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Agrobacterium tumefaciens T-DNA Integrates into Multiple Sites of the Sunflower Crown Gall Genome

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Summary. We analyzed the integration of a tumor inducing (Ti) plasmid into an octopine producing crown gall tumor of sunflower, line PSCG 15955. A continuous Ti plasmid segment (T-DNA) of about 19.5 kilo base pairs (kbp) is transferred and integrated into a small number of sites of the plant DNA.

The number of T-DNA integration sites in our tumor line was estimated by two different methods. First, cloned fragments of the T-DNA were hybridized to tumor DNA and the hybridization patterns were observed. The number of T-DNA integration sites in line PSCG 15955 was found to be approximately eight.

Second, a library was constructed from total DNA of tumor line PSCG 15955 by molecular cloning using the bacteriophage lambda vector Charon 4A. Recombinant phages having sequence homologies to the Ti plasmid were selected. The lower limit on the number of integration sites was three because we obtained three different right hand side genomic clones of plant/T-DNA hybrids. The initial screening of the library also revealed two left hand border clones. Hybridization of these five distinct recombinant clones to uninfected sunflower DNA shows that the cloned T-DNA segments are covalently bonded to plant DNA. The left and right hand plant boundary sequences are homologous to either unique and or repeated plant DNA segments.

Introduction

Agrobacterium tumefaciens strains, harboring tumor-inducing (Ti) plasmids incite cancerous growth, called crown gall, when inoculated into a wide range of gymnosperms and dicotyledonous angiosperms. The genetic determinants for this transformation are carried by large Ti plasmids (Zaenen et al. 1974; Van Larebeke et al. 1974; Watson et al. 1975; and Bomhoff et al. 1976). During infection a segment(s) of the Ti plasmid (T-DNA) is transferred to and stably integrated into the genome of the host plant cell (Chilton et al. 1977b). The T-DNA produces transcription and translation products (Gurley et al. 1979; McPherson et al. 1980; Gelvin et al. 1981; Schröder et al. 1981; Murai and Kemp 1982) resulting in characteristic changes in the physiology of the plant cells. These changes include the ability of transformed cells to grow in the absence of auxin and cytokinin, whereas normal plant cells in culture require phytohormones, and the ability of crown gall cells to produce derivatives of basic amino acids, called opines, which are not synthesized by normal plants (Menagé and Morel 1964 and Goldman et al. 1969). The synthesis of opines such as octopine or nopaline is coded for by the Ti plasmids, which also code for the catabolism of the corresponding opine (Bomhoff et al. 1976; Montoya et al. 1977; McPherson et al. 1980; Murai and Kemp 1982). Since *A. tumefaciens* can utilize the opines as carbon and nitrogen sources, the interaction of the bacteria with the plants is a natural form of "genetic engineering" where the bacterium introduces its genetic information into the plant host, inducing it to synthesize metabolites that only it can utilize.

There are several regions of the Ti plasmid DNA that are common to both octopine and nopaline type plasmids. One of the common DNA sequences, a segment of about 8.5 kbp, is contained within the T-DNA and present in most transformed cells and apparently involved in tumor formation and maintenance (Chilton et al. 1977a; Depicker et al. 1978; Thomashow et al. 1980a; Zambryski et al. 1980; Yadav et al. 1980; Lemmers et al. 1980; and Merlo et al. 1980).

The mechanism by which T-DNA integrates into the host plant genome is unknown. Southern hybridization studies have shown that the T-DNA maintained in several octopine tumors is composed of either one, namely T_L, or two Ti plasmid segments: T_R and T_L (Thomashow et al. 1980a; Merlo et al. 1980; De Beuckeleer et al. 1981). T_L is homologous to the "common" T-DNA segment and has been found in low copy number per cell and having variable edges in different tumor lines. T_R, homologous to a stretch of T-DNA to the right of T_L, was shown to be amplified to a high copy number in one tumor line.

In contrast to the complex T-DNA inserts found in octopine tumors, the T-DNA in nopaline tumors consists of one continuous stretch of T-DNA and appears to be constant in different lines within the resolution of Southern blot analysis technique (Lemmers et al. 1980). Contrary to the reported octopine tumors, nopaline tumors can carry from one to at least four different T-DNA copies, as recognized by their composite border fragments, and the T-DNA copies can be arranged in tandem configuration as was observed in two independent lines (Lemmers et al. 1980; Zambryski e al. 1980).

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A more accurate determination of the T-DNA integration can be achieved by analysis of crown gall clones harboring T-DNA and plant/T-DNA borders. So far, only two octopine (Thomashow et al. 1980b) and two nopaline (Yadav et al. 1980; Zambryski et al. 1980) hybrid border clones have been identified. Studies of these clones provided direct evidence that the T-DNA can be covalently joined to the plant genome. Zambryski et al. (1980) isolated an additional clone derived from a nopaline tumor, which is a "fusion" product of two ends of the left and right copies of T-DNA, suggesting that the T-DNA can either be tandemly repeated in the plant genome or exist as an independent replicon. These results may lead to conclude that the T-DNA integration into the plant varies possibly depending upon virulent Ti plasmid utilized.

The work described in this paper is concerned with the integration of T-DNA into the octopine producing sunflower tumor. Detailed analysis of the T-DNA borders and number of T-DNA integration sites was performed using restriction site mapping and molecular hybridization techniques as well as by construction and analysis of crown gall plant/T-DNA hybrid clones. Our studies suggest that the T-DNA maintained in octopine sunflower tumor line PSCG 15955 appears to be less complex than was previously reported for other octopine tumors.

Material and Methods

Chemicals. Deoxyadenosine 5′-[α-^{32}P] triphosphate and deoxycytidine 5′-[α-^{32}P] triphosphate, specific activity >400 Ci/mmol were purchased from Amersham Corporation and New England Nuclear. The nick translation kit was obtained from Amersham Corporation. Restriction endonucleases were purchased from Biotec, Madison; Bethesda Research Laboratories, Inc. and New England Biolabs. T4 DNA ligase was a product of New England Biolabs. ϕ × 174 digested with *Hin*cII was purchased from Bethesda Research Laboratories, Inc.

Bacterial Strains. Strains p101, p203, p301, p202, p303, p403 and p501 contain recombinant plasmids whose inserts are Bam HI generated fragments B1–2, B4–5 and B5–6 and EcoRI fragments R3–4, R6–7, R7–8 and R8–9, respectively, of pTi-15955 cloned into pBR322 (Fig. 1) (Gurley et al. 1979 and unpublished results). Strains DP50 Sup F: F^- *dap*D8 *lac*Y^- Δ(gal-uvrB) *thy*A *nal*A^r hsds suII and suIII as well as K802: *hsr*$^-$ hsm$^+$ *gal*K^- suII *lac*Y^- met$^-$ were obtained from Dr. O. Smithies, Madison, WI.

Tissue Culture. The establishment of primary sunflower (*Helianthus annus* cv. Mammoth Russian) crown gall tissue culture lines PSCG 15955 and habituated sunflower stem section culture (HSSS) from healthy tissue have been described (Merlo and Kemp 1976). Tissue culture lines were maintained on solid medium as callusses at 27° C in the dark on Linsmaier and Skoog's medium (1965) lacking hormones.

DNA Preparation. We isolated total plant DNA from tissue culture lines PSCG 15955 and HSSS as was described by Bendich et al. (1980). The DNA obtained was larger than 50 kbp and was readily digestible with a number of restriction endonucleases, except for *Sma*I which appears to hydrolyze only T-DNA, and ligatable.

Ti plasmid DNA was isolated from strain *A. tumefaciens* 15955 by the large scale neutral lysis procedure of Ledeboer (1978). Recombinant plasmids containing T-DNA were prepared as described by Ursic and Davies (1979).

Hybridization Analysis. Purified genomic DNA samples of 2 μg/well were digested with restriction endonucleases under conditions recommended by the supplier. For comparison of T-DNA restriction fragment sizes, about 10 pg of Ti plasmid DNA was digested with the appropriate restriction endonucleases. Following digestion, we added 1 μg of calf thymus DNA with an average length of 350 bp and placed this sample in wells adjacent to the correspoinding genomic DNA digest. In all gels, lambda DNA, digested with *Hin*dIII and ϕx174 digested with *Hin*cII were used as markers. The digested DNA was fractionated on horizontal 0.7% agarose gel (Ursic and Davies 1979).

For the experiment of hybridizing the hybrid plant/T-DNA clones to HSSS DNA, the DNA was digested with *Eco*RI and the fragments were separated on a preparative 5 × 20 × 0.6 cm horizontal 0.7% agarose gel. Southern blots were prepared and 0.7 cm vertical strips were used for analysis.

Plasmid DNA was analyzed similarly to genomic DNA studies, except that less than 1 μg of DNA was used. The separated DNA fragments were denatured in the gel and transferred to nitrocellulose membranes as described by Southern (1975). Hybridization probes of the appropriate DNA restriction endonuclease fragments, obtained by electroelution, were prepared by nick translation according to conditions supplied by Amersham. Prehybridization and hybridization conditions were carried out as described by Wahl et al. (1979).

Construction and Screening of Lambda Genomic DNA Collection and Recombinant Phage DNA Isolation. We cloned total genomic DNA of tissue culture lines PSCG 15955 and HSSS by ligating partial *Eco*RI digestion fragments ranging in size from 10 to 22 kbp into Charon 4A lambda vectors as was described previously (Blattner et al. 1978; Slightom et al. 1980). Approximately 2×10^6 lambda plaques were screened using ^{32}P-labeled pTi-15955 as hybridization probe following Benton and Davis (1977) method as was described by Blattner et al. (1978). For recombinant phage growth and DNA isolation we followed the procedures described by Blattner et al. (1977) and Moore et al. (1977).

Results

Restriction Endonuclease Map of T-DNA Region of pTi-15955

To map precisely the T-DNA in our crown gall we constructed a map of the T-DNA region of the virulent *Agrobacterium tumefaciens* plasmid pTi-15955 as shown in Fig. 1. pBR322-T-DNA containing clones were used as probes for the mapping. The clones are shown in Fig. 1 above their corresponding T-DNA position and listed in Table 1.

The nomenclature for the various restriction endonuclease fragments of the T-DNA of octopine type plasmids listed in the literature is not consistent. We standardized the T-DNA region by labeling the restricton endonuclease

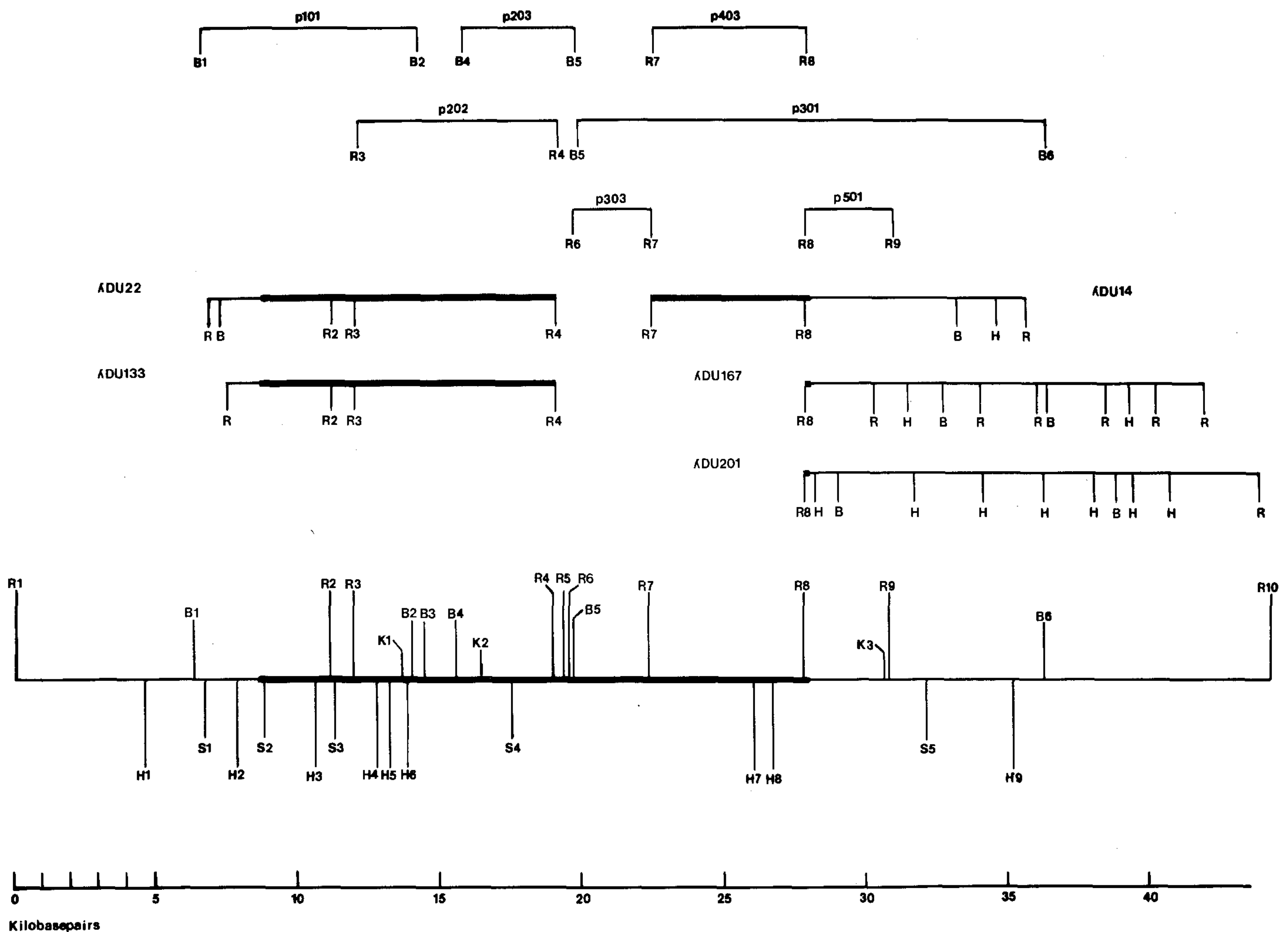

Fig. 1. Restriction endonuclease map of pTi-15955 T-DNA. Restriction endonuclease sites are designated R(*Eco*RI), B(*Bam*HI), H(*Hin*dIII), K(*Kpn*I) and S(*Sma*I) followed by a number. Restriction sites are numbered in order from left to right. T-DNA in sunflower tumor line PSCG 15955 is marked by a bold solid line, unintegrated Ti plasmid DNA by a thin solid line. Crown gall genomic clones, designated λDU22, λDU133, λDU14, λDU167, and λDU201, are shown as they correspond to the T-DNA based on restriction endonuclease analysis and hybridization studies. T-DNA is designated by a bold solid line, plant DNA by a thin solid line. The exact position of plant/T-DNA border has not been determined. Only *Eco*RI restriction endonuclease sites in the T-DNA segment of these clones are marked, the remaining restriction endonuclease sites are the same as shown on the T-DNA map. In clones λDU167 and λDU201 only the plant/T-DNA border *Eco*RI and *Hin*dIII sites, respectively, are precise, the relative locations of the remaining *Eco*RI and *Hin*dIII sites in the plant are marked arbitrarily. pBR322-T-region clones used in our experiments (p101, p203, p301, p403, p202 and p501) are shown above their corresponding T-DNA positions

sites by R, B, H, S, K, for *Eco*RI, *Bam*HI, *Hin*dIII, *Sma*I and *Kpn*I, respectively. Number 1 corresponds to the left most restriction site of the T-DNA region, whereas R10, B6, H9, S5 and K3 correspond to the right side of T-DNA. The relationship of this map to the various restriction endonuclease fragments as designated by different authors is shown in Table 1.

T-DNA is Congruent with a Continuous Segment of the Ti Plasmid

DNAs prepared from uninfected sunflower tissue (HSSS) and from crown gall tumor line PSCG 15955 were digested with *Eco*RI, *Hin*dIII, *Bam*HI and *Sma*I restriction endonucleases. DNA fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose and hybridized against radioactive probes containing specific T-DNA restriction fragments. By varying the restriction endonucleases one can obtain fragments covering the entire T-DNA and each one having DNA in common with those probes. T-DNA clones digested with the appropriate restriction endonucleases were used as markers for internal fragments (not shown). Radioactive probes B1–2 and B5–6 hybridized to PSCG 15955 DNA (Fig. 2b–e and Fig. 3b–d) and not to HSSS DNA (Figs. 2a and 3a). The internal restriction endonuclease fragments are indicated by the lettered sites in these figures.

From these hybridization patterns we conclude that a continuous T-DNA segment extending from a site between *Hin*dIII site H2 and *Sma*I site S2 to the right of *Eco*RI site R8 is present in plant DNA (Fig. 1 and text below). The restriction endonuclease pattern of the T-DNA in our crown gall matches the T-DNA pattern of the Ti plasmid pTi-15955, which was used to induce the tumor line

Table 1. Nomenclature restriction endonuclease fragments of T-region of p-Ti 15955

Ursic	Gurley (3)	Thomashow (4)	Chilton (1)	De Beuckeleer (p-Ti ACH 5) (2)
B1–2	8	8	–	8
B2–3	28	29	–	28
B3–4	–	–	–	30 b
B4–5	17	19	–	17 a
B5–6	2	2	–	2
H1–2	c	–	–	–
H2–3	d	y	–	18 c
H3–4	e	x	–	22 e
H4–5	h	–	–	38 c
H5–6	g	–	–	36 b
H6–7	a	1	–	1
H7–8	f	c	–	–
H8–9	b	5	–	4
R1–2	A	2	–	3
R2–3	F	δ	–	32
R3–4	B	7	–	7
R4–5	H	–	–	–
R5–6	G	–	–	–
R6–7	E	24	–	19 a
R7–8	C	13	–	12
R8–9	D	22	–	20
S1–2	–	–	17	–
S2–3	–	–	16 a	–
S3–4	–	–	10 c	–
S4–5	–	–	3 b	–

Standardization of restriction fragments nomenclature as used by (1) Chilton M-D et al. (1977a); (2) De Beuckeleer M et al. (1981); (3) Gurley WB et al. (1979); (4) Thomashow MF et al. (1980a)

PSCG 15955, indicating that the plasmid DNA is accurately transferred and integrated into plant DNA. The segment of transferred T-DNA is about 19.5 kbp in length, and contains both the T_L and T_R segments.

Estimation of T-DNA Integration Sites by Analysis of Border Fragments

An estimate on the number of integration sites of T-DNA in crown gall tumor line PSCG 15955 was obtained by conducting two sets of experiments. First, analysis of autoradiograms of Figs. 2 and 3 show that the T-DNA has inserted into a limited number of sites as seen by the limited number of bands. The bands represent in addition to the internal T-DNA fragments (as discussed in the previous section) the left (Fig. 2) and the right hand (Fig. 3) border fragments. Assuming that every fragment that is not an internal T-DNA fragment is a plant/T-DNA junction, we found that the T-DNA has inserted into approximately eight different sites. Variation of the number of boundary fragments when different restriction endonucleases are used can be attributed to a number of factors. First, if the border fragments contain very little of the T-DNA, the hybridization of the probe to that border could be too faint to detect. Second, some bands may have been obscured by the internal T-DNA fragments. We therefore used various restriction endonucleases to increase the probability of obtaining border fragments with more T-DNA sequence homology and located at positions which are not obscured by other hybridizing bands. Third, we did not rule out the possibility that some "border" fragments are partial digest products.

A summary of estimated sizes of the left and right hand plant/T-DNA hybrid restriction endonuclease fragments and internal T-DNA fragments with relative intensity of hybridization is shown in Table 2.

Lambda Genomic Clones Show that T-DNA is Integrated into at Least Three Different Sites in Sunflower Crown Gall

A second set of experiments was performed to estimate the number of insertion sites of T-DNA in sunflower crown gall. A library was constructed from total DNA of tumor line PSCG 15955 by molecular cloning using the bacteriophage lambda Charon 4A as a vehicle. Clones hybridizing to nick translated pTi-15955 were isolated and analyzed. The number of different clones harboring plant/T-DNA hybrids set the lower limit on the number of integration sites. We screened for T-DNA content approximately 2×10^6 recombinant λ clones representing approximately 1% of our genomic library. Twenty-five positive phage clones were obtained and nineteen of them were further purified. From these clones we obtained six pure, independent λ clones. The remaining putative T-DNA clones were either false positives or were lost during purification procedure. Thus, in our library about one in every 3×10^5 phage clones contains T-DNA.

Five of these clones were analyzed by restriction endonuclease digestion, followed by hybridization to probes homologous to the right and left hand side of T-DNA. Two clones, λDU22 and λDU133, contained the left hand side of the T-DNA covalently linked to different plant DNA segments. The remaining three clones, λDU14, λDU167 and λDU201 contained the right hand side of the T-DNA and three different pieces of plant DNA.

Figure 4a–f presents digestion and hybridization results obtained with the right hand side T-DNA clone λDU14 which contains a total of 13.3 kbp plant/T-DNA. Upon digestion with *Eco*RI five fragments are generated (Fig. 4a), of which the 7.8 and 5.5 kbp fragments contain crown gall DNA inserts (see below). The 19.8 kbp and 10.9 kbp fragments are the left and right Charon 4A arms, respectively, and the 30.7 kbp fragment is the annealed joined left and right arm of this vector. Analysis of the autoradiogram of Fig. 4b and c show that the 5.5 kbp *Eco*RI fragment shows strong, and the 7.8 kbp fragment weak, sequence homology to the right hand T-DNA segment B5–6 and none to the left end of T-DNA segment B1–2.

We inferred that the 5.5 kbp fragment is the internal R7–8 T-DNA segment and the 7.8 kbp fragment is the plant/T-DNA hybrid extending rightward from the R8 site and containing only a small amount of T-DNA ($\approx$500 bp). This indicates that the right hand T-DNA border is very close to the R8 restriction site. The 5.5 kbp *Eco*RI fragment from λDU14 was found to be equivalent to the internal T-DNA segment R7–8, since it hybridized specifically to the R7–8 recombinant plasmid p403 (data not shown).

To substantiate our conclusions we digested clone λDU14 with *Hin*dIII restriction endonuclease, generating five fragments (Fig. 4d). The 23 kbp and 28 kbp fragments

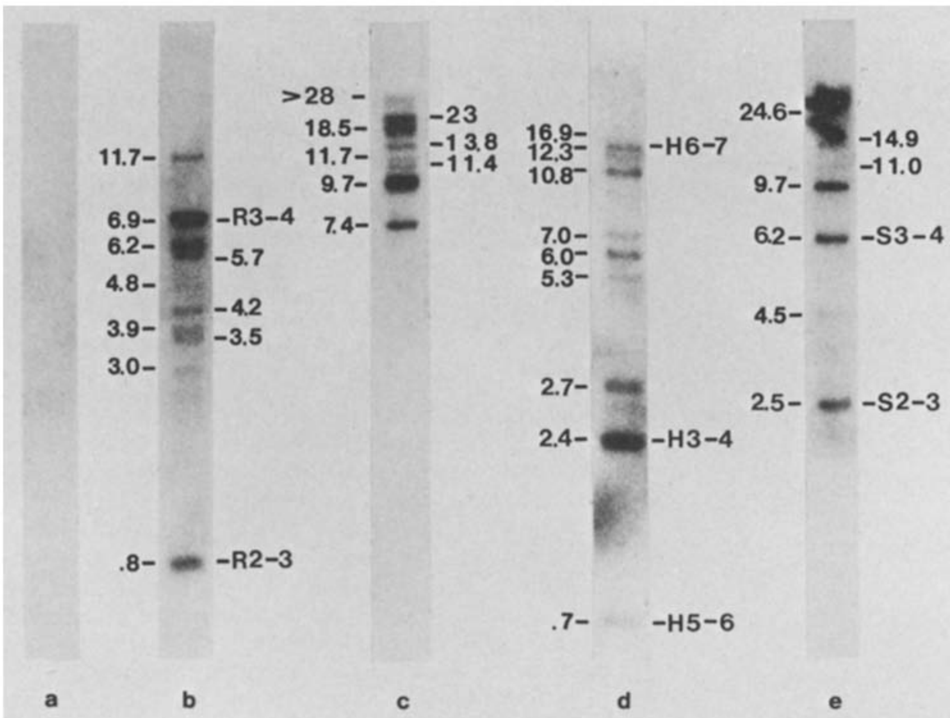

Fig. 2. Hybridization of nick translated probe of segment B1–2 (T-DNA left end) onto Southern blots of *Eco*RI digested HSSS DNA (a), and PSCG 15955 DNA digested with *Eco*RI (b), *Bam*HI (c), *Hin*dIII (d), *Sma*I (e). Fragments labeled R3–4, R2–3, H6–7, H3–4, H5–6, S3–4 and S2–3 are internal T-DNA fragments and illustrated in Fig. 1 and Table 1. Fragment sizes are given in kbp in this and subsequent figures

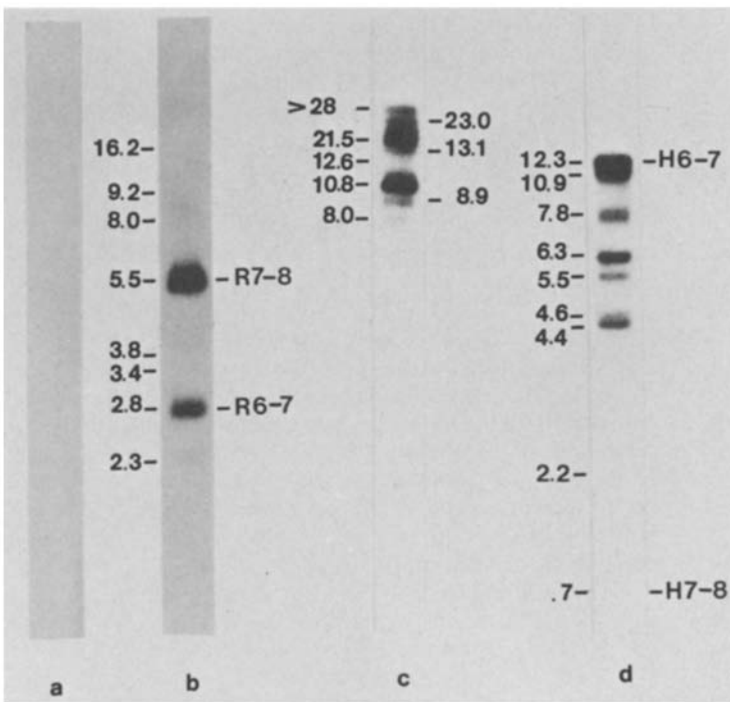

Fig. 3. Hybridization of nick translated probe of segment B5–6 (T-DNA right end) to Southern blots of HSSS-DNA digested with *Eco*RI (a) and PSCG 15955 DNA digested with *Eco*RI (b), *Bam*HI (c), *Hin*dIII (d). Fragments labeled R7–8, R6–7, H6–7 and H7–8 are internal T-DNA fragments and illustrated in Fig. 1 and Table 1

are the left arm and cohesively joined left and right arms of our vector, respectively, containing about 3 kbp of recombinant inserted DNA. The 6.2 kbp fragment is the right arm of the vector plus 1.1 kbp of crown gall DNA. The 5.7 kbp fragment is a λ Charon 4A fragment and the 8.8 kbp band is plant/T-DNA insert. We then hybridized segment B5–6 to the *Hin*dIII digested fragments. Bands of 28 kbp, 23 kbp and 8.8 kbp hybridized to this probe (Fig. 4e). None of the fragments showed sequence homology to probes of B1–2 (Fig. 4f).

Table 2. Restriction endonuclease fragments (in kbp taken as average of 2–3 gels) of PSCG 15955 crown gall DNA homologous to T-DNA. PSCG 15955 total DNA was digested with *Eco*RI, *Bam*HI, *Hin*dIII and *Sma*I. The fragments were separated on agarose gel and transferred to nitrocellulose filters (see Methods). Nick translated T-DNA of strain 15955, as well as T-DNA *Bam*HI fragments (p101) B1–2 (left hand border) and (p301) B5–6 (right hand border) were used as hybridization probes. In the case of *Sma*I digestion, we used Ti plasmid DNA and clone p101 (B1–B2) hybridization to deduce the right hand plant-T-DNA hybrid fragments. The relative intensity of hybridization bands: + + + very strong, + + strong, + weak, ± very weak. Association of some fragments to T-DNA's internal fragments and hybrid clones is indicated. The hybrid clones (border clones) association to the restriction fragments was achieved by matching their sizes

	Restriction enzyme			
	*Eco*RI	*Bam*HI	*Hin*dIII	*Sma*I
Internal fragments	6.9+ + +R3–4 5.5+ + +R7–8 2.8+ + R6–7 0.8+ R2–3	4.8+ + +B4–5 1.1+ B3–4	12.3+ H6–7 2.4+ + +H3–4 0.7± H5–6 0.7± H7–8	6.2+ + +S3–4 2.5+ + +S2–3
Left hand plant-T-DNA hybrid fragments	11.7+ 6.2+ + 5.7+ 4.8± 4.2+λDU22 3.9+ 3.5+λDU133 3.0+	>28 + 23 + + 18.5+ + 13.8+ 11.7+ 11.4+ 9.7+ + + 7.4+ + λDU22	16.9+ 11.8+ 10.8+ 7.0+ 6.0+ 5.3± 2.7+ +	24.6± 14.9+ 11.0+ 9.7+ + + 4.5+
Right hand plant-T-DNA hybrid fragments	16.2±λDU201 9.2± 8.0±λDU14 3.8± 3.4±λDU167 2.3+	>28 ± 23 + 21.5+ + 13.1+ λDU14 12.6+ λDU167 10.8+ + + 8.9+ 8.0+ λDU201	10.9± 7.8+ λDU14 6.3+ + 5.5+ λDU167 4.6+ 4.4+ + 2.2± λDU201	27.7+ + + 12.3+ 7.8+ 5.6+ + 2.9+

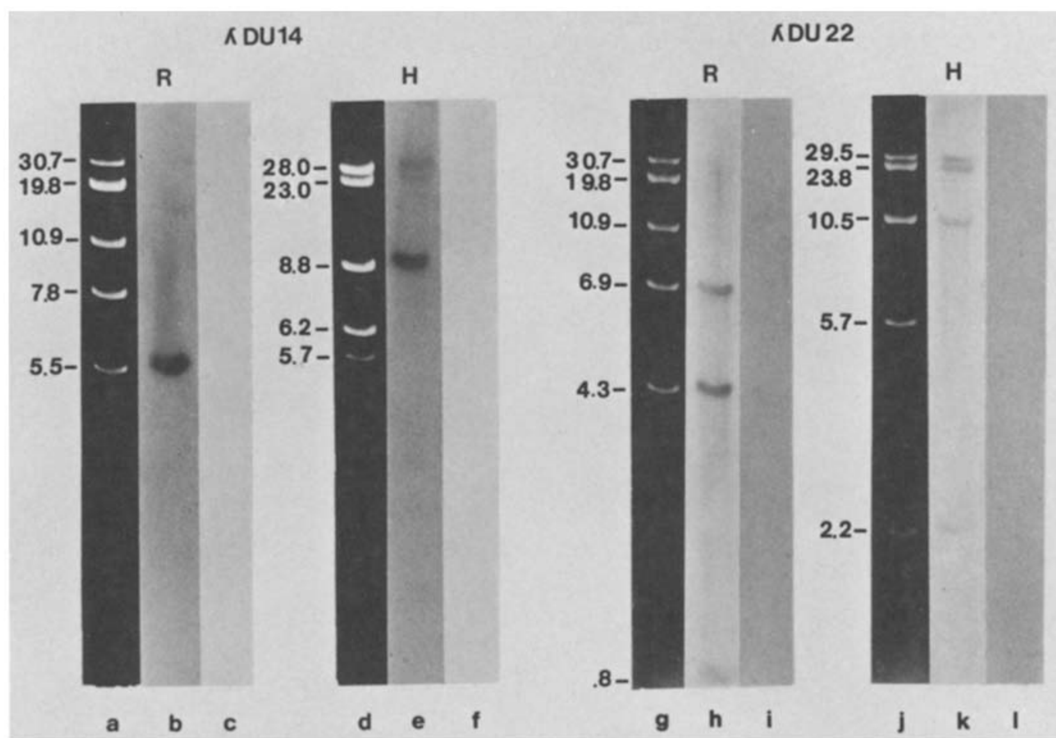

Fig. 4. Restriction endonuclease patterns of clone λDU14 (a–f) and λDU22 (g–l) and its hybridization with purified segments B1–2 and B5–6 (left and right hand side T-DNA, respectively). λDU14 was digested with *Eco*RI (a–c) and *Hin*III (d–f). Southern blots were hybridized to probes of B1–2 (c, f) and B5–6 (b, e). λDU22 was digested with *Eco*RI (g–i) and *Hin*dIII (j–l). Southern blots were hybridized to probes of B1–2 (h, k) and B5–6 (i, l). R and H represent digestions with *Eco*RI and *Hin*dIII, respectively. The 0.5 kbp *Hin*dIII fragment is not shown in this figure

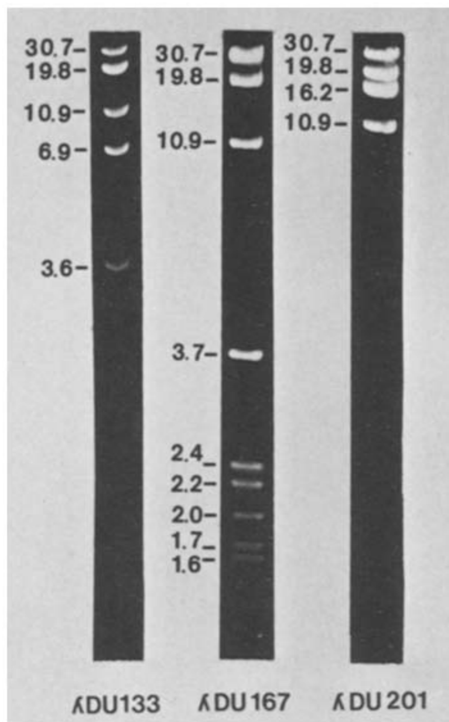

Fig. 5. *Eco*RI restriction endonuclease digestion patterns of clones λDU133, λDU167 and λDU201. Restriction endonuclease fragments of 30.7 kbp, 19.8 kbp and 10.9 kbp correspond to the vector DNA, the remaining fragments are crown gall DNA inserts

Thus, clone λDU14 contains the right hand side of T-DNA, extending from the *Eco*RI site R7 to the right of the R8 site and also containing a 7.8 kbp plant/T-DNA border (see Fig. 1).

For the restriction and hybridization analysis of the left hand boundary we analyzed clone λDU22 (Fig. 4g–l), whose total crown gall DNA insert is 12.3 kbp. From an *Eco*RI digest we obtained in addition to the three vector fragments, three other fragments: 6.9 kbp, 4.3 kbp and 0.8 kbp which represent the recombinant insert (Fig. 4g). Figure 4 h shows that all three crown gall fragments have sequence homology to the left end of T-DNA, segment B1–2 and none to the right end of T-DNA, segment B5–6 (Fig. 4i). However, we observed weak hybridization of the latter probe to the 4.3 kbp fragment in overexposed Southern blots (data not shown). A number of factors could account for these results. First, even though we were unable to detect specific hybridization of *Bam*HI fragments B1–2 to B5–6 in the Ti plasmid (data not shown), the possibility of having sequence homologies between the left and right ends of the T-DNA was not yet ruled out. Second, the weak hybridization of B5–6 to the 4.3 kbp *Eco*RI fragment could be attributed to our hybridization and washing conditions of the filters which, although stringent, still allowed some mismatch. Third, a noncontiguous segment of the Ti plasmid, homologous to the probe could be covalently linked to the left hand T-DNA border as a result of cloning.

We concluded that clone λDU22 contains the left hand side of T-DNA. We attributed the 6.9 kbp and 0.8 kbp *Eco*RI bands to the internal T-DNA segments R3–4, and R2–3, respectively, and fragment 4.3 kbp to the remaining T-DNA plus left handed border plant DNA extending to the left of R2.

To further confirm our conclusion, clone λDU22 was mapped by *Hin*dIII restriction endonuclease digestion which generated six fragments (Fig. 4j). The fragments of 23.8 kbp and 29.5 kbp are the annealed left and cohesively joined left and right arms of the λ Charon 4A vector, respectively, containing 4 kbp of inserted plant/T-DNA. The 10.5 kbp fragment is the right arm of the vector linked to 5.4 kbp of crown gall DNA. The 5.7 kbp fragment is the internal λ Charon 4A vector fragment and the 2.2 kbp and 0.5 kbp fragments (not seen in this figure) were attributed to crown gall DNA insert. All, except for the 5.7 kbp

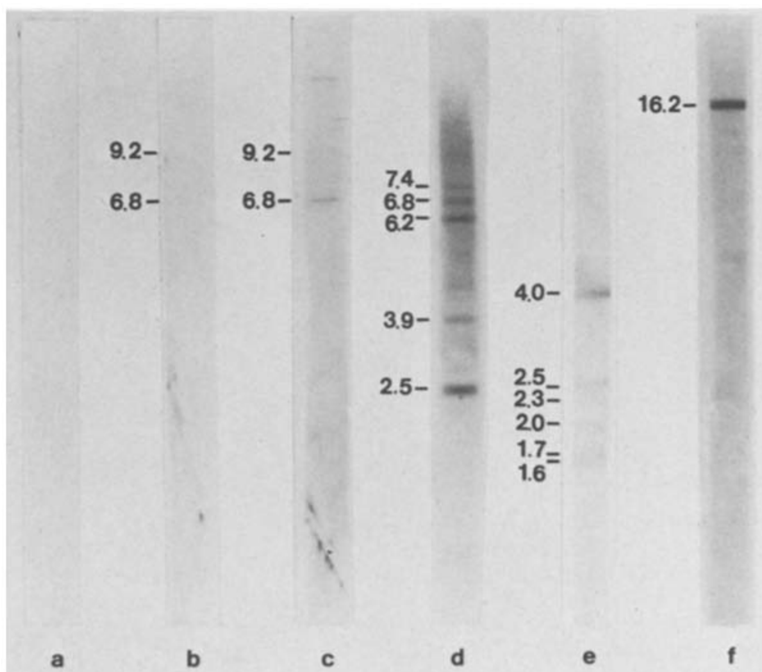

Fig. 6. Hybridization of nick translated λ vector Charon 4A (a), λDU14 (b), λDU22 (c), λDU133 (d), λDU167 (e) and λDU201 (f) to Southern blots of *Eco*RI digested HSSS DNA

Table 3. Recombinant clones of T-DNA-sunflower DNA hybrid (border clones) from crown-gall and bacteriophage lambda Charon 4A DNA. Intensity of hybridization: + + + very strong, + + strong, + weak, ± very weak. L and R stand for left and right hand side of T-DNA, respectively. All values are in kbp

Clone	Size and location of T-DNA insert in clone	Size of *Eco*RI hybrid border fragment	Plant *Eco*RI fragments adjacent to hybrid border fragment in clone	Genomic *Eco*RI fragments of HSSS showing sequence homologies to hybrid border fragments
λDU14	13.3 R	7.8	–	6.8± 9.2±
λDU22	12.3 L	4.2	–	6.8± 9.2±
λDU133	11.7 L	3.6	–	7.4+ + 6.8+ + 6.2+ + 3.9+ + 2.5+ + +
λDU167	13.9 R	2.2	3.7 2.4 2.0 1.7 1.6	4.0+ + + 2.5+ + 2.3+ 2.0+ + 1.7+ + 1.6+ +
λDU201	16.2 R	16.2	–	16.2–16.9+ + +

vector fragment hybridized to the probe of segment B1–2 (Fig. 4k) and none to the segment B5–6 (Fig. 4l). Bands of 2.2 kbp and 0.5 kbp were attributed to segments H3–4 and H5–6, respectively. Segment H4–5 is not visible under our experimental conditions. The 4 kbp segment extending to the left of H3 and the 5.4 kbp fragment from section H6 to R4 were ligated to the left and right hand sides of the vector, respectively.

We concluded that λDU22 contains the left hand side of T-DNA extending to the left from *Eco*RI site R4 to R2 and having a 4.3 kbp boundary fragment. A map of this clone is shown in Fig. 1.

The remaining three clones are λDU133, containing the left hand side, and λDU167, λDU201, both harboring the right hand side of T-DNA. Their *Eco*RI digestion patterns are shown in Fig. 5. We performed the same hybridization experiments as was described for clones λDU14 and λDU22 and established the restriction map for each of these clones. The maps are shown in Fig. 1.

These results substantiate our genomic blot hybridization data that the T-DNA borders extend just left of S2, since we find a *Bam*HI site in clone λDU22 and none in T-DNA in this DNA section. The right hand border is less than 500 bp to the right of R8 since there was very little hybridization to our *Eco*RI digested right hand plant/T-DNA border fragments using clone p501 as a probe (data not shown).

From our restriction maps we know that we obtained five unique clones. Three comprise different right hand borders and two contain different left hand borders of T-DNA. This gave us a lower bound on the number of the insertion sites of T-DNA in plant.

We did not find any clones having T-DNA in tandem arrangement in crown gall as was reported for a nopaline type crown gall (Zambryski et al. 1980). However, this integration mechanism in sunflower could not be ruled out, since we screened only a limited number of clones.

Sunflower Crown Gall Clones Hybridize to Either Unique or Low Repeated Sequences of Uninfected Sunflower DNA

We verified that our hybrid T-DNA containing clones were covalently linked to plant DNA by analysis of an autoradiogram of Fig. 6. It shows that the border clones had sequence homology to a limited number of fragments of *Eco*RI digested HSSS DNA. In previous experiments we had shown (Figs. 2a, 3a) that T-DNA did not hybridize to uninfected DNA. Furthermore, Fig. 6a shows that bacteriophage λ Charon 4A DNA does not anneal to uninfected DNA. Thus, hybridization to uninfected HSSS DNA using the genomic clones as probes can be attributed to sequence homologies in the plant-DNA junctions.

Results presented in Fig. 1 and summarized in Table 3 show that the T-DNA of crown gall tumor line PSCG 15955 is covalently linked to sunflower DNA. In addition, T-DNA has integrated in limited sequences of plant DNA. Some border plant/T-DNA hybrid clones harbor low repeated plant DNA segments.

Discussion

The discovery by Chilton et al. (1977b) that *Agrobacterium tumefaciens* causes crown gall tumors in plants by stably inserting plasmid DNA into the genome of higher plant cells caused considerable interest in the elucidation of this DNA insertion mechanism. Additional impetus in this direction was provided by the finding that the *A. tumefaciens* plasmid responsible for this feat is only one in a family of related plasmids. Some examples are: *Agrobacterium rhizogenes,* which induces hairy root disease of dicotyledonous plants (Smith 1911) and *Rhizobium trifolii* and *Rhizobium leguminosarum*, which fix nitrogen in symbiotic association with legumes such as clover, pea and bean (Hooykaas et al. 1979). The rhizogenic plasmids of *A. rhizogenes* and tumorigenic plasmids of *A. tumefaciens* share sequence homologies

(White and Nester 1980) as do nif plasmids of fast growing *Rhizobium* species and Ti plasmids of *A. tumefaciens* (Prakash and Schilperoort 1982). However, studies so far show that plasmids share little DNA homology with the T-DNA of the Ti plasmids thus they seem to represent separate pathogenic agents.

Tumor formation and rhizogenicity of *A. rhizogenes* carrying Ti plasmids was reported by Costantino et al. (1980). The same group (Hooykaas et al. 1981) observed that introduction of the symbiotic *R. trifolii* plasmid into *A. tumefaciens* results in bacteria that can bring about root nodulation. These findings suggest that these plasmids have similar host range.

Further excitement has been provided by the discovery by Chilton et al. (1982) that similarly to the crown gall tissue induced by *A. tumefaciens, A. rhizogenes* Ri plasmid DNA inserts were detected in the genomes of host plant root cells, which now produce opines.

This system is a natural example of genetic engineering. Very little is known about the chain of events leading to this DNA integration. We addressed our efforts into improving our understanding of the T-DNA transfer from *A. tumefaciens* to the plant genome.

A library was constructed from total DNA of the octopine type crown gall tumor line PSCG 15955 by molecular cloning using the bacteriophage λ Charon 4A as cloning vector. We isolated clones with sequence homology to Ti plasmid DNA. Approximately 2×10^6 plaques were screened. We obtained five clones which were purified and analyzed. They all contained T-DNA and five different stretches of plant DNA covalently bonded to it. Three contained segments of right hand side T-DNA and two a segment of left hand side T-DNA. The ratio of positive clones to the number of screened plaques provides a very rough estimate of the ratio of T-DNA versus plant DNA present. This ratio is 10^5 and is within an order of magnitude in agreement with our remaining data. The amount of DNA per sunflower genome reported in the literature varies (Baetcke et al. 1967; Van't Hoff and Sparrow 1963) by an order of magnitude and hence this rough estimate can only be used to check the overall consistency of our data and cannot provide a more precise estimate of the number of T-DNA copies present per genome.

However, the fact that we found three distinct right hand border containing clones, shows there are at least three distinct integration sites. The fact that all five λ clones were different indicates that, most probably, there are more than three integration sites. These numbers can be updated by screening and identifying additional T-DNA/plant crown gall clones.

An additional interesting fact was provided by hybridizing the five crown gall clones to Southern blots of *Eco*RI digested, normal sunflower DNA. The results indicate that the hybrid plant/T-DNA borders contain unique to low repeated segments of the sunflower genome.

An upper limit on the number of integration sites was estimated by hybridizing cloned T-DNA segments to Southern blots of DNA prepared from uninfected sunflower tissue (HSSS) and from crown gall tumor line PSCG 15955, digested with various restriction endonucleases, and observing the patterns.

We found that a continuous Ti plasmid segment (T-DNA) of about 19.5 kbp was transferred and integrated into the plant genome, containing the "common" T-DNA region (Chilton et al. 1977a). Within the resolution of our mapping analysis the right and left T-DNA borders appear to be constant.

An estimate of the upper limit of T-DNA integration sites was as follows: Assuming that every hybridizing band that is not an internal T-DNA fragment is a plant/T-DNA junction, we inferred from the number of hybridizing bands that there are approximately eight integration sites. Ambiguities arise from the fact that we do not know the restriction patterns of the plant DNA immediately adjacent to the inserted T-DNA, and hence we interpreted the hybridization data considering that all potential hybridization indeed occurred. Potential hybridization bands corresponding to a right or left junction can be lost if the restriction enzyme cleaves close to the plant/T-DNA junction. We compensated for this effect by using more than one enzyme.

In conclusion, PSCG 15955 cells contain from at least three to approximately eight distinct insertion sites, namely three, from our distinct right hand plant/T-DNA clones and eight, from our genomic DNA hybridization studies. We still are unable to state whether these same insertions occur every time *A. tumefaciens* bacteria incite sunflower plants. We hope to settle the issue by repeating the experiment with a series of crown galls resulting from separate infections conducted on a series of cloned sunflower plants. Another approach to study this problem could be the infection of protoplasts with *A. tumefaciens*.

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Note added in proof: Experiments just completed show that each *A. tumefaciens* infection on isogenic sunflower plants inserts T-DNA into a distinct set of sites. Eleven crown gall callus tissue lines show eleven distinct insertion patterns.

References

Baetcke KP, Sparrow AH, Nauman CH, Schwemmer SS (1967) The relationship of DNA content to nuclear and chromosome volumes and to radiosensitivity (LD_{50}). PNAS USA 58:533–540

Bendich AJ, Anderson RS, Ward BL (1980) Plant DNA: long, pure and simple. In: Genome organization and expression in Plants. Leaver CJ (ed) Plenum Press, New York, p 31–33

Benton WD, Davis RW (1977) Screening agent recombinant clones by hybridization to single plaques in situ. Nature 196:180–182

Blattner FR, Blechl AE, Denniston-Thompsom K, Faber HE, Richards JE, Slightom JL, Tucker PW, Smithies O (1978) Cloning human fetal y-globin and mouse -type grobin DNA: preparation and screening of shotgun collections. Science 202:1279–1284

Bomhoff G, Klapwijk PM, Kester HCM, Schilperoort RA, Hernalsteens JP, Schell J (1976) Octopine and nopaline synthesis and breakdown genetically controlled by plasmid of *Agrobacterium tumefaciens*. Mol Gen Genet 145:177–181

Chilton M-D, Drummond MH, Merlo DJ, Sciaky D (1977a) Highly conserved DNA of Ti plasmid overlaps T-DNA maintained in plant tumors. Nature 275:535–536

Chilton M-D, Drummond MH, Merlo DJ, Sciaky D, Montoya

AL, Gordon MD, Nester EW (1977b) Stable incorporation of plasmid DNA into higher plant cells: The molecular basis of crown gall tumorigenesis. Cell 11:263–271

Chilton M-D, Tepfer DA, Petit A, David D, Casse-Delbart F, Tempé F (1982) *Agrobacterium rhizogenes* inserts T-DNA into the genome of the host plant root cells. Nature 295:432–434

Costantino P, Hooykaas PJJ, den Dulk-Ras H, Schilperoort RA (1980) Tumor formation and rhizogenicity of *Agrobacterium rhizogenes* carrying Ti plasmids. Gene 11:49–87

De Beuckeleer M, Lemmers M, De Vos G, Willmitzer L, Van Montagu M, Schell J (1981) Further insight on the transferred-DNA of octopine crown gall. Mol Gen Genet 183:283–288

Depicker A, Van Montagu M, Schell J (1978) Homologous DNA sequences in different Ti-plasmids are essential for oncogenicity. Nature 275:150–153

Gelvin SB, Gordon MP, Nester EW, Aronson AJ (1981) Transcription of the *Agrobacterium* Ti plasmid in the bacterium and in crown gall tumors. Plasmid 6:17–29

Goldman A, Tempe J, Morel G (1969) Quelques particularites de diverses souches d' *Agrobacterium tumefaciens*. CR Acad Sci 162:630–631

Gurley WB, Kemp JD, Albert MJ, Sutton DW, Callis J (1979) Tanscription of Ti-plasmid-derived sequences in three octopine-type crown gall tumor lines. PNAS USA 76:2828–2832

Hooykaas PJJ, Schilperoort RA, Rörsch A (1979) *Agrobacterium* tumor inducing plasmids; potential vectors for the genetic engineering of plants. In: Setlow JK, Hollander A (eds) Genetic engineering, vol 1. Plenum, New York, pp 151–179

Hooykaas PJJ, van Brussel AAN, den Dulk-Ras H, van Slogteren GMS, Schilperoort RA (1981) Sym plasmid of *Rhizobium trifolii* expressed in different rhizobial species and *Agrobacterium tumefaciens*. Nature 291:351–353

Kung S (1977) Expression of chloroplast genomes in higher plants. Ann Rev Plant Phys 28:401–437

Ledeboer AM (1978) Dissertation (University of Leiden, Leiden, The Netherlands)

Lemmers M, De Beuckeleer M, Holsters M, Zambryski P, Depicker A, Hernalsteens JP, Van Montagu M, Schell J (1980) Internal organization, boundaries and integration of Ti-plasmid DNA in nopaline crown gall tumours. J Mol Biol 144:353–376

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127

McPherson JC, Nester EW, Gordon MP (1980) Proteins encoded by *Agrobacterium tumefaciens* Ti plasmid DNA (T-DNA) in crown gall tumors. PNAS 77:2666–2670

Menagé A, Morel G (1964) Sur la presence d'octopine dans les tissus de crown gall. Compt Rend Acad Sci 259:4795–4796

Merlo DJ, Kemp JD (1976) Attempts to detect *Agrobacterium tumefaciens* DNA in crown gall tumor tissue. Plant Physiol 58:100–106

Merlo DJ, Nutter RC, Montoya AL, Garfinkel DJ, Drummond MH, Chilton M-D,Gordon MP, Nester EW (1980) The boundaries and copy numbers of Ti plasmid T-DNA vary in crown gall tumors. Mol Gen Genet 177:637–643

Montoya AL, Chilton M-D, Gordon MP, Sciaky D, Nester EW (1977) Octopine and nopaline metabolism in *Agrobacterium tumefaciens* and crown gall tumor cells: role of plasmid genes. J Bacteriol 129:101–107

Moore DD, Denniston-Thompson K, Furth ME, Williams BG, Blattner FR (1977) Construction of chimeric phages and plasmids containing the origin of replication of bacteriophage lambda. Science 198:1041–1046

Murai N, Kemp JD (1982) Octopine synthase mRNA isolated from sunflower crown gall callus is homologous to the Ti plasmid of *Agrobacterium tumefaciens*. PNAS 79:86–90

Petit A, Delhaye S, Tempe J, Morel G (1970) Mise en evidence d'une relation biochimique specifique entre les souches d'*Agrobacterium tumefaciens* et les tumeurs qu' elles in duisent. Physiol Veg 8:205–213

Prakash RK, Schilperoort RA (1982) Relationship between nif plasmids of fast-growing *Rhizobium* species and Ti plasmid of *Agrobacterium tumefaciens*. J Bacteriol 149:1129–1134

Schröder J, Schröder G, Huisman H, Schilperoort RA, Schell J (1981) The mRNA for lysopine dehydrogenase in plant tumor cells is complimentary to a Ti-plasmid fragment. FEBS Lett 129:166–168

Slightom L, Blechl AE, Smithies O (1980) Human fetal $^{G}\gamma$- and $^{A}\gamma$-globin genes: Complete nucleotide sequences that suggest that DNA can be exchanged between these duplicated genes. Cell 21:627–638

Smith EF (1911) Crown galls of plants. Phytopathology 1:7–11

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Thomashow MF, Nutter R, Montoya AL, Gordon MP, Nester EW (1980a) Integration and organization of Ti-plasmid sequences in crown gall tumors. Cell 19:729–739

Thomashow MF, Nutter R, Postle K, Chilton M-D, Blattner FR, Powell A, Gordon MP, Nester EW (1980b) Evidence for recombination between higher plant DNA and the Ti plasmid of *Agrobacterium tumefaciens*. PNAS 77:6448–6452

Ursic D, Davies J (1979) A cold-sensitive mutant of *Saccharomyces cerevisiae* defective in ribosome processing. Mol Gen Genet 175:313–323

Van Larebeke N, Engler G, Holsters M, Van den Elsacker S, Zaenen J, Schilperoort RA, Schell J (1974) Large plasmid in *Agrobacterium tumefaciens* essential for crown gall inducing activity. Nature 252:169–170

Van't Hoff J, Sparrow AH (19637 A relationship between DNA content, nuclear volume, and minimum mitotic cycle time. PNAS USA 49:897–902

Wahl GM, Stern M, Stark GR (1979) Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. PNAS USA 76:3683–3687

Watson B, Currier TC, Gordon MP, Chilton M-D, Nester EW (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. J Bacteriol 123:255–264

White FF, Nester EW (1980) Relationship of plasmids responsible for hairy root and crown gall tumerigenicity. J Bacteriol 144:710–720

Yadav NS, Postle K, Saiki RK, Thomashow MF, Chilton M-D (1980) T-DNA of crown gall teratoma is covalently joined to host plant DNA. Nature 287:458–461

Zaenen J, Van Larebeke N, Teuchy H, Van Montagu M, Schell J (1974) Supercoiled circular DNA in crown gall-inducing *Agrobacterium* strains. J Mol Biol 86:109–127

Zambryski P, Holsters M, Kruger K, Depicker A, Schell J, Van Montagu M, Goodman HM (1980) Tumor DNA structure in plant cells transformed by *A. tumefaciens*. Science 209:1385–1391

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