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EIGHT DNA INSERTION EVENTS OF AGROBACTERIUM TUMEFACIENS TI-PLASMIDS IN ISOGENIC SUNFLOWER GENOMES ARE ALL DISTINCT*

Doris Ursic

Department of Genetics
University of Wisconsin, Madison, Wi 53705

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SUMMARY. We investigated whether the same or different T-DNA insertions occur every time Agrobacterium tumefaciens, the octopine type strain pTi 15955 str^r, infects genetically identical sunflower plants. Eight newly established crown gall tissue culture lines were analyzed for their T-DNA content. Our data showed that all isogenic crown gall callus DNA produced distinct hybridization patterns. These eight patterns were also different from three standard lines included for comparison. In addition, all the tumor lines analyzed produced octopine, albeit in different quantities, and five produced agropine and mannopine. We concluded, that each A. tumefaciens crown gall tissue line derived from isogenic sunflower plants contained a distinct insertion pattern of T-DNA. Possible causes and reasons for this diversity will be discussed.

INTRODUCTION. Agrobacterium tumefaciens induces a neoplastic disease, crown gall, on dicotyledonous plants. The virulent bacterium harbors a tumor inducing (Ti) plasmid, segments of which (T-DNA) are transferred and inserted into the plant chromosomes (1, 2, 18). The exact mechanism by which T-DNA integrates into the target genome is unknown. No discernible pattern has emerged which would indicate how the insertion locations in the host chromosomes are chosen by the invading T-DNA (15, 16, 21). For reviews on the subject, see (7, 11, 12).

This investigation was initiated to determine if a single strain of A. tumefaciens would produce identical crown galls in separate infection events to genetically identical sunflower plants. The doubt persisted whether this diversity was due to differences in plant genotype, or to other undetermined causes. Part of the problem in settling this issue is the relative slowness in establishing new crown gall tissue lines derived from cloned plants, infected

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with the same virulent A. tumefaciens bacterial strain. The results reported in this paper unequivocally determine that identical genetic contents of plants and bacteria will produce distinct crown galls.

The reasons for this diversity will have to be searched elsewhere. A list of potential mechanisms as yet not investigated is presented.

METHODS. Bacterial strains used were as described in (4, 18).

To obtain isogenic plants, a sunflower plant (Helianthus annus cv. Mammoth Russian) was grown for 2 to 3 weeks until more than five leaves were formed. The apical tip and stem containing at least three leaves were cut off, leaving the base including the hypocotyl and about three leaves. The apical stem and tip were coated with rootonone F (synthetic growth hormone) and replanted. Both portions of the plant were allowed to grow for a few days after which time the root portion acquired a new apical tip. This procedure was repeated. Some of our plants developed between three and four axillary shoots which were excised and replanted. We infected three of these isogenic sunflower plants with the virulent strain A. tumefaciens 15955 str^r at one to three positions of the stem. The sunflowers produced normal crown galls which were excised and separate tissue culture lines from each individual primary gall were established. The tissue culture lines were maintained on solid medium as callusses at 27°C in the dark on Linsmaier and Skooks medium (10) lacking hormones.

Crown gall tissue culture lines a, b and c were obtained from crown galls by infecting the stems of the isogenic sunflower plant #1 at positions top, medium and bottom, respectively. Lines d and e were obtained by infecting plant #2 at top and medium positions and lines f, g and h by infecting isogenic plant #3 at top, medium and bottom positions of the stem, respectively. Analysis of these tissues was less than six month after the lines were established. Tissue culture lines PSCG-15955 (j), PSCG 4-4 (k), PSCG 3-F (l) and HSSS (m) are older established lines and were described in Ursic et al. (18).

Total plant DNA from tissue culture lines, Ti plasmid DNA and recombinant plasmids containing T-DNA was prepared as described previously (16, 18). Hybridization analysis of genomic plant DNA was performed as described in (18).

Isolation of octopine extracts and colorimetric assays were conducted as was described by (8, 9). Mannopine and agropine extracts and assays were carried out following procedures in (5).

RESULTS. Eight newly established independent octopine type crown gall sunflower tissue culture lines a to h, as well as older lines PSCG 15955 (j), PSCG 4-4 (k), PSCG 3-F (l) and nontransformed sunflower line HSSS (m) were analyzed for their T-DNA content. These eleven tumor DNAs were analyzed for their hybridization patterns to the Ti-plasmid used for infection. Figure 1 presents the Ti-plasmid restriction site map of the region of interest (18). Figures 2 and 3 are autoradiograms of Southern blots of EcoR1 digested DNA probed with pTi 15955, and the right side of T-DNA (T_R-DNA) encoding the R7-R8 EcoR1 restriction site, respectively. Tables 1 and 2 summarize our hybridization

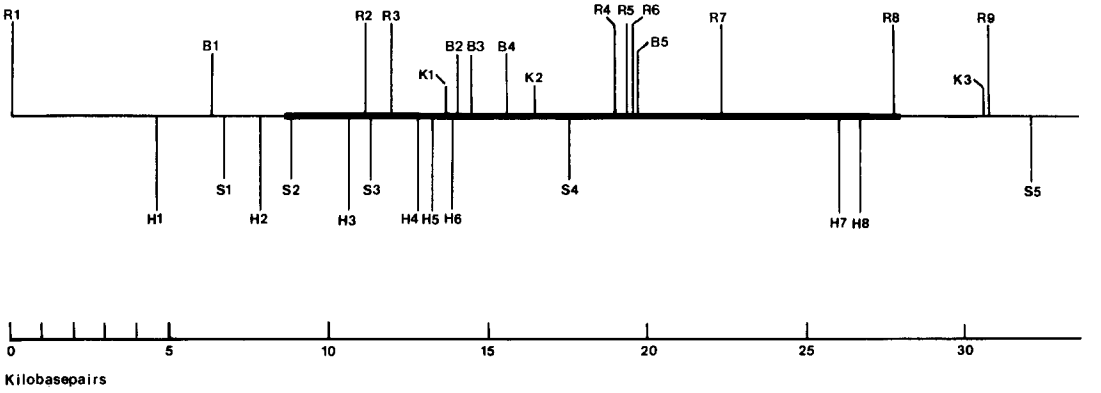

Figure 1. Restriction endonuclease map of pTi-15955 str^r T-DNA. Restriction endonuclease sites are designated R(EcoRI), B(BamHI), H(HindIII), K(KpnI) and S(SmaI) followed by a number. Restriction sites are numbered in order from left to right. T-DNA in sunflower tumor line PSCG 15955 str^r is marked by a bold solid line, unintegrated Ti-plasmid DNA by a thin solid line.

data of HindIII digested tumor DNA to the BamHI fragments B1-B2 and B5-B6 T-DNA (T_R-DNA), respectively.

We found that all eight isogenic crown gall callus DNA produced distinct hybridization patterns. These eight patterns were also different from the three established lines. In contrast to some other octopine tumor lines (7, 11, 12, 15), all of our crown gall DNAs contained sequences homologous to T_R-DNA (19).

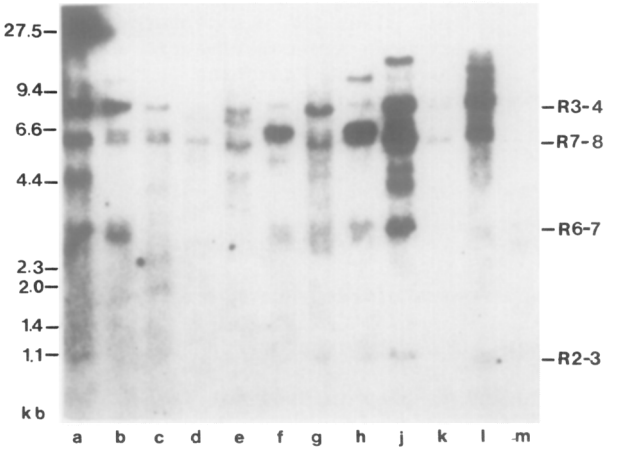

Figure 2. Autoradiogram of Southern blot of crown gall DNA. DNA from isogenic tumor lines (a-h), crown gall tumor line PSCG 15955 (j), PSCG 4-4 (k) and PSCG 3-F (l) and uninfected sunflower tissue HSSS (m) were digested with EcoRI and hybridized to nick translated probe of pTi-15955 str^r. Fragments labeled R7-R8, R6-R7, R3-R4 and R2-R3 are internal T-DNA fragments and illustrated in Figure 1. Fragment sizes are given in kbp in this and the subsequent figure.

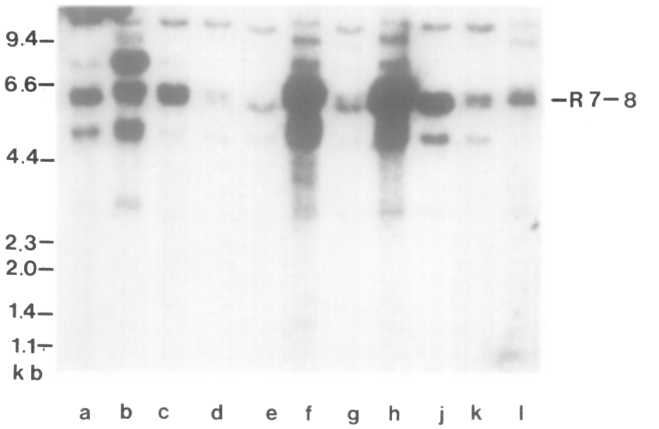

Figure 3. Autoradiogram of Southern blot of crown gall DNA. Headings are as in Figure 2. The DNA was digested with with EcoRI and hybridized to nick translated probe of p403 (R7-R8 Ti-plasmid restriction fragment). Refer to Figure 1 for location of the fragment.

The number of integrations varied widely as judged by the number of bands hybridizing to the various probes. Assuming that every hybridizing band that is not an internal T-DNA fragment was a plant/T-DNA junction, we inferred from the number of hybridizing bands that there were no more than twelve insertion sites. Ambiguities arose from the fact that we did not know the restriction pattern of the plant bordering the T-DNA target site. Potential hybridization

Table 1. Hybridization band intensities of HindIII digested DNA homologous to the left end of T-DNA

FRAGMENT	Kb	a	b	c	d	e	f	g	h	j	k	l
	16.9	*	*	-	-	*	-	-	*	-	*	+
H6-H7	12.3	*	*	+	*	*	+	*	*	+	*	+
	10.8	*	*	*	*	*	+	*	++	-	*	++
	9.8	++	*	*	*	*	*	*	*	*	*	++
	9.4	*	++	-	*	*	+++	+++	++	*	+	++
	8.6	*	*	-	*	*	-	-	-	*	*	+
	7.8	++	*	+	*	*	++	-	*	-	-	++
	7.0	-	+++	++	+	+	+++	-	+++	+++	*	-
	6.5	*	*	*	-	*	*	*	*	*	*	+
	6.0	*	*	-	*	*	+	-	-	+	+	*
	5.3	+	+	*	*	*	-	*	+++	-	-	+
	4.4	*	*	*	*	*	*	*	*	*	*	+
	4.0	*	*	*	*	*	+++	*	++	-	-	+
	2.7	+	-	+	*	*	*	-	-	++	-	+
H3-H4	2.4	++	-	++	*	+	-	++	++	+++	+	+++
H5-H6	0.7	-	*	-	*	*	*	-	-	-	-	-

Summary of autoradiograms of Southern blots of crown gall DNA probed with p101 (BamHI T-DNA restriction fragment B1-B2). Headings are as in Figure 2. H3-H4, H5-H6 and H6-H7 are internal T-DNA fragments and shown in Figure 1. Band intensities are graded from non-visible, *, and weak to strong by -, +, ++, +++.

Table 2. Hybridization band intensities of HindIII digested DNA homologous to the right end of T-DNA

FRAGMENT	Kb	a	b	c	d	e	f	g	h	j	k	l
	15.0	*	*	*	*	*	-	*	-	-	*	-
H6-H7	12.3	*	*	+	*	*	-	*	*	-	*	+
	10.9	*	++	*	-	-	*	*	++	*	+	*
	8.8	*	*	*	*	*	++	*	++	*	*	+
	8.2	-	+++	+	*	*	*	+	*	*	*	*
	7.8	-	++	++	+	-	+++	+	+++	+	+	-
	6.3	-	++	++	*	*	-	*	-	-	+	-
	5.5	-	+	*	*	*	*	*	*	*	*	*
	4.6	*	*	*	*	-	*	*	*	*	*	*
	4.2	*	*	*	*	*	+	+	++	+	+	-
	3.9	*	-	*	*	*	++	*	+	*	*	*
	3.4	-	+	+	+	+	++	+	+	+	+	-
	2.6	*	*	*	*	+	+	*	*	*	*	*
	2.2	*	-	-	*	-	-	-	-	-	*	*
	1.4	*	-	-	*	-	-	-	-	*	-	*
	0.8	*	*	-	*	*	*	*	*	*	*	*

Summary of autoradiograms of Southern blots of crown gall DNA probed with p301 (BamHI T-DNA restriction fragment B5-B6). Headings are as in Figure 2. H6-H7 is an internal restriction fragment as in Figure 1. Symbols as in Table 1.

bands corresponding to the junction fragments can be lost if the restriction enzyme cleaves close to the T-DNA plant target site. Furthermore, the estimation of T-DNA integration sites would be influenced by T-DNA rearrangements which were not excluded.

As shown in Table 3, all tumor lines analyzed produced octopine, although in different amounts, ranging from 126 nmoles/g tissue for PCSG 15955 (j) to 1750 nmoles/g tissue in line c. Thus, the isogenic tissue culture lines as well as the established lines analyzed harbored the octopine synthase gene, which maps to the left of the BamHI B5 restriction site of the T-DNA (T_L-DNA). T_R-DNA encodes genes for agropine and mannopine (14, 19). As seen in Figures 2 and 3, the tumor DNAs contained sequences homologous to segments of T_R-DNA, but

Table 3. Octopine, agropine and mannopine production

TISSUES LINES	a	b	c	d	e	f	g	h	j	k	l
nmoles octop. /g tissue	796	780	1760	418	300	306	186	178	126	228	394
agropine	++	*	++	+++	*	-	*	*	*	++	*
mannopine	+	*	+	++	*	+	*	*	*	++	*

Quantitative and qualitative analysis of octopine, agropine and mannopine, respectively. Intensities of agropine and mannopine stains are graded from weak to strong by -, +, ++, +++. Non-visible stains are indicated with *. Headings of tissues lines are as in Figure 2.

only five of the eleven tumor lines analyzed produced agropine and mannopine (Table 3). Thus, the tumor lines, which did not produce mannopine and agropine either lacked the essential genes for these opines or were under plant regulation or both.

DISCUSSION. We infected isogenic sunflower plants with the same bacterial strain and obtained distinct crown galls. Each of our tumor lines were distinct as judged by their differences in restriction fragment homologies to the T-DNA segments, intensities of hybridization and expression of opines. These differences clearly demonstrated that each crown gall assumed its status with a distinct biochemical heritage. What follows is a list of possible mechanisms for this heterogeneity.

(A) Insertion events are entirely random. A number of organisms insert their DNA into a susceptible host. They include: bacterial transposable elements; transposable elements in Drosophila melanogaster; transposable elements in maize and Antirrhinum majus; transposable elements in yeasts and retroviruses. Many additional examples can be found in the book "Mobile Genetic Elements" (13). Either the selection of sites for the DNA transfer to the host is made at random, or the reasons for a particular choice of integration sites is so complex as to appear random to the available methods of observation.

(B) Insertion events depend on factors outside experimental control. Laboratory conditions may be too variable to assure repeatability of the experiments and provide uniform conditions. It is possibile that minute differences in temperature, illumination conditions, or particular handling environments have a decisive influence in the DNA insertion events.

(C) Heterogeneity of insertion depends on different plant cell types. A plant is constituted of many cell types, and each cell type utilizes and expresses a distinct set of its chromosomal DNA. It is conceivable that distinct insertion events lead to infection of particular cell types, producing dissimilar results. Differences in developmental stages of the various plant cells may also contribute to the observed divergence. It is possible that only

DNA actually active in transcription can be used by the T-DNA as a potential site for insertion.

(D) Different T-DNA copy numbers depend on the number of Ti-plasmids inserted. It is unknown how many T-DNA copies each infected plant cell receives. Variation in T-DNA copy number could be due to multiple bacteria infecting the same cell or to plasmid replication upon plant/bacterium contact.

(E) The insertion is target site specific, but only a few potential sites are colonized. There is evidence that some transposable elements integrate site specifically. As examples we cite: Tn10 insertions in the Salmonella histidine operon and other sites occur about once every thousand base pairs of target DNA (6); the Ac, Dc, Mul, Taml, and Spml transposable elements in maize and Antirrhinum majus (3); a Chi sequence was found on the plant side immediately adjacent to the plant/T-DNA junction (20). These data suggest that there may be a limited number of integration sites that the Ti-plasmid can utilize.

(F) The original insertions are all identical but the plant biochemical defenses delete or modify some of them. This paper conclusively showed that the collection of T-DNA insertions in our transformed lines were different. The causes of this diversity remain to be elucidated. Perhaps the original insertions were all identical, and the plant DNA repair mechanisms only partially succeeded in removing the foreign DNA.

In conclusion, it is certain that our crown galls, although obtained from isogenic plants and using the same A. tumefaciens bacteria, were not identical. They differed in T-DNA contents and expression of opines.

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