Recombination between higher plant DNA and the Ti plasmid of Agrobacterium tumefaciens

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ABSTRACT The Ti plasmid sequences (T-DNA) from the octopine-producing crown gall tumor A6S/2 were isolated by molecular cloning, using the bacteriophage A vector Charon 4A. Analysis of the cloned DNA segments indicates that the Ti plasmid sequences are covalently joined to plant nuclear DNA. These data demonstrate that genetic recombination between a eukaryote and a prokaryote can occur as a natural phenomenon.

Crown gall is a disease of dicotyledonous plants caused by Agrobacterium tumefaciens. The virulence trait of A. tumefaciens is carried on a tumor-inducing (Ti) plasmid, which range in size between about 90 and 150 x 10^6 daltons (1-5). In the course of infection a portion of the Ti plasmid, the T-DNA, is stably transferred to the plant (4) and causes two fundamental changes in the physiology of the plant cells. First, the cells become transformed: whereas normal plant tissue grows in callus culture only when auxin and cytokinin are added to the medium, the growth of crown gall tissue is phytohormone independent (5). Second, crown gall tissues characterize as transforming primary octopine or nopaline, which are not synthesized by normal plant tissues (6, 7). The particular opine produced is coded by the Ti plasmid (8, 9). The Ti plasmid also codes for the catabolism of the corresponding opine (8, 9). Thus the A. tumefaciens-plant interaction is one in which a prokaryote "genetically engineers" a eukaryote to synthesize a compound the bacterium can use as a carbon, nitrogen, and energy source.

We have recently described the organization of T-DNA in four independent crown gall tumor lines by three closely related octopine-type Ti plasmids (10). It was shown that each tumor line contains a "core" T-DNA segment that (i) is apparently responsible for maintaining the transformed state; (ii) is colinear with the Ti plasmid; and (iii) contains the Ti plasmid sequences termed "common DNA"—sequences found in most Ti plasmids and thought to have a central role in crown gall tumorigenesis (11, 12). We also presented data suggesting that the T-DNA is integrated into plant DNA, that preferred regions of the Ti plasmid serve as the points of attachment to plant DNA, and that T-DNA can be linked to more than one site in the plant genome (10). Here we report the molecular cloning of the T-DNA and adjacent plant sequences from the crown gall tumor A6S/2.

MATERIALS AND METHODS

Tobacco Cell Lines. The derivation of the cloned octopine-producing tumor line A6S/2 has been described (13). Nicotiana tabacum strain WBSR was derived from a single root cell of N. tabacum cv. White Burley. WBSR and A6S/2 were maintained on standard Murashige-Skoog medium with and without phytohormones, respectively (14).

Bacterial Strains, Phage, and Plasmids. Escherichia coli K802, N5428, λdg805, and DP50supF were propagated as described (15). Charon 4A and the recombinant phage were propagated as described (15) or from plate lysates. A detailed description of the Ti-A6 plasmid (16) and the recombinant plasmids pNW31C-8, 29-1 and pNW31C-2, 19-1 (ref. 10; see Fig. 1) has been presented. The plasmid vector pBR325 was kindly provided by J. Cosa (University of Washington, Seattle, WA).

DNA Isolation. High molecular weight DNA from tobacco callus (4, 10, 17) and tobacco leaves (18), plasmid DNA from A. tumefaciens (19) and E. coli (10, 20), and chloroplast DNA from tobacco leaves (21) were isolated as previously described. Phage DNA was isolated from CsCl-banded phage (15).

In Vitro Labeling of DNA and Hybridization Conditions. DNA samples were labeled with 32P by a slight modification (10) of the nick-translation procedure (22). Hybridization of nitrocellulose filters containing bound DNA with the 32P-labeled probes and autoradiography were as described (10).

Restriction Enzyme Analysis. Restriction enzymes were used as described (10). DNA digests were fractionated by agarose gel electrophoresis (0.7% agarose unless stated otherwise) and the fragments were transferred from the gels to nitrocellulose by the Southern procedure (23) or recovered from the gels by the method of Tabak and Flavell (24).

Preparation of Packaging Extracts, Phage DNA Arms, and 10- to 20-kilobase (kb) A6S/2 Tumor DNA. Freeze-thaw lysates, sonic extracts, and protein A were prepared according to Blattner et al. (15). Phage DNA arms were isolated as follows: the cohesive ends of Charon 4A DNA were allowed to self-hybridize, the DNA was digested with EcoRI, and the arms were separated from the internal fragments by centrifugation through a 5-20% gradient of NaCl. Ten- to 20-kb fragments of A6S/2 DNA were prepared from EcoRI partial digests by fractionating the digests on a 5-20% gradient of NaCl and combining the appropriate fractions.

Molecular Cloning. Phage DNA arms (20 μg) and the EcoRI fragments of A6S/2 tumor DNA (7 μg) were treated with phage T4 DNA ligase (at 4°C) in a reaction volume of 50 μ1 (25). The DNA was then packaged in vitro into phage coxs as described by Blattner et al. (15), yielding approximately 1.9 x 10^6 plaque-forming units. The phage were plated on E. coli K802 and recombinant phage containing Ti plasmid sequences were located by the plaque-filter hybridization technique (26), using

Abbreviations: T-DNA, Ti plasmid DNA sequences stably transferred to the plant; kb, kilobase(s).

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a mixture of pNW31C-8,29-1 and pNW31C-2,19-1 as probe. The phage that hybridized were picked and cloned, using E. coli DH5α as host. DNA fragments from the recombinant phage were inserted into the plasmid vector pBR322 as follows: EcoRI-digested phage DNA (2.7 μg) and pBR322 DNA (0.19 μg) were mixed and treated with T4 ligase (4°C) in a reaction volume of 10 μl, and the ligated DNA was used to transform (27) E. coli HB101. Ampicillin-resistant chloramphenicol-sensitive clones were isolated and screened for the desired recombinant plasmids by a slight modification (28) of the "cleared-lysat" technique (29). All experiments involving recombinant DNA were conducted with appropriate containment (Federal Register, Dec. 2, 1978).

RESULTS

Detection of T-DNA Sequences in EcoRI-Digested A6S/2 Tumor DNA. We previously defined the T-DNA maintained in the A6S/2 tumor line (10). From the known restriction map of pTi-A6 (Fig. 1), EcoRI digestion of A6S/2 tumor DNA should produce six fragments containing Ti plasmid sequences—two "junction" fragments containing T-DNA joined to plant DNA, assuming T-DNA is integrated, and four "internal" fragments corresponding to pTi-A6 EcoRI fragments 7 (7.4 kb), δ (1.1 kb), β (0.32 kb), and α (0.22 kb). To verify this expectation, EcoRI-digested A6S/2 tumor DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters. The filter-bound DNA was then hybridized with pNW31C-8,29-1 or pNW31C-2,19-1 (Fig. 1). As predicted, two putative junction fragments (8.8 and 3.0 kb) were produced as well as two internal fragments (7.4 and 1.1 kb). The 0.32- and 0.22-kb internal fragments would not have been detected in this gel. The 8.8-kb fragment was presumably the left junction fragment because it hybridized with pNW31C-8,29-1 but not with pNW31C-2,19-1. The putative right junction fragment (3.0 kb) hybridized with pNW31C-2,19-1 as expected, but it also hybridized with pNW31C-8,29-1. Because pNW31C-2,19-1 and pNW31C-8,29-1 do not cross-hybridize (not shown), we conclude that sequences homologous with noncontiguous regions of the Ti plasmid are either fused or in close proximity in the A6S/2 T-DNA (see Discussion).

![Fig. 1. Detection of Ti plasmid sequences in EcoRI-digested A6S/2 tumor DNA. EcoRI-digested tumor DNA was fractionated by agarose gel electrophoresis (7.0 μg well) and transferred to nitrocellulose filters. The filter-bound DNA was hybridized with labeling pNW31C-8,29-1 (probe A; 1.2 x 10⁸ cpmpg) or pNW31C-2,19-1 (probe B; 1.0 x 10⁸ cpmpg) and the fragments containing complementary sequences were detected by autoradiography (2 days with two intensifying screens). Also shown are the Ti plasmid sequences represented in the recombinant molecules pNW31C-8,29-1 and pNW31C-2,19-1 and the T-DNA maintained in the unorganized tumor line A6S/2. The precise left- and right-hand extremes of the T-DNA are not known; they lie somewhere in the region denoted by the boxed hatches. The numbers at the top of the figure refer to fragment size in kb. The size of pTiA6 EcoRI fragments 2, 7, 24, 5, β, and α are 10, 7, 4.2, 1.1, 0.32, and 0.22 kb, respectively.](image1)

Molecular Cloning of T-DNA from A6S/2 Tumor DNA. To establish definitively whether the T-DNA is integrated into plant DNA, we cloned the T-DNA from A6S/2, using the bacteriophage cloning vehicle Charon 4A. In screening approximately 1.5 x 10⁹ plaques we detected three recombinant phage containing T-DNA sequences. EcoRI digestion of the DNA from these phage revealed that all the T-DNA fragments in the EcoRI digest of A6S/2 tumor DNA had been cloned; pCGA5 contained the 7.4- and 3.0-kb fragments (Fig. 2) as well as the α and β fragments (not shown); pCGA3 the 8.8-, 7.4-, and 1.1-kb fragments; and pCGA34 the 7.4-kb fragment. The hybridization characteristics of the cloned fragments were the same as those of the corresponding fragments seen in the EcoRI digests of the A6S/2 tumor DNA; the 3.0- and 7.4-kb fragments hybridized with both pNW31C-8,29-1 (Fig. 2A) and pNW31C-2,19-1 (Fig. 2B), while the 8.8- and 1.1-kb fragments hybridized only with pNW31C-8,29-1 (Fig. 2A). These data suggest that the fidelity of the cloned fragments was maintained through the cloning procedure. However, EcoRI digestion of pCGA5 produced four fragments (see arrows in Fig. 2) that were not detected in the EcoRI-digested A6S/2 tumor DNA and were not present in amounts equimolar with the other fragments of the clone. These fragments have been detected at approximately the same relative amounts in numerous preparations of phage DNA even though the clones have been propagated through many individual plaques. These results suggest that recombinational events occur at a relatively low frequency during phage growth. The proposed recombination apparently does not directly involve λ DNA sequences, because none of the aberrant fragments has detectable homology with the cloning vector (not shown). The rearrangements probably involve the T-DNA sequences because fragments were produced that hybridized only with pNW31C-8,29-1 (Fig. 2A) or

![Fig. 2. Analysis of recombinant phase containing T-DNA. DNA from Charon 4A (lane 1), pCGA34 (lane 2), pCGA31 (lane 3), and pCGA5 (lane 4) was digested with EcoRI, fractionated by agarose gel electrophoresis (0.1 μg well), and transferred to nitrocellulose filters. A photograph of an ethidium bromide (EtBr)-stained gel is presented as well as the autoradiographs of the filters after hybridization with in vitro labeled pNW31C-8,29-1 (A; 1.2 x 10⁸ cpmpg), pNW31C-2,19-1 (B; 1.0 x 10⁸ cpmpg), or WBSR (C; 1.0 x 10⁸ cpmpg). Autoradiography was for 30 min (A and B) or 2 days with two intensifying screens (C). The numbers at the side of the figures refer to fragment size in kb. Arrows point to aberrant fragments (see text).](image2)
pNW31C-2,19-1 (Fig. 2B), whereas the major fragments of the clone, the 7.4-kb fragment and the 3.0-kb putative right junction fragment, hybridized with both probes.

T-DNA Is Covalently Linked to Plant DNA. If the T-DNA is joined to plant DNA, the putative junction fragments should hybridize with plant DNA. To examine this issue, we first inserted the putative junction fragments into the EcoRI site of pBR325, subcloned, and chose two clones for further study: pCG8.8, which contained the 8.8-kb EcoRI fragment and pCG3.0αβ, which contained the 3.0-kb EcoRI fragment as well as the EcoRI α and β fragments of pTi-A6. These recombinant molecules as well as pNW31C-8,29-1, pNW31C-2,19-1, and pBR325 were then labeled and hybridized with EcoRI-digested tobacco DNA (WBSR) that had been fractionated by agarose gel electrophoresis and transferred to nitrocellulose. Both putative junction fragments hybridized with the tobacco DNA (Fig. 3, lanes A and B). Identical hybridization patterns were obtained when the putative left and right junction fragments were isolated from agarose gels and used in place of the junction fragment clones (not shown). The observed hybridization of the junction fragments with tobacco DNA must be due to plant sequences, because neither the plasmid clones nor pBR325 showed detectable hybridization under the conditions of this experiment (Fig. 3, lanes C, D, and E). In addition, both the putative left and right junction fragments hybridized with in vitro labeled tobacco DNA (Fig. 2C, lanes 3 and 4). Again, the observed hybridization must be due to plant sequences, because neither Charon 4A (Fig. 2C, lane 1) nor the Ti plasmid (not shown) hybridized with tobacco DNA. From these data we conclude that the T-DNA in A6S/2 is covalently linked to tobacco DNA. It is also reasonable to assume that the left and right junction fragments represent the ends of the same T-DNA insert because, apparently, all of the T-DNA-containing fragments generated by EcoRI digestion of A6S/2 DNA were cloned and the fragments represented in the clones were found to overlap each other (see Fig. 5).

The observation that both junction fragments produced a "smear" of hybridization when annealed with the EcoRI genomic blots of tobacco DNA (Fig. 3, lanes A and B) suggests that repetitive plant sequences are contained within the cloned segments of tobacco DNA. Consistent with this interpretation is the observed hybridization of labeled tobacco DNA with Southern transfers of both junction fragments (Fig. 2C, lanes 3 and 4). In experiments of similar design we did not detect hybridization of A6S/2 tumor DNA, which contains the T-DNA sequences, with Southern transfers of pTi-A6 (not shown), thus suggesting that the experimental design was not sensitive enough to detect a DNA sequence present at low copy number.

Location of T-DNA Sequences. The hybridization patterns of the junction fragments with EcoRI-digested tobacco DNA indicate that the cloned plant sequences are present in the nuclear DNA (Fig. 3). If the sequences were only in chloroplast or mitochondrial DNA, the observed pattern of hybridization would not have been produced, because EcoRI digestion of these DNAs resulted in a relatively discrete fragmentation pattern (Fig. 4A). This, however, did not rule out the possibility that the plant sequences were also present in mitochondrial or chloroplast DNA and that the T-DNA was joined to one of these DNAs. We therefore isolated chloroplasts from the leaves of N. tabacum cv. White Burley. A typical crude chloroplast fraction contains mitochondria and broken nuclei in addition to chloroplasts (30). The nuclear DNA was removed by treatment with DNase (30), and the remaining DNA was then isolated, digested with EcoRI, and fractionated by agarose gel electrophoresis. The fragmentation pattern reveals the presence of both chloroplast and mitochondrial DNA (Fig. 4A). The major fragmentation pattern is that of chloroplast DNA [compare the pattern from N. tabacum cv. White Burley (Fig. 4A, lane 1) with that of the purified chloroplast DNA from N. tabacum cv. Xanthi (Fig. 4A, lane 2)]. The minor fragmentation pattern represents mitochondrial DNA; the fragments comigrated and cross-hybridized with purified mitochondrial DNA isolated from N. tabacum cv. Havana (not shown). The DNA from such gels was transferred to nitrocellulose and hybridized with the left (pCG8.8) and right (pCG3.0αβ) junction fragment clones (Fig. 4B). Neither junction fragment hybridized with the chloroplast and mitochondrial DNA preparation; as expected, both probes hybridized with pTi-A6 DNA. We conclude that the plant DNA sequences represented in the cloned junction fragments are not homologous with chloroplast or mitochondrial DNA and that the T-DNA is joined to plant nuclear DNA.

Ti Plasmid Sequences Present in the Right Junction Fragment. The hybridization characteristics of the right junction fragment suggest that sequences homologous with noncontiguous regions of the Ti plasmid are either fused or in close proximity at the right end of the T-DNA (Figs. 1 and 2). The right junction fragment clone (pCG3.0αβ) contains sequences homologous with pTi-A6 EcoRI fragment 24 (Fig. 4B) and BstI fragments 2 and 19 (Fig. 4C), as would be expected from the physical map of pTi-A6 (see Fig. 5). However, the right junction fragment clone also contains sequences homologous with pTi-A6 BstI fragment 8 (Fig. 4C) and EcoRI fragment 7 (Fig. 4B) but not with BstI fragment 29 (Fig. 4C) or EcoRI fragments 5 and 2 (Fig. 4B). The same hybridization patterns were observed when the right junction fragment was isolated and used in place of the right junction fragment clone.
FIG. 4. Hybridization of the junction fragments with pTi-A6 and tobacco chloroplast and mitochondrial DNA. Various DNA samples were digested with either EcoRI or Bst I and fractionated by agarose gel electrophoresis. (A) EcoRI-digested chloroplast DNA from N. tabacum cv. Xanthi (lane 1) and chloroplast and mitochondrial DNA from N. tabacum cv. White Burley (lane 2); see text for details. A photograph more clearly showing the large molecular weight fragments of the N. tabacum cv. White Burley sample is also presented (lane 3; the lines indicate the chloroplast DNA fragments). (B) EcoRI-digested pTi-A6 (lanes marked 1; 0.1 µg per well) or N. tabacum cv. White Burley chloroplast and mitochondrial DNA (lanes marked 2; 0.25 µg per well) was fractionated, transferred to nitrocellulose, and hybridized with labeled pCG8.8 or pCG3.0αβ. Numbers at the sides of the figures refer to pTi-A6 EcoRI fragment numbers. This gel did not include fragment α; the photograph shows only fragments ≥0.5 kb and greater. (C) Bst I-digested pTi-A6 was fractionated, transferred to nitrocellulose, and hybridized with labeled pCG3.0αβ. Numbers at the side of the figure refer to pTi-A6 Bst I fragment numbers.

(not shown). These results indicate that the right junction fragment contains sequences homologous with the region of pTi-A6 between the EcoRI 6/7 and Bst I 8/29 recognition sites (see Fig. 5).

FIG. 5. Organization of T-DNA in the A6S/2 tumor. The T-DNA sequences present (solid bars) are homologous with the region of the Ti plasmid shown directly above; the left extreme lies somewhere in the region noted by the diagonally hatched box. The right end of the T-DNA (horizontally hatched box) contains sequences homologous with the indicated region of the T-DNA. The approximate length of this DNA segment has been determined by restriction map analysis (unpublished results). The T-DNA and plant sequences contained in the various clones used in the study are also shown.

DISCUSSION

From the data presented, we conclude that the T-DNA of crown gall tumor line A6S/2 is covalently linked to tobacco nuclear DNA. Thus, the T-DNA is probably integrated into a tobacco chromosome. However, the possibility that the T-DNA is maintained as a free replicon composed of Ti plasmid and plant nuclear DNA sequences is not formally ruled out. Whether the joining of Ti plasmid DNA to plant DNA is required for crown gall formation is not yet known. We have, however, presented data (10) that strongly suggests that the T-DNAs of three additional unorganized tumor lines are integrated into plant DNA. In addition, the results of Yadav et al. (31) indicate that the T-DNA of the teratoma tumor BT-37 is also covalently joined to plant DNA. Thus the linkage of Ti plasmid to plant DNA seems to be a common, if not requisite, feature of crown gall disease.

Our current concept of the organization of T-DNA in the A6S/2 tumor line is graphically represented in Fig. 5. Our data suggest there is one T-DNA insert. The right end of the insert contains a short segment of DNA that is homologous with sequences from the middle of the T-DNA. Analysis by restriction mapping and Southern blotting suggests that these sequences are fused to the main body of the T-DNA (unpublished results). The significance of the arrangement of sequences at the right end of the T-DNA and the mechanism by which the arrangement was produced are unknown. Further, we do not know whether the "repeated" sequences originated from the inte-
igration of the T-DNA or are sequences present in normal plant DNA; we have detected fragments in restriction digests of normal plant DNA that hybridized weakly with Ti plasmid probes (10). Sequence data for the "repeated" DNA and the homologous region of the Ti plasmid should help clarify the situation.

The hybridization of the left junction fragment with a discreet 7.0-kb fragment in EcoRI-digested tobacco DNA (Fig. 3) suggests that the T-DNA in A6S/2 is integrated into a 7.0-kb EcoRI fragment of tobacco DNA. If this is true, it follows that the T-DNA is inserted near the right end of the 7.0-kb fragment; the amount of plant DNA in the left junction fragment is greater than that in the right junction fragment (see Fig. 5). To both the left and right of the site of insertion, there are apparently repetitive plant sequences. This is not surprising, because Zimmerman and Goldberg (32) have shown that at least 55% of the total DNA of N. tabacum is organized in a pattern consisting of single-copy sequences averaging some 1400 nucleotides long alternating with short repetitive sequences approximately 300 nucleotides long. Another 25% of the genome contains long repetitive DNA sequences with a minimal length of 1500 nucleotides. It has been suggested that repetitive sequences play a role in regulating gene expression in eukaryotes (33–36). It would be interesting to know whether the repetitive sequences influence the expression of the T-DNA sequences.

With this report we demonstrate that in crown gall, a naturally occurring system, the physical joining of prokaryotic and eukaryotic genes can occur. As yet, we do not know if the joining process proceeds by way of host-cell recombinant mechanisms or is directed by Ti plasmid genes. Regardless, the process results in the integration and apparent expression (37–41) of foreign genes in a eukaryotic cell. Ultimately, these properties of the Ti plasmid might be exploited to introduce genes of choice into the genomes of higher plants.

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