the right of three other right border repeats (Barker *et al.*, 1983; Depicker *et al.*, 1982; Slightom *et al.*, 1986). We constructed a series of deletions that extended into the *overdrive* to the right of the pTiA6NC right border repeat and demonstrated that efficient T-DNA transmission required this flanking sequence. To determine whether *overdrive* functioned as a separate element, we synthesized this sequence and cloned it to the right of two different border repeats. Although right border repeats function best in one orientation (Wang *et al.*, 1984; Peralta and Ream, 1985a, b), *overdrive* functioned in either orientation. *Overdrive* also retained its activity when moved either 10 bp closer to or 7 bp farther from the border repeat than it normally occurs. Thus, *overdrive* formed a discrete element distinct from the border repeat. These results indicate *overdrive* may distinguish right and left T-DNA borders, and they suggest a model for the early events in T-DNA transmission. The relationship of T-DNA right borders to *overdrive* resembles that of inverted repeats to recombinational enhancers in several site-specific inversion systems (Johnson and Simon, 1985; Plasterk and van de Putte, 1985; Kahmann *et al.*, 1985; Huber *et al.*, 1985).

Results

Border assay system

To identify sequences required for T-DNA transmission, we developed an assay system for border sequence function (Figure 1; Peralta and Ream, 1985a). The octopine-type Ti plasmid pTiA6NC contains two adjacent but non-contiguous T regions designated TL and TR; TL, the left T-DNA, contains all the genes required for tumour maintenance (Thomashow *et al.*, 1980; Ooms *et al.*, 1982). A deletion mutant (pWR113 in WR3095) of pTiA6NC lacks the three border repeats that lie to the right of TL (the TL right border and both TR borders) without affecting the tumour maintenance genes (Peralta and Ream, 1985a). This right border deletion renders WR3095 essentially avirulent on all plants tested. A wide host range shuttle vector, pWR64, contains sequences from *Eco*RI fragment 1 (DeVos *et al.*, 1981) to the right of TL in pWR113 (Figure 1; Peralta and Ream, 1985a). To assay the border function of restriction fragments containing border repeats, we insert them into the shuttle vector at the single *Hin*dIII site, introduce the border fragments into pWR113 by homologous recombination in *A. tumefaciens* (Ruvkun and

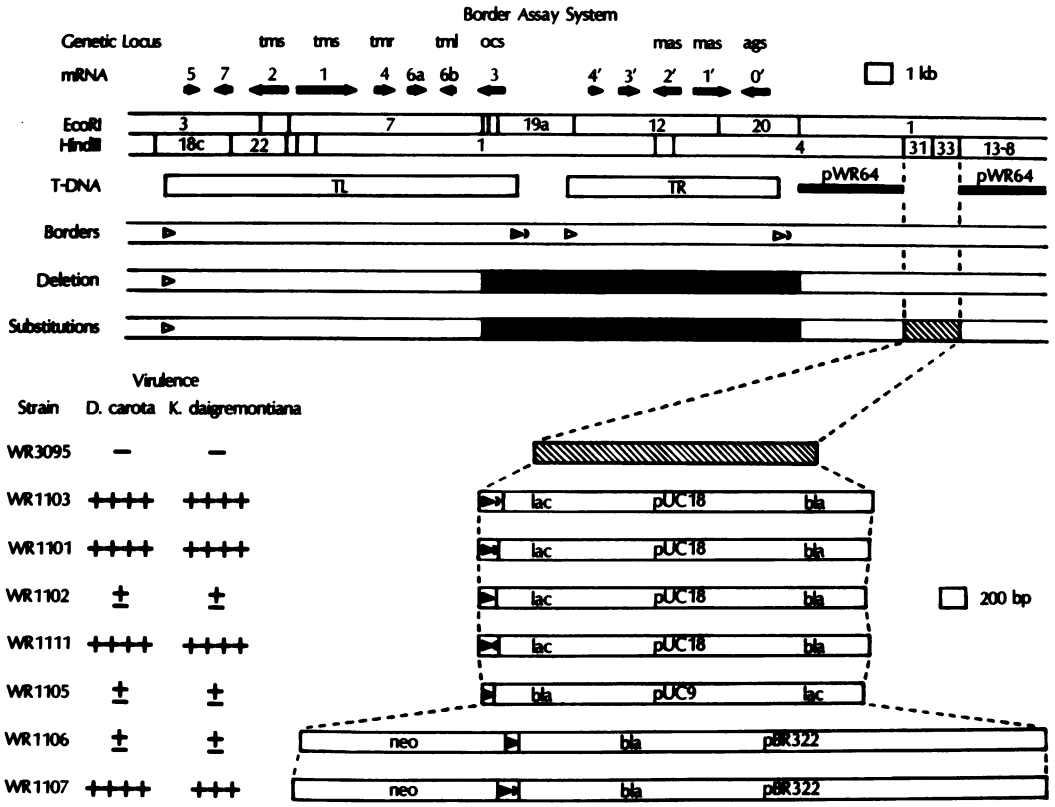

Fig. 1. Border assay system. The symbols indicate: ++++, fully virulent; +++, almost fully virulent; +/−, weakly virulent; −, avirulent; □, pTi DNA; ■, deletion; ▨, site of substitutions; ▬, homology between pTiA6NC and the shuttle vector pWR64; ▶, pTiA6 TL right border repeat; ▷, pTiT37 T-DNA right border repeat; ▷, other border repeats; ◗, *overdrive* (from pTiA6NC); ◖, inverted *overdrive*. *tms, tmr* and *tml* symbolize *t*umour *m*orphology *s*hoot, *r*oot and *l*arge. *ocs, mas,* and *ags* symbolize *oc*topine, *ma*nnopine, and *ag*ropine *s*ynthases. *lac* indicates β-galactosidase, *bla* indicates β-lactamase and *neo* indicates neomycin phosphotransferase. Arrows indicate locations and directions of transcription.

Ausubel, 1981) and test their ability to restore virulence. Since deletions that remove *Eco*RI fragment 1 do not affect virulence (Ooms *et al.*, 1982), our reintroduced construct will not interfere with other sequences important for T-DNA transmission. To determine whether a T-DNA right border could function fully when placed in new surroundings 6.9 kb to the right of its normal location, we tested a restriction fragment containing the TL right border repeat surrounded by normal flanking sequences (67 bp to the left and 1035 bp to the right). Since this restriction fragment fully restores virulence to WR3095 in our border assay system (Peralta and Ream, 1985a), the new location does not adversely affect efficient right border function. This right border repeat functions poorly when we reduce the normal righthand flanking sequences to 4 bp (Peralta and Ream, 1985a). Thus, our previous work indicates that sequences normally lying to the right of the border repeat greatly stimulate its function.

Deletion analysis of the T-DNA transmission enhancer

To identify the Ti plasmid sequences that stimulate border repeat activity, we constructed a series of deletions that extended into the Ti plasmid sequences present to the right of the TL right repeat (Figures 2 and 3). Strains which contained the TL right border repeat flanked on the right by at least 40 bp of Ti sequences exhibited full virulence (strains WR1101, WR1103; Figures 2 and 3). An otherwise identical strain (WR1102) that contained the TL right repeat flanked on the right by only 25 bp of Ti sequences exhibited greatly reduced virulence (Figure 2). Strains (WR1105) that contained a synthetic TL right repeat (without flanking sequences) showed similar weak virulence (Peralta and Ream, 1985a). Therefore, efficient T-DNA transmission required sequences located within 40 bp to the right of the TL right repeat; the sequences within 25 bp to the right of the repeat did not include all of the stimulatory sequences. A conserved sequence, 5′ TAAPuTPy-CTGTPuT-TGTTTGTTTG 3′, begins 17 bp to the right of the pTiA6NC TL right border repeat

Table I. Conserved core sequence

Plasmid	Border[a]	bp from repeat[b]	Core sequence	Core sequence coordinates[c]
pTiA6NC	TL right	31	TGTTTGTT	14 112–14 119
pTiA6NC	TR right	30	TGTTTGTT	23 810–23 817
pTiT37	T37 right	76	TGTaTGTg	−403 to −410[d]
pRiA4	TL right	1	TGTTTGTT	19 989–19 996

[a]The pTiA6NC TL right border repeat extends from coordinate 14 059 to 14 081, and the TR right border repeat extends from 23 758 to 23 780 (Barker *et al.*, 1983). The pRiA4 TL right border repeat extends from 19 996 to 19 988 (Slightom *et al.*, 1986). The pTiT37 T-DNA right border repeat extends from −305 to −327 on the complement of the published strand which presents the border repeat sequence in an inverted orientation (Depicker *et al.*, 1982).

[b]The numbers indicate the distance (in bp) from the rightmost base pair of each 23-bp right border repeat to the leftmost base pair of the core sequence.

[c]Coordinates correspond to those of Barker *et al.* (1983) for pTiA6NC, Depicker *et al.* (1982) for pTiT37 and Slightom *et al.* (1986) for pRiA4.

[d]Since the article cited presents the nopaline synthase and border repeat sequences in an inverted orientation, the complement of the published sequence contains the core sequence.

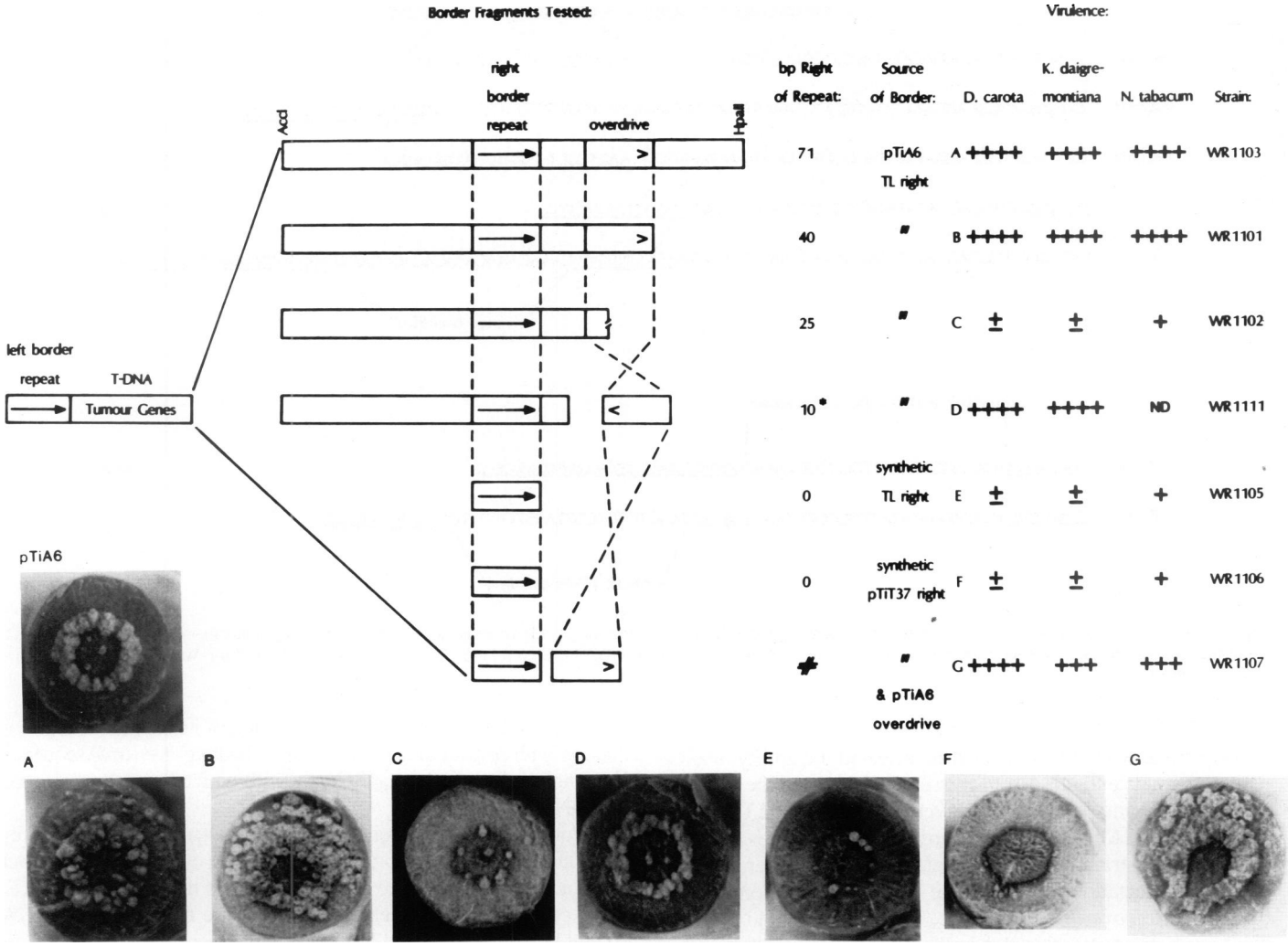

Fig. 2. Border fragment assay. We inoculated the carrot slices shown as follows: **pTiA6** with wild-type strain A348 [A136(pTiA6NC)], **A** with WR1103, **B** with WR1101, **C** with WR1102, **D** with WR1111, **E** with WR1105, **F** with WR1106 and **G** with WR11007. Symbols indicate: ++++, fully virulent, + to +++, partially virulent; +/−, very weakly virulent; *, WR1111 contains the pTiA6NC TL right border repeat flanked on the right by 10 bp of normal Ti plasmid sequences and a 24-bp synthetic *overdrive* oligonucleotide (from the pTiA6 TL right border) in an inverted orientation: #, WR1107 contains a 24-bp synthetic *overdrive* oligonucleotide (from the pTiA6 TL right border) in its wild-type orientation; ⟶, T-DNA border repeat; [ ], Ti plasmid sequences; [>], *overdrive*; [<], inverted *overdrive*.

and 16 bp to the right of the TR right repeat (Barker *et al.*, 1983). Sequences sharing 75−100% homology with an 8-bp core sequence (5′ TGTTTGTT 3′; underlined above) lie to the right of T-DNA right border repeats (Table I) in the nopaline-type Ti plasmid pTiT37 (Depicker *et al.*, 1982) and the *A. rhizogenes* Ri plasmid pRiA4 (Slightom *et al.*, 1986). The deletion which removed 15 bp (including the core sequence) from the right end of the conserved sequence also eliminated the stimulatory effect of flanking sequences on TL right border repeat function (Figures 2 and 3).

Efficiency of other border repeats

To determine whether other border repeats with slightly different sequences function efficiently without the conserved flanking sequence, we tested the ability of two other T-DNA border repeats to promote T-DNA transmission. We tested a synthetic pTiT37 right border repeat that lacked Ti plasmid flanking sequences (WR1106) and a 767-bp restriction fragment containing the pTiA6NC TL left border repeat (not shown). The sequences that flank left border repeats do not contain the conserved sequence found near right border repeats (Barker *et al.*,1983). Each border repeat, without the specific flanking sequence, promoted T-DNA transmission poorly (Figures 2 and 3).

Synthetic T-DNA transmission enhancer: (overdrive)

To determine whether the conserved flanking region identified by deletion analysis contained all the sequences required to fully stimulate T-DNA border repeat function, we synthesized an oligonucleotide comprising the conserved flanking sequence found to the right of the pTiA6NC TL right border repeat (Figure 3). We inserted this sequence in its normal orientation only 6 bp to the right of a synthetic pTiT37 right border repeat (Figures 2 and 3). The resulting strain (WR1107) exhibited much greater virulence than the parental strain (WR1106) which contained only the synthetic pTiT37 right border repeat (Figures 2 and 3). This synthetic oligonucleotide enhanced the activity of a heterologous border repeat when positioned 10 bp closer than normal to a right border repeat. Thus, this conserved region contained the sequences needed to stimulate border repeat function, and the sequence retained its function even when moved 10 bp closer than normal to the border repeat. We call this conserved flanking sequence *overdrive*.

Fig. 3. Right border constructions. Large letters represent right border repeats and the *overdrive* sequence. Small letters represent other Ti plasmid sequences. Underlined small letters represent *Escherichia coli* plasmid sequences (pUC18 for WR1101, WR1102, WR1103, WR1111; pUC9 for WR1105; pBR322 for WR1106, WR1107).

Orientation independence of overdrive

To determine whether its orientation affected the ability of *overdrive* to stimulate T-DNA transmission, we inserted the synthetic *overdrive* oligonucleotide in an inverted orientation 23 bp to the right of the pTiA6NC TL right border repeat (Figures 2 and 3). The resulting strain (WR1111) contains a right border repeat in its normal orientation (relative to the T-DNA) flanked on the right by an inverted *overdrive* sequence. This strain (WR1111) exhibited much greater virulence than the parental strain which lacked *overdrive* (Figures 2 and 3). Thus, the *overdrive* sequence stimulated T-DNA transmission regardless of its orientation relative to the right border repeat, and *overdrive* retained its function when positioned 7 bp farther than normal from a right border repeat.

Discussion

Our experiments demonstrate that efficient T-DNA transmission requires two discrete sequences, the 23-bp T-DNA right border repeat and a second sequence (*overdrive*) lying to the right of right border repeats. Border repeats and *overdrive* apparently constitute separate elements that interact to promote efficient T-DNA transmission. These elements presumably do not form a single contiguous site because: (i) a border repeat can function inefficiently without *overdrive*; (ii) the exact spacing and sequence between a border repeat and *overdrive* can vary slightly without reducing border function, and (iii) *overdrive* functions fully in either orientation whereas the right border repeat promotes T-DNA transmission best in one direction.

The specific sequence of a border repeat did not restrict it to function as either a 'left' or 'right' border repeat. The three border repeats tested (the pTiA6NC TL left and right border repeats and the pTiT37 right border repeat) all promoted T-DNA transmission at low efficiency when placed in the active orientation (Peralta and Ream, 1985a; Wang *et al.*, 1984) to the right of the T region in our assay system. Thus, a border repeat promoted T-DNA transmission as a function of its location and orientation with respect to the T-DNA; the flanking *overdrive* sequence strongly influenced the efficiency with which a repeat promoted T-DNA transmission.

Our 24-bp *overdrive* oligonucleotide contains all the sequences required for wild-type *overdrive* activity in our assay system, but full activity may require only a portion of this 24-bp sequence. We compared the sequences that flank the four known T-DNA right border repeats and found the 8-bp *overdrive* core sequence (5′ TGTTTGTT 3′) at slightly different locations to the right of each border repeat. Two mismatches occur within the core sequence that flanks the pTiT37 right border repeat, but the other three right borders (TL and TR of pTiA6NC and TL of pRiA4) each contain the exact core sequence. Comparisons between any two putative *overdrive* sequences reveal regions of homology longer than the 8-bp core sequence. For example, the pTiA6NC TL and TR right borders have homology in 19 of 24 positions in the *overdrive* region (5′ TAA-T--CTGT-T-TGTTTGTTTG 3′; core sequence underlined), the TL right borders of pTiA6NC and pRiA4 share homology in 15 of 17 positions in the *overdrive* region (5′ ATGTTTGTT--ATTGTT 3′), and the right borders of pTiT37 and TR in pTiA6NC share homology in nine of 10 positions in the *overdrive* region (5′ ATTTGT-TGT 3′). However, the 8-bp core sequence constitutes the region most highly conserved in all four potential *overdrive* regions. *Overdrive* activity may require only the 8-bp core sequence, or larger *overdrives* with somewhat different sequences may function in each plasmid due to variations in *vir*-encoded proteins. The two pTiA6NC *overdrives* share the greatest homology possibly because both interact with identical *vir*-encoded proteins. Alternatively, some *vir* proteins may promote efficient T-DNA transmission without an *overdrive*.

Our results differ from those of Wang *et al.* (1984): in the nopaline-type plasmid pTiC58, the nopaline-type right border repeat alone promotes efficient T-DNA transmission, but in the octopine-type Ti plasmid pTiA6NC, we observe only partial ac-

tivity with either octopine or nopaline-type right border repeats alone (Peralta and Ream, 1985a; this work). Jen and Chilton (in preparation) identified a region to the right of the nopaline-type (pTiT37) right border repeat which exhibited *overdrive* activity when an octopine-type Ti plasmid supplied the *vir* functions. The region with *overdrive* activity includes the core sequence identified in our study, and the nopaline-type repeat exhibits only weak activity in their system. Thus, efficient T-DNA transmission may require *overdrive* when an octopine-type Ti plasmid provides the *vir* genes but not when a nopaline-type Ti plasmid supplies the *vir* functions. Further mutagenesis will define the sequences important for *overdrive* activity in pTiA6NC, and complementation tests with *vir* genes from pTiC58 (a nopaline-type Ti plasmid) will determine whether these *vir* genes can render the pTiA6NC right border repeat fully active without *overdrive*.

Our experiments, together with data from other studies, suggest a possible function for *overdrive*. Efficient T-DNA transmission requires only the right border repeat in its wild-type orientation (Peralta and Ream, 1985a; Wang *et al.*, 1984) and the *overdrive* sequence in *cis* (in pTiA6NC; Peralta and Ream, 1985a). *Overdrive* itself does not promote T-DNA transmission in the absence of a right border repeat (Shaw *et al.*, 1984), but *overdrive* greatly stimulates T-DNA transmission. Apparently, a directional T-DNA transmission process begins at the right border and moves leftward through the T region. Presumably, some of the Ti plasmid-carried *vir* (virulence) genes (Klee *et al.*, 1983), induced by exudates from wounded plant cells (Stachel *et al.*, 1985a, b; Okker *et al.*, 1985), encode proteins which act at the right border repeat to initiate T-DNA transmission. Since both left and right border repeats (without *overdrive*) exhibit weak unidirectional right border activity in our assay, we propose that *overdrive* greatly enhances interaction between the right border and the appropriate *vir* proteins. Therefore, most T-DNA transmission events will initiate at the right border and move leftward through the T-DNA.

Other site-specific recombination systems resemble the T-DNA border repeat−*overdrive* system. Several genetic recombination pathways, for example phage lambda integration (Nash, 1981) and Tn*3* resolution (Kitts *et al.*, 1983), require specific flanking sequences in addition to repeat sequences directly involved in a cross-over site. Site-specific inversions that mediate tail fiber switching in bacteriophages (e.g. Mu, P1, P7) and variation of *Salmonella typhimurium* flagellar antigens require inverted repeat sequences and specific flanking sequences in *cis* for efficient inversion (Plasterk and van de Putte, 1985; Kahmann *et al.*, 1985; Johnson and Simon, 1985; Craig, 1985; Huber *et al.*, 1985). These flanking sequences, called recombinational enhancers, stimulate site-specific inversion independent of their position and orientation relative to the inverted repeats. We will continue to test whether the *overdrive* sequence can also enhance T-DNA transmission in a similar fashion.

Materials and methods

Bacterial strains

Figures 2 and 3 list the *A. tumefaciens* strains used.

Border fragment constructions

WR1103 contained the pTiA6NC TL right border on a 161-bp *Acc*I−*Hpa*II restriction fragment (coordinates 13 991−14 152; Barker *et al.*, 1983) inserted into the *Hin*cII and *Acc*I sites of pUC18 to create pER121. We used exonuclease *Bal*31 to create deletions in this *Acc*I−*Hpa*II fragment. A *Bam*HI site occurs in pUC18 to the right of the *Acc*I−*Hpa*II fragment and a *Hin*dIII site occurs to the left. We cleaved pER121 with *Bam*HI, incubated the linear DNA with *Bal*31 (International Biotech. Inc., slow form) at 30°C for 10−27 min, created blunt ends with Klenow polymerase, ligated unphosphorylated *Bam*HI linkers (5′ CCGGATCCGG 3′; Bethesda Research Labs) to the blunt ends and circularized the linker-containing fragments as described (Lathe *et al.*, 1984). We recloned our deletion mutant borders as *Hin*dIII−*Bam*HI fragments into intact pUC18. Thus, the strains (WR1101, WR1102) that contained these deletion derivatives differed from the parental strain (WR1103) only in the amount of Ti plasmid sequences present to the right of the border repeat. WR1111 contained the pTiA6 TL right border repeat on a 100-bp *Acc*I−*Sst*I restriction fragment (coordinates 13 991−14 091) inserted into the *Hin*cII and *Sst*I sites of pUC18; this plasmid also contained a synthetic pTiA6NC *overdrive* oligonucleotide (coordinates 14 098−14 121; tailed with *Bam*HI cohesive ends) inserted in an inverted orientation into the *Bam*HI site immediately to the right of the border repeat. WR1105 contained a synthetic TL right repeat (coordinates 14 059−14 083) with *Pst*I and *Eco*RI cohesive ends cloned into these sites in pUC9. WR1106 contained pEB10, a pBR322 derivative that contains *neo* (kanamycin resistance) and a synthetic pTiT37 right border repeat (constructed by Drs E.Bach, M.-D.Chilton and A.Montoya). WR1107 contained a derivative of pEB10 with a synthetic pTiA6NC *overdrive* oligonucleotide (coordinates 14 098−14 121; tailed with *Bam*HI cohesive ends) inserted into the *Bam*HI site immediately to the right of the border repeat in pEB10. L.Washington used an Applied Biosystems 380A to synthesize the oligonucleotides.

DNA sequencing

We determined the nucleotide sequences of the T-DNA right border regions as described by Maxam and Gilbert (1977) or by dideoxy sequencing of plasmid DNA (Chen and Seeburg, 1985).

Border fragment translocations

To assay restriction fragments with border repeats for border function, we used homologous recombination to translocate each cloned fragment from a broad host range shuttle vector (pWR64) into a right border deletion mutant of pTiA6NC (pWR113, in WR3095, an A136 derivative; Peralta and Ream, 1985a). We cloned border sequences into plasmids that contain *bla* (carbenicillin resistance): pUC18 (Yanisch *et al.*, 1985; WR1101, WR1102, WR1103, WR1111), pUC9 (Yanisch *et al.*, 1985; WR1105), or pBR322 (Bolivar *et al.*, 1977; WR1106, WR1107). We cleaved these plasmids with *Hin*dIII and ligated them into pWR64 which contains a single *Hin*dIII site flanked by Ti plasmid sequences (the portions of *Eco*RI fragment 1 that overlap *Hin*dIII fragments 4 and 13−8; Peralta and Ream, 1985a; DeVos *et al.*, 1981). We transformed WR3095 with these shuttle vector clones and selected cells resistant to carbenicillin. WR3095 harbors pPH1JI (Beringer *et al.*, 1978; gentamicin resistance) a plasmid incompatible with the incoming pWR64 clones; recombinants that translocated border fragments from the shuttle vector to the Ti plasmid exhibited resistance to both gentamicin and carbenicillin. From each recombinant we isolated total *A. tumefaciens* DNA and confirmed the structure of each recombinant by Southern analysis (Gillen *et al.*, 1981; Maniatis *et al.*, 1982; Currier and Nester, 1976).

Virulence assays

We performed virulence tests on *Kalanchoe daigremontiana* leaves and *Nicotiana tabacum* cv. Xanthi-nc stems as described (Peralta and Ream, 1985a). To quantitate virulence on *K. daigremontiana* leaves, we measured the area of each wound covered by tumour tissue 3 weeks after inoculation. All fully virulent strains reproducibly induced tumours that covered 100% of the wound with tumour tissue. Strains that lacked *overdrive* induced tumours that covered < 10% of the wound with tumour tissue. To compare the virulence of different strains, we inoculated nearby wounds on the same leaf. To quan'itate virulence on *N. tabacum* stems, we examined the decapitated plants 2 weeks and 4 weeks after inoculation. Fully virulent strains induced tumours over the entire surface of the wound in 2 weeks whereas strains without *overdrive* showed little or no response. After 4 weeks these weakly virulent strains induced small galls over a portion of the wound surface; stems inoculated with fully virulent strains produced large galls in 4 weeks. The galls grew normally on the plants and in tissue culture regardless of the virulence of the inciting strain; therefore, *overdrive* influences the efficiency of tumour induction and not the rate of subsequent tumour growth.

We surface sterilized fresh *Daucus carota* roots with 20% bleach for 20 min, rinsed with sterile water, sliced the root into 6−8 mm sections, placed the apical surface of each slice on water agar, and inoculated the basal surface with 0.025 ml of *A. tumefaciens* cells harvested from a logarithmic phase YEP broth culture and suspended in phosphate-buffered saline (Pueppke and Benny, 1981) at 10^9 cells/ml. To compare the virulence of different strains we inoculated serial sections from a single carrot (placed on the same agar plate). The slices pictured represent typical results that we observed reproducibly in at least 15 separate trials. We photographed the *D. carota* tumours 10−20 days after inoculation. To quantitate virulence on carrot slices, we counted the individual tumour foci 2 weeks after inoculation. Fully virulent strains generally produced 70−120 foci per slice (average 80 ± 10) while strains without *overdrive* produced 0−10 foci per slice (average 5 ± 2). As on tobacco, once tumours formed they grew equally rapidly regardless of the virulence exhibited by the inciting strain.

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