Virulence genes *A*, *G*, and *D* mediate the double-stranded border cleavage of T-DNA from the *Agrobacterium* Ti plasmid

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ABSTRACT *Agrobacterium tumefaciens* transfers the T-DNA portion of its Ti plasmid to the nuclear genome of plant cells. Upon cocultivation of *A. tumefaciens* strain A348 with regenerating tobacco leaf protoplasts, restriction endonuclease fragments of the T-DNA were generated that are consistent with double-stranded cleavage of the T-DNA at the border sequences. The T-DNA border cleavage was also induced by acetosyringone, a compound that induces many of the virulence genes. T-DNA cleavage did not occur in *Agrobacterium* strains harboring Tn3-HoHo1 insertions in the *virA*, *-D*, or *-G* genes. Insertion mutations in *virB*, *-C*, or *-E* did not have any effect on the T-DNA cleavage. Complementation of the mutations in *virA*, *-D*, or *-G* with cosmids containing the respective wild-type genes restored the T-DNA cleavage. Since *virA* and *-G* are essential in regulating the expression of other *vir* genes in response to plant signal molecules, the *virD* gene product(s) appear to mediate double-stranded T-DNA border cleavage.

The ability of *Agrobacterium tumefaciens* to transfer the T-DNA (transferred DNA) portion of its tumor-inducing (Ti) plasmid to the nuclear genome of plant cells has been extensively used to engineer desirable genes into plants (1–4). At least three components of *Agrobacterium* appear to play an important role in this complex process of T-DNA transfer: (i) the T-DNA border sequences, (ii) the chromosomal virulence (*chv*) loci, and (iii) the Ti plasmid virulence (*vir*) loci. The T-DNA of a typical wide host range octopine-type Ti plasmid consists of two noncontiguous sections called T left (T_L) and T right (T_R) (5). Each of these segments is flanked by 25-base-pair (bp) imperfect direct repeat sequences called borders, which appear to define the sequences of the Ti plasmid destined for transfer (6–8). The presence of the right border in its correct orientation together with its flanking sequences (overdrive) has been suggested to be important for T-DNA transfer by *Agrobacterium* to many plant species (9–12). The border sequences are cis-acting components in the T-DNA transfer, whereas the chromosomal virulence region and the Ti plasmid virulence region act in trans (13, 14). A chromosomal virulence region, consisting of the *chvA* and *chvB* loci, specifies the binding of *Agrobacterium* to the plant cells and is constitutively expressed in the bacterium (15). The 40-kbp virulence region of the Ti plasmid is organized into six complementation groups designated *virA*, *-B*, *-G*, *-C*, *-D*, and *-E* (16–18). *virA* and *-G* are constitutively expressed, while the expression of other *vir* genes is induced when *Agrobacterium* is cocultivated with actively growing plant cells (18, 19). A small group of phenolic compounds secreted by plant cells serve as the signal molecules in mediating the induction of the *vir* genes in the bacteria (20, 21). Circular copies of the T-DNA generated from the Ti plasmid can be rescue-cloned from *Agrobacterium* into *Escherichia coli* after induction by cocultivation of

Agrobacterium with plant cells (22). Nucleotide sequence analysis of the junction sequences of the T-DNA circles indicated that all the analyzed recombinant plasmids contained a single copy of a hybrid 25-bp border sequence derived from both left and right border sequences. Model systems have recently been developed to study the formation of T-region circles in both *E. coli* and in *A. tumefaciens* to facilitate the study of the role of the *vir* genes in the generation of the T-DNA circles (23–25). These studies suggest that the excision and religation of the T-DNA ends, leading to the generation of T-DNA circles, may be among the early steps of the T-DNA transfer process.

Our objective has been to study directly the early events of the T-DNA excision and transfer within *Agrobacterium*. We have addressed the question of whether double-stranded T-DNA cleavage occurs at or around the borders upon cocultivation of the bacteria with plant cells and whether this cleavage is regulated by the *vir* genes. We present evidence that the cleavage of the T-DNA borders is mediated by the *vir* genes *A*, *G*, and *D*.

MATERIALS AND METHODS

Bacterial Strains. *A. tumefaciens* strain A348 (26) harboring pTiA6 was used in our studies. Strains containing mutations in the virulence region of pTiA6 were generated by transposon mutagenesis using Tn3-HoHo1 (19). The plasmids pTi237, pTi243, pTi364, pTi311, pTi341, and pTi321 harbor Tn3-HoHo1 insertions in the *virA*, *-B*, *-C*, *-D*, *-E*, and *-G* genes of pTiA6, respectively (18). pVK224, pVK227, and pVK257, carrying overlapping segments of pTiA6 *vir* region, were previously constructed by cloning *Sal* I partial digests of pTiA6 into the unique *Sal* I site of the broad host range vector pVK102 (26). These cosmids were separately introduced into *A. tumefaciens* strain A348 or into the *vir* mutant strains by triparental mating using pRK2013 as a helper plasmid (27). *A. tumefaciens* strains were grown at 30°C on either AB minimal medium plus 0.5% glucose or on YEP medium (28). The antibiotic concentrations used were rifampicin, 10 µg/ml; kanamycin, 100 µg/ml; and carbenicillin, 100 µg/ml.

Isolation of Plant Protoplasts. Protoplasts were isolated from the leaves of aseptically grown *Nicotiana tabacum* cv. Wisconsin 38 plants (29). Leaves from 6- to 8-week-old plants were cut into small pieces in a Petri dish (15 × 100 mm) with 15 ml of K₃ medium (29) containing 0.5% cellulase (Worthington), 0.5% Macerase (Calbiochem), and carbenicillin (50 µg/ml). The Petri dish was wrapped with parafilm and incubated in the dark with gentle shaking (20 rpm). After 6–8 hr of digestion, the suspension was passed successively through 30-, 100-, and 200-mesh steel screens. The protoplasts were concentrated by centrifugation for 5 min at 100 × g, which resulted in their accumulation at the surface. The protoplasts were collected, mixed with 5 additional vol of K₃

medium, and centrifuged again. The protoplasts were collected, counted using a hemacytometer, and the appropriate volume of K_3 medium was added to attain a concentration of 10^5 protoplasts per ml. Ten-milliliter portions of protoplasts were added to plastic Petri dishes (15×100 mm), wrapped with parafilm, incubated in the dark at 25°C for 24 hr, and then transferred to moderate light (500 lux), where the incubation was continued for 60 hr. After this treatment, they were used for cocultivation with bacteria.

Cocultivation of *A. tumefaciens* with Plant Protoplasts. *A. tumefaciens* strains were grown in 20 ml of AB minimal medium plus 0.5% glucose at 30°C in Klett flasks. When the bacteria reached a concentration of 10^9 bacteria per ml (≈ 100 Klett units) a 5-ml portion was saved for DNA analysis (referred to as B_0 bacteria) and a 10-ml portion was centrifuged at $6000 \times g$ for 10 min. The bacteria were resuspended in 10 ml of K_3 medium at a concentration of 10^9 bacteria per ml. One milliliter of bacteria was added either to 10 ml of K_3 medium (referred to as K_3 bacteria) or to 10 ml of protoplasts (referred to as P bacteria), resulting in an approximate final concentration of 10^5 protoplasts and 10^8 bacteria per ml of medium. The Petri dishes were wrapped with parafilm and incubated in the dark at 25°C with gentle shaking (20 rpm). After 24 hr, the mixture of protoplasts plus bacteria was filtered through Whatman no. 1 paper, which retained all the plant cells. The bacteria were centrifuged at $6000 \times g$ for 10 min and resuspended either in 10 mM Tris-HCl plus 0.1 mM Na_2EDTA (pH 8) for DNA analysis or in Z buffer for measuring the β -galactosidase activity (19).

Induction of *A. tumefaciens* with Acetosyringone. Acetosyringone (Aldrich) was dissolved in dimethyl sulfoxide to prepare a 1 mM stock. Bacteria were prepared as described for cocultivation with plant cells. Acetosyringone (final concentration, $10 \mu\text{M}$) was added to a 5-ml culture containing 5×10^8 bacteria per ml of K_3 medium in culture tubes. The cultures were shaken at 300 rpm at room temperature (25°C) for 24 hr, after which the bacteria were analyzed for T-DNA border cleavage or for *vir* gene induction.

Analysis of the DNA. DNA from *A. tumefaciens* was extracted using lysozyme (Sigma) and NaDodSO_4 . The bacterial culture was centrifuged at $6000 \times g$ for 10 min and resuspended in 0.2 ml of 10 mM Tris-HCl/0.1 mM Na_2EDTA , pH 8, followed by the addition of 0.4 ml of 10 mM Tris-HCl/10 mM Na_2EDTA , pH 8, containing 1 mg of lysozyme per ml. The mixture was left on ice for 5 min, after which $50 \mu\text{l}$ of 20% NaDodSO_4 was added, mixed well, and subjected to four neutral phenol/chloroform (1:1) extractions. The aqueous phase was extracted twice with ethyl ether, and the DNA was precipitated by addition of 0.1 vol of 3 M sodium acetate and 2 vol of 100% ethanol. The nucleic acids were collected by centrifugation, dissolved in 0.4 ml of 10 mM Tris-HCl/10 mM Na_2EDTA , pH 8, and subjected to a second cycle of ethanol precipitation. The DNA was finally dissolved in 0.4 ml of 1 mM Tris-HCl/0.1 mM Na_2EDTA , pH 7, and the DNA concentration was determined fluorimetrically by using diaminobenzoic acid (30). The DNA was digested with various restriction endonucleases using 10 units of enzyme per μg of DNA under the conditions recommended by the supplier (Bethesda Research Laboratories). Five hundred nanograms of each DNA sample was fractionated by electrophoresis through 0.7% agarose gels in TBE buffer (31). Southern blot hybridizations were performed as described (32). Nick-translations of gel-purified restriction endonuclease fragments (31) were performed using Amersham nick-translation kits. DNA specific activities of $50\text{--}150 \times 10^6$ cpm/ μg were routinely achieved. Recombinant DNA techniques were performed under P1 containment conditions as specified by the National Institutes of Health guidelines.

RESULTS

Induction of *vir* Genes. To evaluate the effectiveness of our cocultivation conditions, we studied the levels of induction of a set of *vir* genes in *A. tumefaciens* cocultivated with regenerating tobacco leaf protoplasts. β -Galactosidase activity was measured in strains in which a promoterless *lacZ* gene was placed under the control of the *vir* gene promoters of pTiA6 by Tn3-HoHo1 insertions (19). β -Galactosidase activity increased 125-, 40-, and 20-fold upon cocultivation with protoplasts in strains harboring pTi243 (*virB*), pTi341 (*virE*), and pTi219 (*pinF*), respectively. In a *virG* mutant (pTi321), an 8-fold increase in β -galactosidase activity was seen upon transfer of the bacteria from AB glucose medium (pH 7) to K_3 medium (pH 5.6), and there was no further induction in K_3 medium plus protoplasts (data not shown). This is in contrast to the previous observations in which *virG* was found to be plant inducible (18, 33). The *virB* and *pinF lac* fusions were also induced by acetosyringone (data not shown).

T-DNA Is Cleaved at the Borders. DNA from *A. tumefaciens* strains grown in AB glucose medium (B_0) and incubated in K_3 medium (K_3) or K_3 medium plus protoplasts (P) was digested with *EcoRI*, *Pst* I, or *HindIII*; subjected to Southern analysis; and probed with a 2.8-kbp *HindIII* fragment Y encompassing border A (Fig. 1). As shown in the restriction map (Fig. 1A), this probe normally hybridizes to an 11.25-kbp *EcoRI* fragment, a 9.8-kbp *Pst* I fragment, and a 2.8-kbp *HindIII* fragment. As expected, these are the only fragments detected in the B_0 and K_3 bacteria that were not exposed to the protoplasts (Fig. 1B). However, cocultivation with the protoplasts (P) resulted in the generation of additional 7.7-, 3.6-, and 2.6-kbp *EcoRI* fragments; an 8.3-kbp *Pst* I fragment; and a 2.5-kbp *HindIII* fragment (Fig. 1B). The generation of most of these fragments is consistent with the double-stranded cleavage of the T-DNA at border A. For instance, cleavage at border A would generate 3.6- and 7.7-kbp *EcoRI* fragments, an 8.3-kbp *Pst* I fragment, and a 2.5-kbp *HindIII* fragment, all of which will hybridize to the *HindY* probe (Fig. 1A). All fragments expected from border A cleavage are generated only upon cocultivation of the bacteria with protoplasts, suggesting that cleavage of the T-DNA occurs in bacteria in contact with plant cells. The 2.6-kbp *EcoRI* fragment generated in induced bacteria was susceptible to S1 nuclease and was transferred to nitrocellulose under nondenaturing blotting conditions (data not shown), suggesting that this fragment may represent a single-stranded T-DNA structure in induced bacteria. A *BamHI/Kpn* I fragment to the left of border A (Fig. 1A) was also used to ascertain the nature of the newly generated fragments. As would be consistent with border A cleavage, the 3.6-kbp *EcoRI* fragment and the 8.3-kbp *Pst* I fragment did not hybridize to the *BamHI/Kpn* I probe (data not shown). The 3.6-kbp *EcoRI* fragment expected from the border A cleavage was also generated when *A. tumefaciens* was incubated with $10 \mu\text{M}$ acetosyringone (Fig. 1C). However, the frequency of border cleavage was very low compared to that obtained by cocultivation with protoplasts. Moreover, it was necessary to increase the copy number of *vir* genes by introducing the cosmid pVK224 (see Fig. 3A) to see the T-DNA cleavage with acetosyringone.

Cleavage of the T-DNA at borders B, C, and D was analyzed by digesting the DNA with *HindIII* and probing with restriction endonuclease fragments close to the respective borders. The restriction map of the entire T-DNA and the sequences used as probes are shown in Fig. 2A. Cocultivation of *A. tumefaciens* strain A348 with protoplasts resulted in the generation of new DNA fragments expected from cleavage of the T-DNA at borders B and C but at a very low frequency. Therefore, we attempted to increase the frequency of border cleavage by increasing the copy number of *vir* genes. Introduction of pVK224, a cosmid containing *virG*, -C, -D, and -E

FIG. 1. T-DNA cleavage at border A. (A) Restriction endonuclease map of the T-DNA at the border A region and the sizes and positions of the *EcoRI*, *Pst I*, and *HindIII* fragments expected from border A cleavage. The sequences used as hybridization probes are indicated as solid bars. (B) Restriction endonuclease analysis of DNA from *A. tumefaciens* strain A348 grown in AB minimal medium with glucose (B₀) and incubated for 24 hr in K₃ medium (K₃) or in K₃ medium with protoplasts (P). The DNA was digested with the indicated restriction enzyme and probed with *HindIII* fragment Y. R, reference lane in which purified pTiA6 was digested with *EcoRI* and probed similarly. (C) Results of acetosyringone treatment. *A. tumefaciens* strain A348 into which pVK224 was introduced was incubated in K₃ medium (-AS) or in K₃ medium with 10 μ M acetosyringone (+AS). The DNA was digested with *EcoRI* and probed with *HindIII* fragment Y.

(Fig. 3A), resulted in increased border cleavage. A 4.7-kbp *BamHI* probe for border B hybridized only to the native 12.6-kbp *HindIII* fragment of the Ti plasmid in the B₀ and K₃ bacteria (Fig. 2B). However, two additional fragments of 9.3 and 7.4 kbp were generated upon cocultivation with protoplasts. As shown in Fig. 2A, the 9.3- and 7.4-kbp fragments are consistent with T-DNA fragments resulting from cleavages at borders C and B, respectively. The *Xho I/EcoRI* fragment, a probe for border C, hybridized to the expected 12.6-kbp *HindIII* fragment in the B₀ and K₃ bacteria (Fig. 2B). In the bacteria cocultivated with protoplasts, three additional fragments of 9.3, 5.2, and 3.3 kbp were observed. As shown in the restriction map (Fig. 2A), the generation of 9.3- and 3.3-kbp fragments would be consistent with border C cleavage, whereas the 5.2-kbp fragment is expected from border B

cleavage. An *Nco I/EcoRI* fragment, a probe for border D, hybridized to the expected 8.65-kbp *HindIII* fragment in the B₀, K₃, and P bacteria (Fig. 2B). However, it hybridized to an additional 4.8-kbp fragment in the induced bacteria, consistent with a cleavage at border D. A 2.5-kbp fragment detected in DNA from the B₀, K₃, and P bacteria was also seen in reconstructions prepared from the *HindIII*-digested purified pTiA6 plasmid (data not shown). This result suggests that this band results from cross-hybridization to a related sequence in some other portion of the Ti plasmid. Thus, restriction endonuclease fragments arising from cleavages at or near each of the four borders were detected. In all cases, the border cleavage occurred only upon cocultivation with protoplasts, and the new fragments represented 1–10% of the total hybridization signal.

FIG. 2. T-DNA cleavage at borders B, C, and D. (A) *HindIII* restriction endonuclease map of the T-DNA region. The positions of borders A, B, C, and D are indicated. The sizes and positions of the *HindIII* fragments expected from cleavage at the four borders are shown. The sequences used as hybridization probes are indicated as solid bars. (B) Restriction endonuclease analysis of the DNA from *A. tumefaciens* containing additional copies of *vir* genes. pVK224, a cosmid containing *virG*, *-C*, *-D*, and *-E* was introduced into *A. tumefaciens* strain A348 and the transconjugant was used for cocultivation. The *HindIII*-digested fragments were probed with a 4.7-kbp *BamHI* fragment (border B), an *Xho I/EcoRI* fragment (border C), or an *Nco I/EcoRI* fragment (border D).

FIG. 3. The effect of mutations in different *vir* genes on border A cleavage. (A) Restriction endonuclease map of the *vir* region (18), positions of the Tn3-HoHo1 insertions (19), and the regions covered by the cosmids pVK224, pVK227, and pVK257 (26) used for complementation analysis. (B) Restriction endonuclease analysis of DNA from *A. tumefaciens* strain A348 and the *vir* mutants. The bacteria were grown in AB minimal medium plus glucose (B_0) and incubated in K_3 medium (K_3) or in K_3 medium plus protoplasts (P). The DNA was digested with *Eco*RI and hybridized with *Hind*III fragment Y.

T-DNA Cleavage Occurs Specifically at the Borders. If the cleavage of the T-DNA resulting from cocultivation with protoplasts occurs specifically at the border sequences, one should not see cleavage of the T-DNA region in sequences internal to the borders. To test this, two internal T-DNA segments, *Eco*RI 7 from T left and *Eco*RI 13 from T right (5), were used as hybridization probes to analyze *Eco*RI restriction endonuclease fragments. Both probes hybridized only to the respective native T-DNA fragments in bacteria exposed to all three (B_0 , K_3 , and P) environments (data not shown), suggesting that the observed T-DNA cleavage occurred specifically at or near the borders.

The Role of the *vir* Genes in the T-DNA Cleavage. Since the *vir* genes have been postulated to play an important role in the T-DNA transfer process, we studied the effect of mutations in individual *vir* genes on the T-DNA cleavage. These mutations were generated by Tn3-HoHo1 insertions (19). Each mutant strain was cocultivated with protoplasts, and the DNA was analyzed for border A cleavage by digesting with *Eco*RI and probing with *Hind*III fragment Y. The 3.6-kbp fragment expected from border A cleavage was generated in the wild-type strain (A348) and *virB*⁻, *virC*⁻, and *virE*⁻ mutants upon cocultivation with protoplasts (Fig. 3B). The 3.6-kbp fragment was not generated in the *virA*⁻, *virG*⁻, and *virD*⁻ mutants. These results suggest that the *virA*, *virG*, and *virD* genes play an important role in the events leading to the cleavage of the T-DNA. The 2.6- and 2.2-kbp fragments generated in induced bacteria were susceptible to S1 nuclease digestion and were transferred to nitrocellulose under nondenaturing blotting conditions (data not shown) and therefore may represent single-stranded T-DNA structures.

Since mutations of the *virA*, *virG*, and *virD* genes resulted in the loss of the ability to cause T-DNA cleavage, an attempt was made to restore the normal activity of these genes by complementation in trans with cosmids containing the wild-type genes. The cosmid pVK257 was used to complement the *virA* mutation, pVK224 was used to complement the *virG* and

virD mutations, and pVK227 was used to complement the *virD* mutation (Fig. 3A). The cosmids were transferred to *A. tumefaciens* harboring the respective mutation on the Ti plasmid. The transconjugants were cocultivated with protoplasts, and the DNA was analyzed for border A cleavage. The complementation of *virA*⁻ with pVK257, *virG*⁻ with pVK224, and *virD*⁻ with pVK224 or pVK227 resulted in the restoration of border A cleavage, leading to the generation of the 3.6-kbp fragment in bacteria cocultivated with protoplasts (data not shown). These results suggest that *virA*, *virG*, and *virD* genes mediate the T-DNA cleavage at the borders.

DISCUSSION

The results we have presented in this paper provide evidence for the double-stranded cleavage of the T-DNA specifically at or near the borders in *A. tumefaciens* induced by cocultivation with protoplasts or by incubation with acetosyringone. The cleavage at border A has been particularly well-characterized using three different restriction endonucleases and two different probes. We have observed that the cleavage at border A (left border) is more frequent than the cleavage at border B (right border). This is particularly intriguing in light of the evidence that the right border and adjacent sequences (overdrive) of the T-DNA are more important than the left border, as judged by virulence assays (9–12).

Using two independent methods, λ *in vitro* packaging and plasmid rescue, Koukolikova-Nicola *et al.* (22) demonstrated that circular forms of the T-DNA generated in *A. tumefaciens* upon cocultivation with plant cells can be recovered in *E. coli*. It was also proposed that these circular T-DNA copies may serve as early intermediates in the T-DNA transfer process. More recently, Alt-Moerbe *et al.* (23) demonstrated that circles containing T-DNA border sequences are generated in *E. coli* when a portion of the *vir* region including the *virC* and *virD* genes is also present in the same bacterium. A genetic system has also been developed to study the gener-

ation of circular copies of T-DNA in *Agrobacterium* (24, 25). The promoter and the coding sequences of a kanamycin-resistance gene (*NPTII*) were separately placed close to the left and right T-DNA borders. Circular forms of T-DNA generated by recombination at the borders would thus result in a plasmid containing the promoter and the coding sequences adjacent to each other, allowing the expression of kanamycin resistance. With this approach, it was shown that circular forms of the T-DNA are generated upon incubation of the bacteria with protoplasts or in a protoplast-free conditioned medium. Evidence from all of these investigations therefore suggested that T-DNA excision and possibly religation may be one of the mechanisms by which the T-DNA portion of the Ti plasmid is transferred from the bacteria to the plant cell.

Stachel *et al.* (34) have recently reported that the induction of *A. tumefaciens* by acetosyringone generates a single-stranded linear T-DNA molecule (T-strand). T-DNA fragments with cuts at both DNA strands were obtained only after S1 nuclease treatment. We have not investigated the generation of such T-strands corresponding either to T left or T right. However, we have detected fragments resulting from double-stranded cuts without S1 nuclease treatment. The differences in these observations could be due to the following differences in experimental conditions: (i) We have cocultivated bacteria with regenerating protoplasts since *vir* gene induction and the frequency of border cleavage (Fig. 1) were much higher under these conditions compared to induction with acetosyringone, and (ii) our DNA analysis does not select for single-stranded DNA intermediates. In the *EcoRI*-digested DNA from induced bacteria, in addition to the 3.6-kbp fragment expected from double-stranded border A cleavage, two other fragments of 2.6 and 2.2 kbp were also present (Figs. 2 and 3). The 3.6-kbp fragment was not digested by S1 nuclease, whereas the 2.6- and 2.2-kbp fragments were (data not shown), suggesting the double-stranded nature of the 3.6-kbp fragment and the possible single-stranded nature of the 2.6- and 2.2-kbp fragments. The double-stranded border cleavage we observe may therefore represent a mechanism distinct from the generation of the T-strand (34).

Genetic studies by transposon mutagenesis and complementation analyses have revealed that a chromosomal virulence locus consisting of two genes (*chvA* and *chvB*) and Ti plasmid virulence loci consisting of 6 complementation groups (*virA*, *-B*, *-G*, *-C*, *-D*, and *-E*) are important for the virulence of the bacterium on many plant species (13, 15–18). Although there is abundant evidence for the role of *vir* genes in mediating all of the events that together lead to the excision, transfer, and integration of the T-DNA, the relative role of each of the *vir* genes in the individual events is not well understood. *virC* has been implicated in specifying, in part, the plant host range for virulence of different *A. tumefaciens* strains (35). Two independent studies have indicated that the *virE* gene product(s) could function external to *Agrobacterium*, possibly in directing the integration of T-DNA into the plant genome (36, 37). Mutations of *virA* and *virG* affect the induction of other *vir* genes, suggesting that *virA* and *virG* are the regulatory genes of the entire *vir* regulon (21, 33).

Our results indicate that *virA*, *-G*, and *-D* are required for the cleavage of T-DNA at the borders. Taken together with the fact that *virA* and *-G* are regulatory genes for the entire *vir* regulon, it would appear that *virD* encodes enzyme(s) required for cleaving the T-DNA portion of the Ti plasmid. This conclusion is supported by other recent studies in which it was shown that *virC* and *-D* are sufficient for the generation of T-region circles in *E. coli* (23) and *virA*, *-G*, and *-D* are required for the generation of T-region circles in *A. tumefaciens* (25). We have recently confirmed this hypothesis by demonstrating that expression of the 5' proximal open reading frames of the *virD* locus are sufficient for cleavage of T-DNA borders in *E. coli* (unpublished data).

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