Unusual plasmid DNA organization in an octopine crown gall tumor

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ABSTRACT

A cloned tobacco crown gall tumor, 1595501, incited by A. tumefaciens strain 15955 was studied. Molecular analysis of the organization of the T-DNA by means of Southern transfer and hybridization techniques indicated that the 1595501 tumor has about 10 copies of T_L DNA, five of which are complete T_L DNA, whereas most octopine tumors have only one to two copies of complete T_L DNA. Hybridization studies and genomic cloning indicated that some segments of the T-DNA have undergone deletions. One of the clones contained two copies of T-DNA which were inverted in orientation with respect to each other.

Two left ends of T_L DNA from the 1595501 tumor line and the corresponding region of the octopine plasmid were sequenced. Comparison of the various cloned T-DNA sequences with Ti-plasmid sequence indicated that while there is an association with a 25 base pair direct repeat, there is no specific set of base pairs in the T-DNA at which divergence from Ti-plasmid sequences occurred.

INTRODUCTION

The soil bacterium, Agrobacterium tumefaciens, causes crown gall tumor, a neoplastic disease of many dicotyledonous plants. All oncogenic strains of Agrobacterium have a large tumor-inducing (Ti) plasmid with a molecular weight in the range of 150kb to 800kb (1; L. Unger, personal communication). Tumor formation results from the transfer and stable incorporation of a portion of the Ti-plasmid, called the T-region, into the plant cell. The transferred DNA (T-DNA) (2) encodes enzymes which synthesize tumor specific compounds, opines, and phytohormones (3, 4, 5). A restriction endonuclease map of the T-DNA of the Ti-plasmid pTi15955 is shown in Figure 1A.

The T-DNA organization of both octopine and nopaline tumors has been elucidated by Southern transfer and hybridization experiments (6, 7). Several conclusions have been drawn from these studies: (a) The T-DNA in the tumor is generally colinear with the Ti-plasmid. (b) Preferred sequences of the plasmid DNA are integrated into the plant genome. (c) In octopine tumors, the T-DNA can be composed of two separate pieces of T-DNA, T_L and T_R,

Figure 1

Organization of the T-region of an octopine type plasmid and T-DNA from different octopine type tumors.

- Restriction map of the T-region of pTi15955. The arrows indicate the four 25bp sequences (see text for description).
- T-DNA sequences present in A6S/2 and E9 tumors.
- Plasmid clone pNW31c-8,29-1 contains BamHI fragments 8, 29, and 29'. Clone pNW31c-2,19-1 contains BamHI fragments 2 and 19. Clones HindIII A and HindIII B, contain HindIII fragment A, HindIII fragment B, respectively, and clone HindIII C has the DNA fragment bounded by the HindIII restriction site on the right hand side of HindIII fragment B and the BamHI site on the left hand side of BamHI fragment 29.
- Left end T_L clones from the tumor 1595501. Clones p21 and e6 were isolated from a genomic library of 1595501 DNA. The open bar is the region homologous to the Ti-plasmid. The hatched box indicates regions which diverge from the Ti-plasmid.

respectively. The T-DNA organization of the octopine tumor lines A6S/2 and E9 is shown in Figure 1B.

Analysis of the DNA sequences of the plant plasmid junctions has shown that the T-DNA/plant junctions are in close proximity to a 25 bp direct repeat at either end of the T-region of the Ti-plasmid (8, 9, 10, 11, 12). In the case of octopine tumors, four of these 25 bp sequences can be found. The first one is in HindIII Y, which is the left junction of T_L DNA. Two more are in EcoRI 24, which contains the right junction of T_L T-DNA and the

left junction of T_R. The final 25 bp sequence is found in EcoRI 22, the right end of T_R (Figure 1).

The mechanism of T-DNA integration remains unknown. More detailed information on the structure of the integrated T-DNA may be useful in establishing the mechanism by which T-DNA is integrated. In the present study, we report the T-DNA organization of an octopine type tumor with an unusually high copy number for T_L T-DNA. We show that some of the T_L DNA has undergone rearrangement and deletion. Large inverted repeats of the T-DNA are also observed.

MATERIALS AND METHODS

Tissue Culture

Clone 1595501 was derived from a tumor incited by *A. tumefaciens* strain 15955 on *Nicotiana tabacum* var. Xanthi nc. The tumor was grown on Murashige-Skoog medium in the absence of plant growth hormones.

DNA Preparation

High molecular weight DNA from tobacco tissue was prepared using the procedure of Cilton, *et al.* (13) or Thomashow, *et al.* (6). Plasmids containing recombinant DNA were isolated from *E. coli* strains by the method of Birnboim and Doly (14). Ti-plasmid from *Agrobacterium* was isolated as described by Currier and Nester (15). Charon 4A DNA and recombinant phage DNA were isolated from phage banded in cesium chloride as described by Blattner, *et al.* (16).

Hybridization Analysis

DNA digests were separated by electrophoresis on a 0.7% agarose gel with a Tris acetate buffer (13). The DNA was denatured *in situ*, transferred to nitrocellulose filters and hybridized to ³²P-DNA labelled by nick translation (6). The filters were then autoradiographed at -70°C using Kodak XRP5 film and DuPont Cronex intensifying screens.

DNA Sequence Analysis

The DNA to be sequenced was cloned into the M13 phage vectors of Messing (17) and then sequenced by the dideoxy method of Sanger, *et al.* (18). The reaction products were separated by electrophoresis for one or four hours on an 8% or 6% acrylamide-8M urea gel (43 cm x 10.5 cm x 0.35 mm) at 45 watts.

DNA Cloning

Molecular cloning into the vector Charon 4A was performed as described (5). Cloning of T-DNA fragments into the single stranded phage M13mp7 vector was performed as described by Messing, *et al.* (17).

Figure 2

Detection of DNA fragments containing Ti-plasmid sequences in 1595501 tumor. 1595501 DNA was digested with EcoRI, fractionated by electrophoresis in a 0.7% agarose gel, transferred to a nitrocellulose filter and hybridized with ^{32}P labelled probes.

- Hybridization with ^{32}P labelled pTi15955. (a) 4 copy reconstruction of Ti-plasmid. The equivalent of 4 copies of Ti-plasmid per diploid genome was added to salmon DNA followed by EcoRI digestion. (b) EcoRI digestion of Tumor 1595501 DNA.
- Hybridization of the filter with a mixture of ^{32}P labelled pNW31c-8,29-1 and pNW31c-2,19-1. Lanes a, b, and c contain 7, 4 and 1 copy equivalents of the pTi-plasmid, respectively. (d) EcoRI digestion of tumor 1595501 DNA.

Electron Microscopy of Inverted Repeats

DNA was denatured, neutralized and renatured as described by Ohtsubo and Ohtsubo (19). The DNA was then dialyzed. Ten nanograms of DNA were spread in 50ul of hyperphase according to the procedure of Davis, *et al.* (20). Grids were stained in uranyl acetate, shadowed with Pt-Pd, and examined in a Philips 201 transmission electron microscope.

RESULTS

T-DNA Organization in the 1595501 Tumor

DNA from the tumor line 1595501 was digested with the restriction

Table I. Size and copy number of DNA fragments containing Ti-plasmid sequences produced upon EcoRI digestion of 1595501 DNA.

<u>EcoRI Fragments</u>	<u>Copy Number per Diploid Genome</u> *
7.4kb	2
7.1kb	5
6.4kb	5
5.3kb	5
3.7kb	8-10
1.8kb	5
1.04kb	5

* Copy numbers were determined from microdensitometer reading of Figure 2B.

endonuclease EcoRI, fractionated on agarose gels, denatured and transferred to nitrocellulose filters. When the filter-bound DNA was hybridized to radiolabelled pTi15955 DNA, fragments migrating with the sizes of EcoRI fragments 7, 13, and were detected, along with additional fragments of 7.4, 6.4 and 3.7 kb (Fig. 2). The absence of EcoRI fragment 24 as an internal fragment suggests the T-DNA is present in separate segments, corresponding to T_L and T_R. Thus, the internal organization of the T-DNA of the 1595501 tumor is similar to some other octopine type tumors studied. Similar results were obtained when clones pNW31c-8,29-1 and pNW31c-2,19-1 (Fig. 1) were used as probes. The copy number of individual fragments was estimated by microdensitometer readings of the intensities of the autoradiogram shown in Fig. 2B (Table I).

Deletion of Sequences in 1595501 Tumor T-DNA

Using smaller cloned pieces of Ti-plasmid as probes, additional T-DNA fragments of 1.8kb, 4.9kb and 5.7kb were observed which hybridized only with HindIII fragments A and B, and did not hybridize with HindIII fragment C and BamHI fragment 29 (see legend to Figure 1, footnote C for the definition of HindIII fragment C). The simplest interpretation of these data is that the central and right portion of the EcoRI fragment 7 has been deleted (Figure 4).

Cloning and Characterization of T-DNA in 1595501 Tumor

The results of Southern transfer and hybridization experiments indicated that the integration pattern of T-DNA in tumor line 1595501 is complex. In order to analyze the structure of T-DNA in more detail, the 1595501 DNA was partially digested with EcoRI, and DNA fragments with sizes ranging from 10kb

Figure 3

Detection of DNA fragments containing Ti-plasmid sequences in 1595501 tumor DNA. 1595501 DNA was restricted with EcoRI, fractionated by electrophoresis in an agarose gel, transferred to a nitrocellulose filter and hybridized to various ^{32}P labelled probes.

The lanes were (a) and (b) 1595501 DNA, (c) 5 copy $_{32}$ reconstruction of Ti-plasmid, (d) 1 copy reconstruction, (a-d) probed with ^{32}P labelled HindIII A; (e) and (f) 1595501 DNA, (g) 5 copy reconstruction, (h) 1 copy reconstruction, (e-h) probed with ^{32}P labelled HindIII B; (i) and (j) 1595501 DNA, (k) 5 copy reconstruction, (l) 1 copy reconstruction, (i-l) probed with ^{32}P labelled HindIII C, (m) and (n) 1595501 DNA, (o) 5 copy reconstruction, (p) 1 copy reconstruction, (m-p) probed with ^{32}P labelled BamHI 29.

to 20kb were cloned into Charon 4A (16). Hybridization with probes pNW3lc-8, 29-1 and pNW3lc-2,19-1 identified clones containing Ti-plasmid sequences.

A. Recombinant Clone cpTL823. EcoRI digestion of cpTL823 DNA produced nonvector fragments of 7.1, 6.4 and 1.04kb. Hybridization of ^{32}P labelled pNW3lc-8, 29-1 and pNW3lc-2, 19-1 with Southern transfers of cpTL823 demonstrated that the 7.1kb and 6.4kb fragments hybridized with both probes, while the 1.04kb fragment was homologous only to pNW3lc-8,29-1. Subcloning of the EcoRI fragments and restriction enzyme analyses showed that the EcoRI 7.1kb fragment was identical to EcoRI fragment 7, while the 1.04kb fragment was identical to EcoRI fragment Δ . Since the 6.4kb fragment has exactly the same restriction endonuclease sites as the left region of EcoRI fragment 7, the EcoRI 6.4kb fragment appears to be EcoRI fragment 7 with the right end deleted. The restriction map of cpTL823 is shown in Figure 4B. These results and the results of other mapping experiments (data not shown) suggested that the T-DNA in cpTL823 is an inverted repeat. The 11kb HindIII

Figure 4
Recombinant clones cpTL823 and cpTL5A2.

- Restriction map of the T-region of the 15955 Ti-plasmid and plasmid clones pNW31c-8,29-1 and pNW31c-2,19-1.
- Restriction map of T-DNA in clone cpTL823. 3* and 8* are restriction fragments which have part of HpaI fragment 3 and BamHI fragment 8 respectively.
- Restriction map of T-DNA in cpTL5A2. 7* is restriction fragment which has part of EcoRI fragment 7.
- Possible structures of the 1.8 kb and 4.9 kb fragments. The hatched box indicates plant DNA.

fragment and 2.3 kb BamHI fragment present in cpTL823 were also detected in 1595501 genomic DNA, indicating that the observed arrangement does not result from a cloning artifact.

The structure of the clone cpTL823 was also confirmed by electron microscopy. The DNA was denatured and then annealed under conditions permitting only intramolecular hybridization. The formation of a stem and loop structure confirms the presence of an inverted repeat (Figure 5). There is no detectable hybridization between the inverted repeat and plant DNA.

Figure 5A
Electronmicrograph of the intramolecular duplex formed from the DNA of clone cpTL823. **Figure 5B** - Tracing of Figure 5A.

First line: 15955 plasmid sequence
 Second line: 1595501 tumor sequence from clone p21
 Third line: 1595501 tumor sequence from clone e6

5' ← 3'

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15955  GGGTGC AAAG CGGCAGCGGC GGCAGGATAT ATTCAATTGT AAATGGCTTC ATGTCCGGGA AATCTACATG GATCAGCAAT GAGTATGATG GTCAATATGG AGAAAAAGAA
p21   gaactttcgt aaaaggagaa ataccaATAT ATTCAATTGT AAATGGCTTC ATGTCCGGGA AATCTACATG GATCAGCAAT GAGTATGATG GTCAATATGG AGAAAAAGAA
e6    tcaatgccca aatgatattt tacacctcga tatatccTGT AAATGGCTTC ATGTCCGGGA AATCTACATG GATCAGCAAT GAGTATGATG GTCAATATGG AGAAAAAGAA
  
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Figure 6

Comparison of pTil5955 plasmid sequence and various left T-DNA junction sequences. Plasmid sequences are in block letters and DNA sequences in the tumor which diverge from the Ti-plasmid sequences are in small letters. The arrow marks the left member of the 25bp sequences.

B. Recombinant Clone cpTL5A2. Digestion of cpTL5A2 DNA with EcoRI produced nonvector fragments of 6.4kb, 5.7kb and 4.1kb. ^{32}P labelled normal N. Xanthi leaf DNA hybridized to all three EcoRI fragments. These EcoRI fragments did not hybridize with pNW3lc-2,19-1, but the 5.7kb fragment did hybridize to pNW3lc-8,29-1. The 5.7kb fragment in this clone is probably the 5.7kb fragment described in the previous section. HindIII fragment B was the only internal fragment that can be cut out from this 5.7kb fragment. To determine if the 5.7kb fragment was a plasmid DNA/plant DNA junction fragment, it was hybridized to an EcoRI digest of normal N. Xanthi DNA. The signal appeared as a smear, suggesting that the fragment contains repetitive DNA. A restriction map of this clone is shown in Figure 4C. We conclude that the 5.7kb fragment is a junction fragment in which the left region of EcoRI fragment 7 is fused to repetitive DNA.

Analysis of the Left End of T_L by DNA Sequencing

Since the left end of T_L in octopine tumors usually ends in HindIII Y (2,6), two 1595501 recombinant clones, p21 and e6, which contain HindIII Y sequences were identified as left end junction clones. (We have also determined that one of these clones has unique plant DNA sequences attached to it, while the other one was linked to the right end of T_R). In order to determine whether there is any specific DNA sequence in these two clones at which the DNA diverged from the Ti-plasmid sequences, the sequences of these two clones, as well as part of HindIII Y of pTil5955 were determined. The restriction enzyme maps of the two left end T_L T-DNA clones and the regions of DNA which were sequenced are shown in Figure 1D. Sequence data are given in Figure 6. The results show that there is no specific set of base pairs in the T-DNA which diverge from the Ti-plasmid sequence.

DISCUSSION

In this paper, we investigate an unusual octopine tumor, 1595501. The morphology of this tumor is similar to that of other unorganized tumors; however, an unusually high number of copies, ca. 10, of T_L DNA is present. T_L DNAs with deletions are also found in this tumor. This T-DNA rearrangement, which involves deletion of a central portion of T_L DNA, is not common in octopine tumors, although a nopaline tumor produced in flax also has an unusual organization (21). The possible origin of deleted T-DNA can either be due to integration of defective T-DNA or to the integration of intact T-DNA which then undergoes deletion. The high copy number of T-DNA in this tumor may facilitate a DNA recombination event to generate deletions. Our studies of the T-DNAs with deletions in this tumor indicated that deletions usually start at the left end of HindIII fragment B and extend toward the right.

One characteristic of the T-DNA organization of this tumor is the presence of large inverted repeats. Each of the inverted repeats is composed of an EcoRI 6.4kb fragment and an EcoRI 7.1kb fragment. These two EcoRI fragments, when denatured and renatured, form a stem of 5.9kb and a loop of 1.7kb. There are about 5 copies of these inverted repeats per diploid tobacco genome. One possible mechanism for the formation of copies of the inverted repeat involves several steps. The first step is the integration of a fragment of the T-DNA into the plant genome. This is followed by the integration of another fragment of T-DNA in the opposite orientation next to the first one. The T-DNA repeat introduced into the plant genome then undergoes amplification to generate multiple copies. Botchan, et al. and Roberts, et al. (22, 23) have proposed a model for DNA amplification involving a localized burst of DNA replication at an origin of replication which has escaped normal control mechanisms. Since wounding of the plant is necessary for tumor formation, the cell being transformed is probably in an active stage of DNA synthesis. If the plasmid T-DNA is integrated into a site very close to an origin of replication within the plant genome, and if aberrant replication occurs, this could account for the amplification of the T-DNA. At the present stage of our investigations, we cannot rule out the possibility of the amplification of the entire chromosome.

The high copy number of T-DNA in the 1595501 tumor is also associated with high levels of transcription. Studies utilizing northern blots have shown that the levels of T-DNA transcripts in the 1595501 tumor are much higher than in the A6S/2 tumor, which has only one copy of T_L DNA (data not

shown). All seven T_L transcripts, which are detected in most unorganized tumors (3), are also detected in the 1595501 tumors, and no aberrant transcripts were detected. Thus, either the T-DNA with deletions gives rise to truncated mRNAs which are degraded rapidly or the T-DNA with deletions was not transcribed.

Production of octopine is a characteristic of octopine crown gall tumors. When this tumor was assayed for octopine, more octopine was detected in this tumor than in other tumor lines (W. Kwok, unpublished data). The level of octopine synthase, the enzyme responsible for synthesis of octopine, is also higher than in other tumor lines (K. Taylor, personal communication).

Elevated levels of cytokinins and dimethylallylpyrophosphate-AMP transferase, the enzyme responsible for the synthesis of cytokinin, were also found in the 1595501 tumor line (24). The high levels of all of the metabolic products and enzymes described above may be due to the high copy numbers of T-DNA sequences present in this tumor.

Simpson, *et al.*, Zambryski, *et al.*, Gielen, *et al.*, Barker, *et al.*, and Yadav, *et al.* (8, 9, 10, 11, 12) have reported that there is a 25bp imperfect direct repeat at each end of the T-DNA in both octopine and nopaline plasmids, and they suggest that this repeat is involved in the T-DNA transfer process. The use of disarmed plasmids in genetic engineering also shows that the 25bp repeats are important (25, 26). Both of the two T_L junctions sequenced in this study diverge from plasmid sequence within the left member of this 25bp repeat (Fig. 6). The fact that HindIII Y is 2.8kb long, and the sites of integration for both junctions are within this 25 bp, indicate that this 25 bp sequence probably plays a role in T-DNA integration. The observation that the left junctions end at different sites within this 25bp repeat may result from DNA rearrangements that involve excision and repair after an initial precise integrative event. Alternatively, these junctions may reflect an imprecise integrative event.

Since the T-DNA in the Ti-plasmid is bounded by 25bp repeats, it has a structure similar to a transposon. However, in a typical transposition process, the repeated sequence remains intact at both ends of the transposon. Our sequence data show that the left end of the T-DNA did not end at a definite sequence. The right end of T_L DNA is also variable. The T_L right junction of the tumor line 1595501 can end in EcoRI 24, EcoRI 7 or HindIII B.

In summary, 1595501 tumor cells contain a high copy number of T_L DNA. Some copies of the T_L DNA are smaller in size than the T_L DNA usually found in unorganized octopine tumors (6, 8). These smaller T_L DNAs may have arisen

by deletion of the normal T_L . T_L DNA in 1595501 is also found as an inverted repeat. Our sequencing data of the left junctions of this tumor are consistent with the proposal that a 25bp imperfect direct repeat in the Ti-plasmid plays a role in the integration process, although the sites of integration are not exact.

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