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T-DNA and Opine Synthetic Loci in Tumors Incited by *Agrobacterium tumefaciens* A281 on Soybean and Alfalfa Plants

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We report here the molecular characterization of transferred DNA (T-DNA) in leguminous tumors incited by *Agrobacterium tumefaciens* A281 harboring the tumor-inducing plasmid pTiBo542. The T-DNA is composed of two regions named T_L (left portion)-DNA and T_R (right portion)-DNA, in accordance with the nomenclature for the octopine strains. T_L-DNA is defined by several internal *Hin*dIII restriction fragments totaling 10.8 kilobase pairs (kbp) in uncloned soybean and alfalfa tumors. Alfalfa tumor DNA may contain one more *Hin*dIII fragment at the left end of T_L-DNA than does soybean tumor DNA. T_R-DNA has a 5.8-kbp *Bam*HI-*Eco*RI internal fragment. All borders other than the left border of T_L-DNA appear to be the same within the detection limits of Southern blot hybridization experiments. The two T-DNA regions are separated by 16 to 19 kbp of DNA not stably maintained in tumors. The distance from the left border of T_L-DNA to the right border of T_R-DNA is approximately 40 kbp. Loci for the mannityl opines are situated in T_R-DNA, based on genetic and biochemical criteria.

Agrobacterium tumefaciens is a bacterial plant pathogen that causes crown gall (43). A sector of DNA from the tumor-inducing (Ti) plasmid carried by the bacterium is transferred to the plant nucleus, where it is integrated into and stably maintained primarily in plant chromosomal DNA (10), although it has also been documented in chloroplast DNA (16). This transferred DNA (T-DNA) contains genes that are transcribed in the plant nucleus (4), encoding synthetic functions for opines (17, 20, 29) and the plant growth regulators auxin (28, 46) and cytokinin (1, 2). *Agrobacterium* strains (and their Ti plasmids) are categorized by their host range (either wide or limited) and the characteristic types of opines synthesized in their respective plant tumors (45). The bacterium inciting a tumor can specifically catabolize the opines synthesized in those tumors by using enzymes encoded by the Ti plasmid (7, 34, 38).

A. tumefaciens is of interest not only because it is a plant pathogen but because the Ti plasmid has become useful as a plant genetic engineering vector. Dominant selectable and scorable markers made up of bacterial genes with plant regulatory sequences have been successfully introduced into and expressed in plants by using disarmed T-DNAs as vectors (5, 22, 24, 26). This technology will be useful for engineering desirable traits into agronomically important plants. Ti plasmid-derived vectors are also used to study plant gene regulation by introduction of developmentally regulated, tissue-specific, or organelle-related genes into a foreign system (35, 36, 40, 41, 49).

Of particular interest in this regard is *A. tumefaciens* A281. This strain harbors pTiBo542 and incites large, fast-growing tumors (compared with A277, A208, and Bo542; 26a) on several solanaceous plants. Strain A281 is also virulent on alfalfa and soybean, host range traits not exhibited by Bo542, the wild-type strain which was the Ti plasmid donor for A281 (42). The hypervirulence of strain A281 will likely be a useful trait to exploit for a plant genetic engineering vector (26a).

pTiBo542 has been physically characterized (27), and its opines have been identified (9, 13, 15, 21, 23). This strain was originally referred to as an agropine-type strain, an opine produced by a number of different *A. tumefaciens* strains (23). However, recent evidence shows that an iminodiacid, either L,L-succinamopine (L,L-SAP) or leucinopine (LOP), is the unique opine produced by tumors incited by this strain. Although L,L-SAP and LOP are produced by several pTiBo542-induced tumors (9, 13), LOP is a poor metabolic substrate for strain A281 (12), and for this reason we will refer to strain A281 as an L,L-SAP strain. In this paper we report the molecular organization of T-DNA in tumors incited by strain A281 on alfalfa and soybean. We also present a detailed restriction endonuclease map of the T region, as well as genetic and biochemical evidence for the location of mannityl opine synthetic loci.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are described in Tables 1 and 2. Growth conditions in Luria broth or on L agar or nutrient agar were as described previously (27).

Plant material, growth conditions, and culture of tumor tissue. Soybean, *Glycine max* cv. Wayne, and alfalfa, *Medicago sativa* cv. RA-3, were greenhouse grown. Plants, when approximately 6 to 10 in. (1 in. = 2.54 cm) tall, were inoculated with agar-grown bacteria and 26-gauge needles as described previously (27). Tumors were cultured on hormone-free MS (Murashige and Skoog [37]) medium supplemented with 500 μg of carbenicillin per ml.

Mapping restriction endonuclease sites in plasmid DNAs. Restriction endonucleases were purchased from either New England BioLabs, Inc., or Bethesda Research Laboratories, Inc., and used as recommended by the supplier. DNAs from T-region clones (Table 2) were prepared by the method of

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TABLE 1. *A. tumefaciens* strains used in this work

Strain	Relevant plasmid(s)	Opine	Source or reference
A281	pTiBo542	LOP or L,L-SAP, mannityl	42
Bo542	pTiBo542	LOP or L,L-SAP, mannityl	42
A479(pAL4404::Tn*903*)(pCS277)	pAL4404::Tn*903*, pCS277	LOP or L,L-SAP, mannityl	26a
A479(pAL4404::Tn*903*)(pCS65)	pAL4404::Tn*903*, pCS65	LOP or L,L-SAP	26a
LBA4404(pCS65)	pAL4404, pCS65	LOP or L,L-SAP	26a

Birnboim and Doly (6) as described by Maniatis et al. (32). These DNAs were digested with one, two, or three restriction endonucleases to locate recognition sites relative to *Bam*HI restriction endonuclease sites. Submerged agarose gel electrophoresis was performed as described previously (27). Restriction enzymes that cut frequently in certain *Bam*HI fragments, such as *Hin*dIII in *Bam*HI fragments 5, 10, 16, and 7b, were mapped precisely by a modification of the procedure of Rackwitz et al. (39) originally designed to map lambda clones. Clones of individual *Bam*HI fragments were digested to completion with *Bam*HI and then partially digested with *Hin*dIII (1 to 2 U/μg) at room temperature. Samples were taken every 15 min for approximately 2 h and added directly to loading dye. After separation of these fragments on agarose gels, Southern (44) blots of these gels were made and probed with a gel-isolated fragment from one end of the *Bam*HI fragment being mapped. The fragments used as probes in these experiments are illustrated above the restriction endonuclease map in Fig. 1. Blots were prehybridized in 15 ml of a solution of 6× SSC (1× SSC contains 8.76 g of sodium chloride and 4.41 g of sodium citrate dihydrate per liter) and 10× Denhardt solution (18). Hybridization was carried out in 15 ml of a solution containing 3× SSC, 5× Denhardt solution, 20 mM Tris hydrochloride (pH 7), 0.1% sodium dodecyl sulfate, 2 mM EDTA, 20 μg of denatured calf thymus DNA per ml, and 5×10^6 to 10×10^6 dpm/μg of nick-translated DNA as a probe. Blots were washed four times at 65°C in a solution of 2× SSC–0.5% sodium dodecyl sulfate.

Isolation of DNA from agarose. Fragments were isolated from agarose gel slices as described by Maniatis et al. (32). After elution from the gel slice by electrophoresis, the DNA was precipitated with ethanol and redissolved in 100 μl of TE buffer (10 mM Tris hydrochloride pH 8, 1 mM EDTA). The DNA was extracted three times with isoamyl alcohol, twice with phenol equilibrated with TE buffer, and once with chloroform and then precipitated with ethanol a second time. DNA treated in this manner was labeled to high specific activity by nick translation.

DNA blot hybridization of plant DNA. Plant DNA was isolated as described by Chilton et al. (11) and digested with 5 to 10 U of the indicated restriction enzymes per μg for 6 h at 37°C in a buffer containing 50 mM NaCl, 100 mM Tris hydrochloride (pH 7.9), 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol. Fragments were separated on submerged agarose gels (0.8% SeaKem agarose) in Tris-borate buffer (10.8 g of Tris base, 0.9 g of disodium EDTA, 5.5 g of boric acid per liter). Blots of these gels were prepared by the method of Southern (44) as described by Maniatis et al. (32). Hybridization, nick translation of probes, and wash conditions for these blots (see Fig. 2) were as described previously (27). Each of these blots was exposed to Kodak X-Omat film for 10 days with one intensification screen. Southern blots (see Fig. 3 and 4) were prehybridized overnight at 65°C in a solution of 5× Denhardt solution–5× SSC. These blots were then hybridized at 65°C for 7 h in a 10% dextran sulfate solution (51) also containing 6× SSC, 5× Denhardt solution, 0.25% sodium dodecyl sulfate, 167 μg of denatured salmon sperm DNA per ml, and at least 10^8 dpm/μg of probe DNA. Probes were nick translated as described by Maniatis et al. (32) by using 0.3 to 0.4 μg of gel-isolated DNA fragment and 20 μCi each of [α-^{32}P]dCTP, [α-^{32}P]dATP, and [α-^{32}P]TTP plus 0.1 mM unlabeled dGTP. Blots were washed in a solution of 2× SSC–0.1% sodium dodecyl sulfate at 65°C four times for 1 h each. Each of these blots was exposed to Kodak X-Omat film for 1 to 8 days with two intensification screens. Copy number reconstructions on genomic Southern blots were made from A281 Ti plasmid DNA digested with the indicated enzymes. Calf thymus carrier DNA (10 μg) was added to each reconstruction lane to ensure equal migration of reconstruction and plant DNA fragments. Calculations of copy number equivalents were made from the genome size estimations of Bennett and Smith (3).

Opine analysis. Plant tumor tissues were ground in 95% ethanol at a 1:1 (wt/vol) ratio. Cellular debris was removed by centrifugation. The extract (4 to 20 μl) was assayed for various opines by paper electrophoresis. Agropine and mannopine or mannopinic acid contents were determined by

TABLE 2. Plasmids used in this work

Plasmid	Cloned fragment	Source of fragment	Source or reference
pEHC277	*Bam*HI-5, 10, 13b, 33, 26, 16, 22, 18, 7b	pTiBo542	27
pCS277	Cointegrate of pEHC277 with a wide-host-range shuttle vector		26a
pEHC65	*Bam*HI-5, 10, 13b, 33, 26, 16, 22	pTiBo542	27
pCS65	Cointegrate of pEHC65 with a wide-host-range shuttle vector		26a
pEHC018	*Bam*HI-18	pTiBo542	27
pEHB34	*Bam*HI-16	pTiBo542	27
pEHB107	*Bam*HI-10	pTiBo542	27
pEHB126	*Bam*HI-10, 13b, 33, 26	pTiBo542	27
pEHB140	*Bam*HI-10, 13b	pTiBo542	27
pEHB144	*Bam*HI-5	pTiBo542	27
pEHB146	*Bam*HI-7b	pTiBo542	27

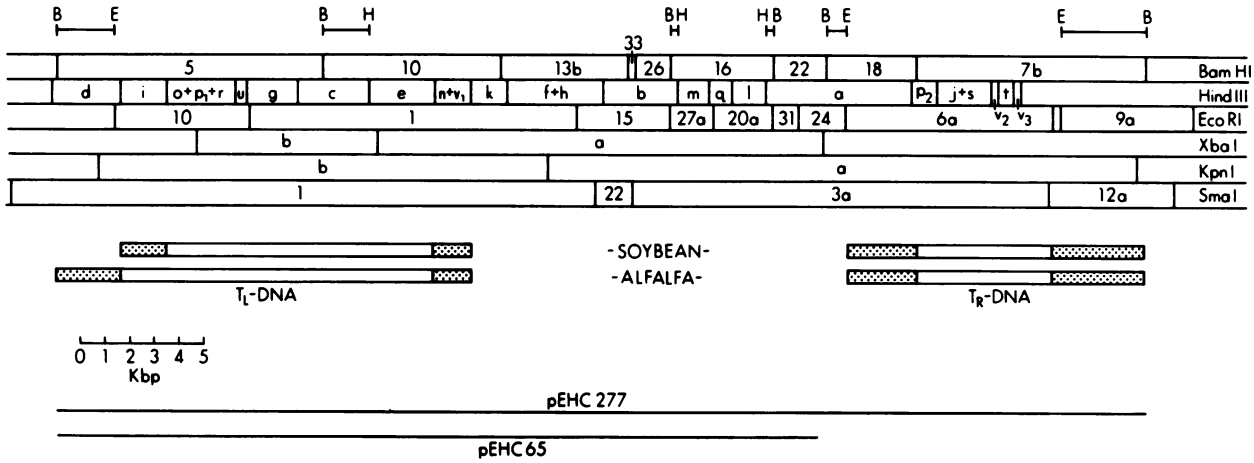

FIG. 1. Restriction endonuclease map of the T region of pTiBo542. The bars above the map identify the probes used in mapping *Hin*dIII partial-digest fragments (see the text for details). *Bam*HI fragments 5, 10, 18, and 7b had been shown previously to have homology to octopine and nopaline T-DNAs (27). These fragments were used as probes on genomic Southern blots (Fig. 2, 3, and 4). The bars below the map represent the extent of T-DNA internal fragments in soybean and alfalfa tumor tissue. The stippled ends represent border regions. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. The lowercase letters are alternate names of restriction endonuclease fragments.

electrophoresis at pH 1.8 and development in silver nitrate without mannitol as described by Chilton et al. (14).

RESULTS

Restriction endonuclease mapping of the pTiBo542 T region with *Hin*dIII, *Eco*RI, *Xba*I, and *Kpn*I. Figure 1 shows the fragment map of the T region of pTiBo542 for several restriction endonucleases. The *Bam*HI and *Sma*I recognition sites shown were determined as described previously (27). *Eco*RI, *Xba*I, and *Kpn*I sites were determined by analysis of products of single, double, and triple digestions of plasmid DNAs from clones containing one or several of the *Bam*HI fragments shown in Fig. 1. The plasmids used for these determinations are listed in Table 2. The *Xba*I and *Kpn*I fragments are so large and similar in size that cosmid DNAs (pEHC277 and pEHC65) were double digested with these two enzymes to determine the relative order of these fragments on the map. In contrast, the *Hin*dIII fragments shown on the map are quite small and were difficult to order by the double-digest method. Therefore, the *Hin*dIII sites in *Bam*HI fragments 5, 10, 16, and 7b were determined by complete *Bam*HI and partial *Hin*dIII digestion of clones of individual *Bam*HI fragments as described in Materials and Methods. *Hin*dIII fragment sizes were determined from complete digests of these clones visualized on stained agarose gels (data not shown). These data were coordinated with data from the partial digest procedure to complete the map as shown.

*Bam*HI fragment 18 has one *Hin*dIII site and one *Eco*RI site. It was important to determine whether these sites were on the same or opposite sides of fragment 18. Single, double, and triple digests of pEHCO18 were done by using *Bam*HI, *Eco*RI, and *Hin*dIII, fragments were separated on an agarose gel, and the gel was blotted onto nitrocellulose paper. The blot was probed with the *Bam*HI-*Eco*RI fragment shown in Fig. 1. The probe hybridized to itself and to the larger double-digest fragments in each case but did not hybridize to the small *Bam*HI-*Hin*dIII fragment (data not shown), indicating that these enzymes cut on opposite sides of the *Bam*HI fragment.

Table 3 lists the sizes of the restriction enzyme fragments shown in Fig. 1. Each *Bam*HI, *Eco*RI, or *Sma*I fragment is numbered according to its size in a digest of the whole Ti plasmid. *Hin*dIII, *Xba*I, and *Kpn*I fragment sizes were determined from clones of the T region and are lettered in descending order from the largest fragment that each enzyme makes in the T region.

Molecular characterization of T-DNAs in soybean and alfalfa tumors. Several lines of A281-incited tumors were established in culture. These lines included sunflower and tomato tumors which were incited on inverted, sterile stem segments in culture and *Datura stramonium*, tobacco, soybean, and alfalfa tumors which were incited on stems of young greenhouse-grown plants. The uncloned tumor tissues grew well in culture on hormone-free MS medium. However, all attempts to clone A281-incited tumor tissues were unsuccessful. Therefore, we analyzed the T-DNAs in DNAs isolated from uncloned tumor lines.

The presence of T-DNA-related sequences in soybean tumor tissue had been determined previously by using clone pEHC277 (Table 2) as a probe in a DNA blot hybridization experiment (27). When the whole Ti plasmid was used as a probe on similar blots, the same pattern was seen (data not shown), indicating that no other regions of the Ti plasmid are represented in soybean tumor DNA. The pattern of hybridization is quite complex with these large probes. Therefore, to further define this T-DNA, we used individual *Bam*HI fragments as probes on blots of tumor DNAs from soybean and alfalfa plants. The plant DNAs were digested with various restriction endonucleases to determine which Ti plasmid fragments were found intact in tumor tissues.

Figure 2 shows the results of Southern blot experiments on soybean tumor DNA digested with *Bam*HI and probed with the individual *Bam*HI fragments 5, 10, and 7b from pTiBo542. The latter two fragments were implicated as possible internal fragments by Hood et al. (27) when using the general probe pEHC277. However, it is clear from the blots in Fig. 2 that sequences homologous to these probes are present but none of the fragments is found intact in *Bam*HI-digested soybean tumor DNA. In addition, *Bam*HI fragments 13b and 22 showed no hybridization to tumor

TABLE 3. Sizes of restriction endonuclease fragments from the T-DNA region of pTiBo542

Fragment no.	Size (kb) of fragment produced by cleavage with:					
	*Bam*HI	*Eco*RI	*Sma*I	*Hin*dIII[a]	*Kpn*I[a]	*Xba*I[a]
1		13.25	24.0	5.9 (a)	24.0 (a)	18.0 (a)
2				3.0 (b)	18.2 (b)	7.3 (b)
3			17.0	2.9 (c)		
4				2.8 (d)		
5	10.8			2.6 (e)		
6		8.3		2.1 (f)		
7	9.2			2.05 (g)		
8				1.9 (h)		
9		5.7		1.85 (i)		
10	7.2	5.5		1.55 (j)		
11				1.43 (k)		
12			5.0	1.4 (l)		
13	5.3			1.3 (m)		
14				1.2 (n)		
15		3.8		1.15 (o)		
16	4.1			1.0 ($p_{1,2}$)		
17				0.85 (q)		
18	3.65			0.7 (r)		
19				0.65 (s)		
20		2.4		0.6 (t)		
21				0.43 (u)		
22	2.1		1.55	0.3 (v_{1-3})		
23						
24		1.9				
26	1.4					
27		1.7				
31		1.1				
33	0.3					

[a] Letters in parentheses are alternate designations for T-DNA fragments produced by cleavage with the indicated enzyme. Fragments are so designated in descending order by size.

DNA (data not shown). These results clearly indicated the presence of a split T-DNA configuration in tumors incited by strain A281.

The limits of T-DNA in tumors were defined more precisely by using other enzymes that digest the T region into smaller fragments (Fig. 1). The blots shown in Fig. 3 define the limits of the left portion of T-DNA (T_L-DNA) in *Hin*dIII-digested soybean and alfalfa tumor DNAs. Figures 3A and B were probed with *Bam*HI fragments 5 and 10, respectively. The reconstructions represent the equivalent of one copy per cell in soybean or 1.8 copies per cell in alfalfa genomic DNA. The internal *Hin*dIII fragments present in soybean and alfalfa tumor DNAs were c, g, o, p_1, and r, which were 2.9, 2.05, 1.15, 1.0, and 0.7 kilobase pairs (kbp), respectively, in size (Fig. 3A, lanes 2 and 4). In addition, alfalfa tumor DNA contained a fragment that comigrated with *Hin*dIII fragment i in a copy number equivalent to the other internal fragments, indicating that alfalfa tumor DNA may have a slightly longer T_L-DNA. BamHI fragment 10 hybridized to two internal *Hin*dIII fragments 2.9 (c) and 2.6 (e) kbp in size in transformed soybean and alfalfa DNAs (Fig. 3B, lanes 2 and 4). No hybridization to control alfalfa and soybean DNAs was seen in either case.

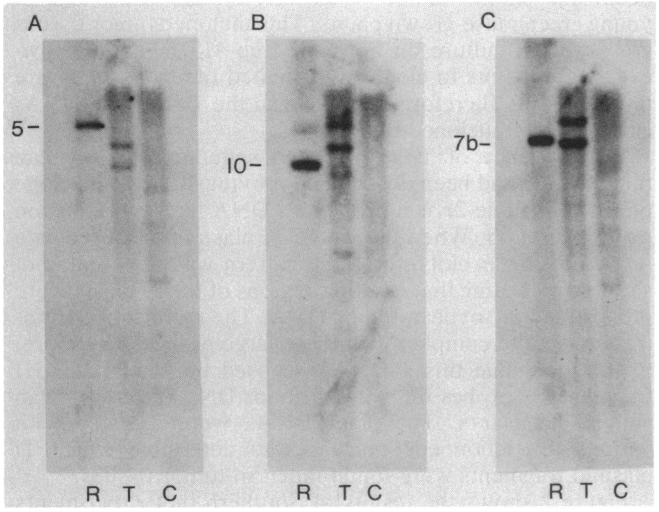

FIG. 2. Detection of T-DNA sequences in soybean tumor and control DNAs digested with *Bam*HI. DNAs were digested and fractionated, and gels were blotted to nitrocellulose filters as described in Materials and Methods. The filters displayed in A, B, and C were prepared from three identical sections of the same agarose gel. All probes were nick-translated, gel-purified fragments. The blots were probed with (panels): A, *Bam*HI fragment 5; B, *Bam*HI fragment 10; C, *Bam*HI fragment 7b. Lanes: R, one copy per cell reconstruction; T, transformed soybean DNA; C, control soybean DNA.

The results in Fig. 3 define a segment of internal *Hin*dIII fragments in T_L-DNA at least 10.8 kbp in size for soybean and alfalfa tumor DNAs. The right border of T_L-DNA is in either the 0.3- or 1.2-kbp *Hin*dIII fragment (n or v_1) of *Bam*HI fragment 10. The left border of T_L-DNA in soybean tumor tissue is in *Hin*dIII fragment i, the 1.85-kbp fragment to the left of *Hin*dIII fragments o, p_1, and r of *Bam*HI fragment 5. The left border of alfalfa tumor T_L-DNA appears to be in the 2.65-kbp *Bam*HI-*Hin*dIII fragment on the left side of *Bam*HI fragment 5. The possibility exists that the alfalfa tumor DNA fragment that comigrated with *Hin*dIII fragment i is a border fragment rather than an internal fragment. However, the left border of T-DNA has been shown to be less precise than the right border (L. W. Ream

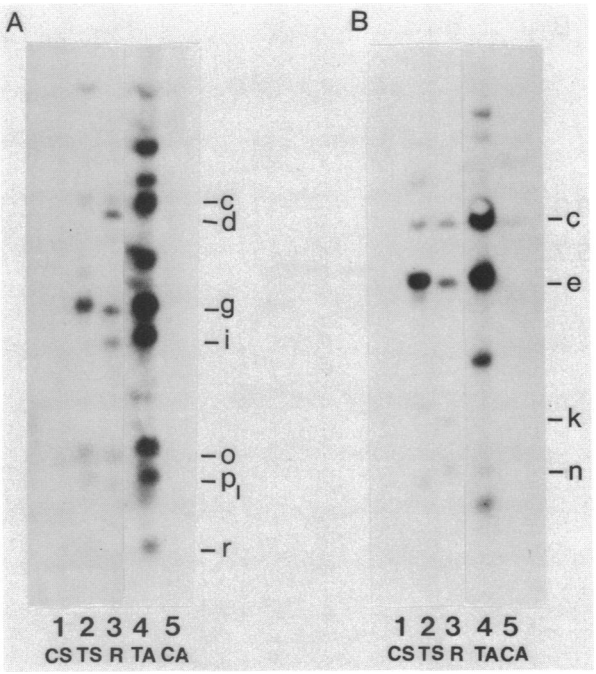

FIG. 3. Detection of T_L-DNA sequences in soybean and alfalfa tumor and control DNAs digested with *Hin*dIII. The filters displayed in A and B were prepared from two sections of the same agarose gel. Probes were nick-translated, gel-purified fragments. The blots were probed with either *Bam*HI fragment 5 (A) or *Bam*HI fragment 10 (B). The lanes on both blots contained DNAs from: 1, control soybean (CS); 2, transformed soybean (TS); 3, 1 (soybean) or 1.8 (alfalfa) copies per cell construction by using *Hin*dIII-digested A281 Ti plasmid DNA (R); 4, transformed alfalfa (TA); and 5, control alfalfa (CA). The reconstructions on these blots did not contain calf thymus carrier DNA, and thus the bands in the reconstructions ran slightly ahead of the bands in the plant DNA-containing lanes. The letters on the side refer to the *Hin*dIII fragments in Fig. 1.

and E. G. Peralta, Abstr. UCLA Symp. Plant Genet., 1985, J. Cell. Biochem. **9C**:262), and we favor the interpretation that this tumor tissue displays a longer T-DNA.

Similar experiments were carried out to define the limits of the right portion of T-DNA (T_R-DNA). *Bam*HI fragment 18 was used as a probe for either *Eco*RI-digested soybean DNA or *Eco*RI-*Bam*HI-digested soybean and alfalfa DNAs (Fig. 4A). Neither of the two *Eco*RI fragments in the reconstruction (lane 3) was found intact in soybean tumor DNA (lane 2). The same was true for the tumor DNAs double digested with *Eco*RI and *Bam*HI. Although there was a fragment of slightly larger size than the 2.6-kbp reconstruction fragment in both soybean and alfalfa tumor DNAs (lanes 5 and 7), a fragment of similar size was also present in control DNAs (lanes 4 and 8) and cannot be considered an internal fragment in T-DNA.

When *Bam*HI fragment 7b was used as a probe (Fig. 4B), once again neither of the two *Eco*RI fragments in the reconstruction (lane 3) was found intact in soybean tumor DNA. However, the larger *Eco*RI-*Bam*HI fragment in the reconstruction (lane 6) was found intact in *Eco*RI-*Bam*HI-digested soybean and alfalfa tumor DNAs (lanes 5 and 7). No hybridization to control DNAs was seen with this probe (lanes 1, 4, and 8).

A 5.8-kbp *Eco*RI-*Bam*HI fragment of *Bam*HI fragment 7b is an internal fragment of T_R-DNA in A281-incited tumors on soybean and alfalfa (Fig. 4). The left border of T_R-DNA lies in *Bam*HI fragment 18 to the right of the internal *Eco*RI site which defines the left edge of *Eco*RI fragment 6a. The right border of T_R-DNA is in the 3.3-kbp *Eco*RI-*Bam*HI fragment at the right edge of *Bam*HI fragment 7b. The enzymes that were mapped in the T region (Fig. 1) do not cut often in the T_R-DNA region and, thus the limits of this T-DNA were not defined further.

Genetic and biochemical evidence for opine synthetic loci in T_R-DNA. We have reported DNA homology studies that indicated that the synthetic loci for the mannityl family of opines are in *Bam*HI fragments 7b and 18 of pTiBo542 (27). Here we present genetic and biochemical evidence to support this conclusion (Table 4). Two clones from the T region, pCS277, containing both T_L-DNA and T_R-DNA, and pCS65, containing only T_L-DNA (Table 2), were used in *trans* complementation experiments with an octopine virulence region to incite tumors on tobacco and tomato plants (26a). The resultant tumor tissues were assayed for agropine and mannopine or mannopinic acid. The mannityl opines were present only in those tumors that were incited by wild-type strains or strains containing T_R-DNA (as on plasmid pCS277) (Table 4). These data showed that the structural organization of this split T-DNA is quite similar to that of octopine-type plasmids with the mannityl opine synthetic genes in T_R-DNA (29).

DISCUSSION

Characterization of a number of Ti plasmids and their T-DNAs leads to a better understanding of the mechanism of transfer and stabilization of T-DNA in plant cells. This study addressed the question of stable T-DNA transfer from a virulent strain of *A. tumefaciens* into two leguminous species.

Several conclusions about the T-DNA of strain A281 can be drawn from the data presented above. (i) The T-DNA of pTiBo542 has two components: T_L-DNA and T_R-DNA. (ii) T_L-DNA has at least 10.8 kbp of internal *Hin*dIII fragments in soybean and alfalfa tumors, although alfalfa tumors may contain an additional 2 kbp. T_R-DNA has an *Eco*RI-*Bam*HI internal fragment of 5.8 kbp. These two regions are separated by approximately 16 to 19 kbp of DNA not found to be stably maintained in tumors. (iii) The synthetic loci for the mannityl opines were determined to be in T_R-DNA by genetic and biochemical criteria.

T-DNAs from several octopine and nopaline Ti plasmids have been analyzed in a number of different tumor tissues, both cloned and uncloned (25, 31, 33, 47, 48, 50, 54). The structure of T-DNA differs between these two types of Ti plasmids (see reference 4 for a review). Octopine T-DNA is divided into two components: T_L-DNA and T_R-DNA of approximately 12 and 6 kbp, respectively. These are separated on the Ti plasmid by 1 to 2 kbp of nontransferred region. T_L-DNA contains the oncogenes necessary for tumor maintenance and octopine synthesis (30), whereas T_R-DNA contains genes for agropine synthesis (29). In contrast, nopaline T-DNA is one continuous piece of approximately 20 kbp and contains all of the genes necessary for tumor maintenance and opine synthesis (31). T-DNAs from several root-inducing plasmids of *A. rhizogenes* have also been analyzed (8, 19, 52). T-DNAs from the agropine-type plasmids pRi1855 and pRiA4b also are composed of two parts, but these are widely separated on the root-inducing plasmid. Agropine synthetic loci (19) and a region homologous to the Ti plasmid auxin synthetic loci (52) have been localized in

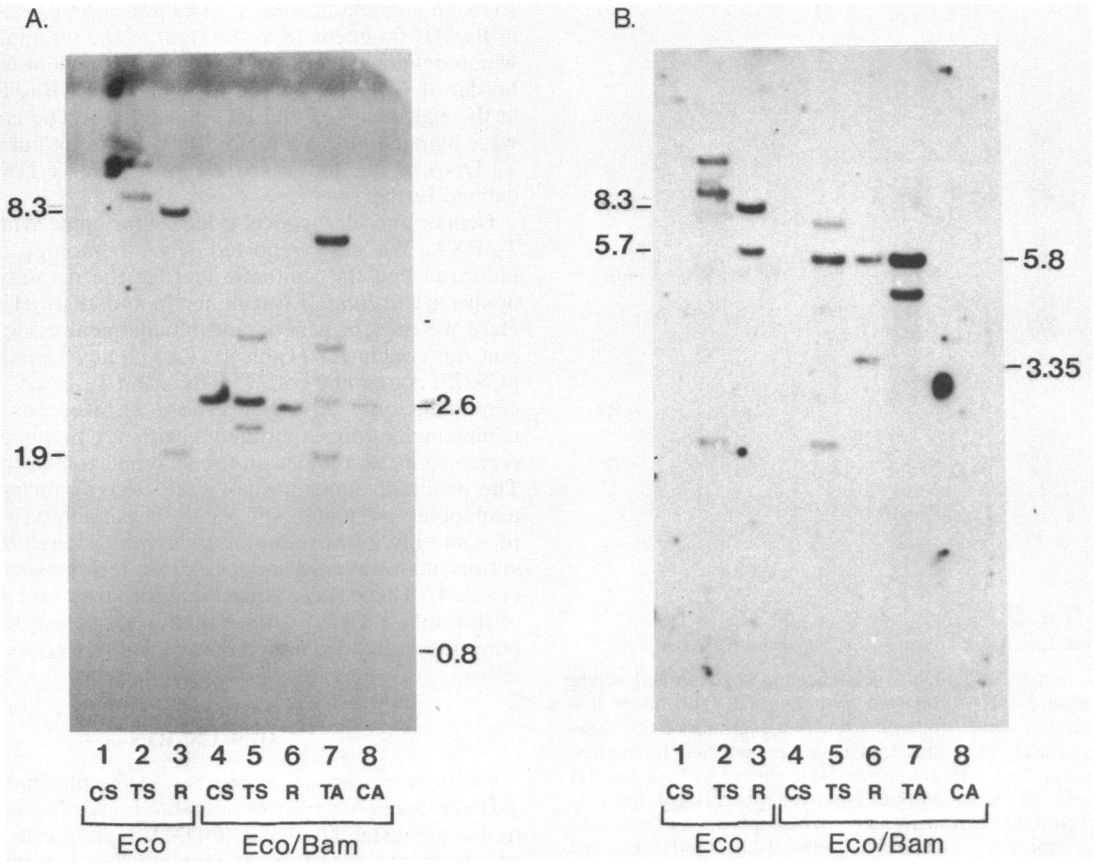

FIG. 4. Detection of T_R-DNA sequences in soybean and alfalfa tumor and control DNAs digested with *Eco*RI or *Eco*RI plus *Bam*HI. The filters displayed in A and B were prepared from different agarose gels. Probes were nick-translated, gel-purified fragments. Blots were probed with either *Bam*HI fragment 18 (A) or *Bam*HI fragment 7b (B). The DNAs in lanes 1 to 3 in each case were digested with *Eco*RI; the DNAs in lanes 4 to 8 were digested with *Eco*RI plus *Bam*HI. The lanes on both blots contained DNAs from (lanes): 1, control soybean (CS); 2, transformed soybean (TS); 3, one copy per cell reconstruction by using A281 Ti plasmid DNA (R); 4, control soybean (CS); 5, transformed soybean (TS); 6, 1 (soybean) or 1.8 (alfalfa) copies per cell reconstruction by using A281 Ti plasmid DNA (R); 7, transformed alfalfa (TA); 8, control alfalfa (CA). The numbers on the side represent the sizes of fragments in kilobase pairs.

T_R-DNA. Here we report the structure of T-DNA from *A. tumefaciens* A281, an L,L-SAP strain. It is like the *A. rhizogenes* and octopine T-DNAs in that it has T_L- and T_R-DNA components. However, its intervening region of 16 to 19 kbp is like that of the root-inducing plasmid and much larger than that found on octopine Ti plasmids. This wide separation of T-DNAs is also seen in limited-host-range Ti plasmids (54). However, the oncogenes on the limited-host-range and root-inducing plasmids are split between the two T-DNAs as shown by hybridization and functional studies (52, 54), an organization differing from the wide-host-range Ti plasmids as discussed above. We have shown by hybridization (27) and genetic experiments (26a) that T_L-DNA contains the oncogenes necessary for tumor formation. Hybridization data also indicated that T_R-DNA contains the synthetic loci for the mannityl family of opines (27), an organization much like that of octopine Ti plasmids. We confirmed these latter data by using genetic and biochemical tests.

The core T-DNA in most octopine and nopaline tumors is constant, containing the full complement of tumor oncogenes and opine genes, as well as a number of unidentified loci. The borders also appear to be fairly constant within the detection limits of Southern blot analyses. However, the exact end(s) of T-DNA can differ as seen in two tobacco tumor lines incited by the same octopine Ti plasmid (47), an octopine tumor on sunflower (48) and a nopaline tumor on flax (25). The A281-incited soybean and alfalfa tumor lines analyzed in these experiments appear to have defined colinear internal T-DNA segments, although the alfalfa tumor may have approximately 2 kbp of additional DNA at the left end of T_L-DNA. The remaining boundary fragments appear to be the same within the detection limits of Southern blot hybridization. In addition, preliminary studies on a tomato tumor line incited by strain A281 showed the complement of internal *Hin*dIII fragments to be somewhat shorter than the soybean tumor DNA at the left end of T_L-DNA. This observation is being investigated further.

T-DNA in these uncloned legume tumors was present at approximately one copy per cell for soybean and 5 to 10 copies per cell for alfalfa DNA (Fig. 3 and 4). The tumor tissue was uncloned and presumably contained transformed and untransformed cells. The number of copies could be the result of a large number of insertions per cell in a few cells or a single insertion in most cells of the tumor. The stable state of these cultured tumors may not reflect the original ratio of transformed to untransformed cells if a selection takes place for transformed cells as the tumor is cultured in vitro. There

TABLE 4. Opines in tumor tissues incited by strains of *A. tumefaciens* derived from A281

Strain and plant	Presence of opine[a]: MOP or MOA	AGR
A281		
Alfalfa	+	+
Soybean	+	+
Tomato	+	+ + +
Tobacco	+	+ +
Bo542		
Tobacco	+	+ +
None		
Tobacco	−	−
Tomato	−	−
A479(pAL4404::Tn*903*)(pCS65)		
Tobacco	−	−
Tomato	−	−
LBA4404(pCS65)		
Tobacco	−	−
Tomato	−	−
A479(pAL4404::Tn*903*)(pCS277)		
Tobacco	+	+ +
Tomato	+	+ +

[a] MOP or MOA = Mannopine or mannopinic acid; AGR = agropine.

appeared to be only a few major border fragments in these soybean and alfalfa tumor lines (Fig. 3 and 4), indicating that probably only a few insertions took place. Therefore, it appeared that these few cells proliferated preferentially while the tissue was being cultured.

The data presented here represent the first detailed molecular characterization of a virulent T-DNA in leguminous species. The principles of T-DNA transfer appear to be followed for this T-DNA in that a core of common DNA encoding hormone independence, as well as a second DNA segment encoding synthetic loci for several opines, is present. It will be interesting to determine the sequence of the T-DNA borders in these tumors to see whether they also conform to the 25-base-pair repeat structure seen in octopine and nopaline T-DNAs (53, 55).

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