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Tumor induction by *Agrobacterium rhizogenes* involves the transfer of plasmid DNA to the plant genome

(hairy root/plant tumorigenesis/root-inducing plasmid)

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ABSTRACT **The DNA from tumors of *Nicotiana glauca* initiated by strains of *Agrobacterium rhizogenes* was shown to contain sequences that are homologous to the root-inducing (Ri) plasmid of the bacterium. Two independently established tumor lines contained a similar portion of the Ri-plasmid. The Ri-plasmid also hybridized to DNA fragments from uninfected *N. glauca*. A cosmid clone of the Ri-plasmid encompassing the region containing the Ri-plasmid sequences that are stably transferred to the plant also hybridized to the Ri-plasmid-related fragments found in uninfected plants. Five of six tumor lines tested produced a tumor-specific compound that is similar to agropine.**

Members of the genus *Agrobacterium* incite tumors when inoculated into wounds on susceptible dicotyledonous plants. In the case of *Agrobacterium tumefaciens*, the etiological agent of crown gall tumors, oncogenicity is invariably associated with diverse tumor-inducing (Ti) plasmids (1, 2). A portion of the Ti-plasmid is transferred to and stably integrated into the plant cell genome and this transferred DNA (T-DNA) determines two fundamental characteristics of crown gall tumor cells: (*i*) the cells grow in the absence of the phytohormones that are required for normal cell growth in callus culture, and (*ii*) the cells synthesize unusual tumor-specific compounds, called "opines," including octopine (3), nopaline (4), and agropine (5). The Ti-plasmid also confers on the bacterium the ability to catabolize the particular opine that is produced in the tumor (6, 7).

Agrobacterium rhizogenes incites hairy root tumors when inoculated onto a plant. The tumors characteristically have less callus than do crown gall tumors but are even more clearly distinguished by the extensive proliferation of roots from the wound site. Hairy root tumorigenicity of *A. rhizogenes* is conferred by a large plasmid of approximately 260×10^6 daltons (8). The tumorigenicity traits are also encoded by another plasmid of approximately 160×10^6 daltons which results from the dissociation of the larger plasmid (8). Conjugative transfer of either form of the root-inducing (Ri) plasmid to a nonpathogenic strain confers on the transconjugant the ability to induce hairy root symptoms which are identical to those induced by the wild-type strains.

It is not known whether the phenomenon of hairy root tumorigenicity is associated with the transfer of plasmid DNA to plant cell DNA. Two facts suggest that plasmid DNA is transferred. First, a close genetic relationship exists between strains of *A. rhizogenes* and *A. tumefaciens*. The Ri-plasmids show a high degree of sequence homology with a region of the Ti-plasmid which is concerned with oncogenicity. This suggests that both Ri- and Ti-plasmids evolved from a common ancestorial plasmid and implies that these plasmids have similar biological functions (9). The Ri-plasmid genes can, in fact, complement some *onc* mutations in the Ti-plasmid which affect oncogenicity (unpublished data). It is important to note, however, that Ri-plasmids do not contain the T-DNA sequences that are common among many other Ti-plasmids (10–12). Second, it has recently been reported that axenic root cultures from hairy root tumors synthesize agropine (13). Octopine and nopaline genes of the T-DNA direct the synthesis of the opines in the tumor cells and it is suspected that agropine synthesis is similarly controlled. We report here our initial analysis of axenic hairy root tumor tissue for Ri-plasmid sequences.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Tumors were initiated with *A. rhizogenes* strains A4 and 15834. The plasmid content of these strains has been described (14). Plasmid pRiA4b was obtained from a derivative of the strain A4T (15). Plasmids from strains of *Agrobacterium* were isolated according to the procedure of Currier and Nester (16). The cosmid vector pHC79 (17) was isolated from *Escherichia coli* by the method of Blair *et al.* (18). *E. coli* strain HB101 was used as the recipient in cloning experiments (19). Cosmids from the cloning experiments were screened by using the procedure of Birnboim and Doily (20).

Plant Tumor Lines. Tumors were induced on *Nicotiana glauca* by inoculating plant stems with the appropriate strain of *A. rhizogenes*. The inoculation site was wrapped with paraffin film and the tumors were grown for 1 month. Tumor tissue was excised from the plant and prepared for *in vitro* cultivation in the following manner. Tumors were immersed in a 20% commercial bleach solution for 10 min and rinsed with five changes of sterile water. The tumors were incubated overnight with shaking in Murashige–Skoog medium (21) containing 0.4 mg of Benomyl (Charles H. Lilly, Portland, OR) per ml and 1 mg of carbenicillin (Geopen; Pfizer, New York) per ml. On the following day the tumors were soaked for 30 sec in 70% ethanol, rinsed once with sterile distilled water, and then soaked for 1 min in a 20% bleach solution. The tissue was rinsed thoroughly (five times) with sterile distilled water. The surface tissue was removed with a scalpel, and sections of the tumors were placed on B5 medium (22) containing 0.1% Casamino acids (B5/CAA medium) with carbenicillin at 1 mg/ml. After 4 weeks the tissue was transferred to B5/CAA medium without antibiotics. Subsequently, any tissue that showed no sign of bacterial or fungal contamination was passaged on either B5/CAA or Murashige–Skoog medium without phytohormones. The tumor lines were checked for bacterial contamination by crushing samples of tissue, plating the exudate on nutrient agar (Difco), and incubating the plates at 28°C.

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Abbreviations: Ti-plasmid, tumor-inducing plasmid; Ri-plasmid, root-inducing plasmid; T-DNA, Ri- and Ti-plasmid sequences that are stably transferred to the plant; kb, kilobase(s).

Agropine Assay. One gram of plant tissue was macerated in 1 ml of 95% ethanol and centrifuged at 6,000 rpm in a SS-34 rotor C (Sorvall) for 25 min. The supernatant was collected and concentrated to 100 μl. A 20-μl sample was applied to and dried on a 45-cm sheet of chromatography paper (Whatman no. 1). A 15-μg sample of agropine (kindly supplied by J. L. Firmin) was run in a separate lane with the plant extracts. The paper was wetted and subjected to electrophoresis in a 5:15:80 mixture of formic acid, acetic acid, and water for 4 hr at 10 V/cm. The chromatogram was air-dried and the location of agropine was determined by using the alkaline silver nitrate reagent (23).

Isolation of Plant DNA. Plant tissue (usually 10–20 g) was weighed, frozen in liquid N_2, and ground to a powder with a mortar and pestle. Liquid N_2 was added periodically during the grinding process. The powder was added to a volume of lysis buffer (0.020 M Tris·HCl, pH 8.0/5 mM EDTA/1% Na$DodSO_4$) equal to twice the tissue weight. One volume of phenol/chloroform, 1:1 (vol/vol), was added immediately and the mixture was mixed vigorously. Mixing was continued intermittently at ambient temperature until the ground tissue had thawed. Then the mixture was placed on ice. The aqueous phase was collected and the phenol/chloroform interface was reextracted with 1 vol (versus the tissue weight) of lysis buffer. The two aqueous phases were pooled and the DNA was precipitated with an equal volume of cold absolute ethanol. The DNA was resuspended in 1 vol of TES buffer (0.05 M Tris·HCl, pH 8.0/0.05 M NaCl/5 mM EDTA) and extracted with 0.5 vol of phenol/chloroform. The aqueous phase was again ethanol precipitated. The DNA was resuspended in TES buffer and banded twice in cesium chloride/ethidium bromide gradients. The ethidium bromide was extracted with isopropyl alcohol which was equilibrated with 20× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate) and the DNA was precipitated with ethanol. The pellet was resuspended in 1 ml of 0.2 M sodium acetate/0.01 M Tris·HCl, pH 8.0/1 mM EDTA, extracted twice with phenol/chloroform, precipitated twice in ethanol, and finally resuspended in 1 ml of 0.01 M Tris·HCl, pH 8.0/1 mM EDTA.

Restriction Enzymes and Gel Electrophoresis. All restriction endonucleases were used under the conditions recommended by the supplier (Bethesda Research Laboratories). Generally, 10 μg of plant DNA was digested in 50 μl of reaction buffer. Reaction mixtures were incubated overnight (8–10 hr), and the reactions were stopped with 10 μl of 20% (wt/vol) Ficoll/1% Na$DodSO_4$/0.05% bromophenol blue/5 mM EDTA. The DNA was loaded into wells in a submerged 0.7% agarose gel (0.5–0.7 cm thick by 38 cm long) and subjected to electrophoresis at 1.0–1.3 V/cm for approximately 36 hr. Electrophoresis buffer was either Tris/acetate (0.08 M Tris, pH 8.0/0.04 M sodium acetate/4 mM Na_2EDTA) or Tris/borate (0.089 M Tris, pH 8.0/2.5 mM Na_2EDTA/8.9 mM boric acid).

Plasmid Reconstruction Mixtures. Reconstruction experiments used mixtures of normal *N. glauca* DNA and Ri-plasmid DNA based on the ratio of the estimated genome of *N. glauca* and the molecular weight of the Ri-plasmid. Because the M_r of the *N. glauca* genome has not been reported, it was estimated from the known genome size of other *Nicotiana* species. Diploid species have genome M_r in the range $2–3 \times 10^{12}$ whereas the tetraploid species *Nicotiana tabacum* has a genome M_r of 6×10^{12} (24, 25). Therefore, *N. glauca*, which is a diploid species (24, 25), was estimated to have a genome M_r of 3×10^{12}. The ratio of molecular weights for *N. glauca* and pRiA4c (M_r, 2.6×10^8) is 1.2×10^4. Therefore, 830 pg of pRiA4c was added to 10 μg of *N. glauca* leaf DNA for a one-copy reconstruction mixture. When pRiA4b (M_r, 1.6×10^8) was used, 530 pg of pRiA4b was added to 10 μg of *N. glauca* leaf DNA for a one-copy mixture.

Preparation of Nitrocellulose Filters, *in Vitro* Labeling of DNA, and Hybridization Conditions. Nitrocellulose filters were prepared according to the method of Southern (26) as modified by Thomashow *et al.* (27). Plasmids were labeled with [^{32}P]deoxyribonucleotides by the nick-translation procedure (28) as described by Thomashow *et al.* (27). Unless otherwise stated, all chemicals were reagent grade.

Molecular Cloning. Plasmid pRiA4b was partially digested with *Hin*dIII to yield predominantly large linear DNA fragments [>24 kilobases (kb)] and were cloned into *Hin*dIII-cleaved pHC79 according to Blattner *et al.* (29). The recombinant DNA experiments were performed under P1 containment as specified by the National Institutes of Health guidelines.

RESULTS

Axenic Hairy Root Tissue Culture. Our initial attempts at culturing axenic roots directly from plant wounds infected with *A. rhizogenes* failed, although we were able to isolate axenic callus tissue from crown gall tumors incited by *A. tumefaciens*. Therefore, we screened different plant species in order to obtain a host plant from which a callus tissue could be isolated after infection with *A. rhizogenes*. *A. rhizogenes* strains A4 and 15834 caused typical hairy root symptoms on various plants (Fig. 1*b*). Seven to 10 days after inoculation, a slight callus developed at the wound site on the plant; 1–2 days later, roots began to emerge from the callus; and by 14 days, extensive root development was clearly visible. However, tumors on *N. glauca* induced by strains A4 and 15834 had a markedly different appearance. The tumor was predominantly a soft callus (Fig. 1*a*) with few roots. *N. glauca* provided an excellent source of tumor tissue for studying the events in *A. rhizogenes* tumorigenicity. Tumors incited with strains A4 and 15834 were excised from plants and cultured on plant tissue culture medium (Fig. 1*c*). The axenic tissues continued to grow in the absence of bacteria when cultured on media lacking auxin and cytokinin.

Agropine Production by *N. glauca* Tumor Lines. If the tumors incited on *N. glauca* resulted from the transformation of plant cells by *A. rhizogenes*, the axenic tumor lines might be expected to synthesize agropine. Six independently derived tumor lines were analyzed and at least five of the lines contained a compound with characteristics similar to those of agropine. The compound had the same mobility as purified agropine after paper electrophoresis and reduced the silver nitrate reagent (Fig. 2). A similar compound was detected in crown gall tumor line E9, a known producer of agropine. The compound was not detected in either the nopaline-producing tumor line T37 or in uninfected *N. glauca* leaf tissue.

Presence of Ri-Plasmid Sequences in Tumor Tissue. If tumor DNA contained Ri-plasmid sequences, plasmid DNA should hybridize with DNA isolated from such tumors. To determine if hairy root tumors contained plasmid sequences, the Ri-plasmids were used as probes in hybridization experiments with plant tumor DNA. Strain A4 harbors a 260×10^6 dalton plasmid (pRiA4c) which dissociates into two smaller plasmids. Tumorigenicity functions reside on the 160×10^6 dalton plasmid (pRiA4b). Plasmid pRiA4c was used first as the probe in order to detect the maximal amount of plasmid DNA incorporated into the plant genome. Then pRiA4b was used as the

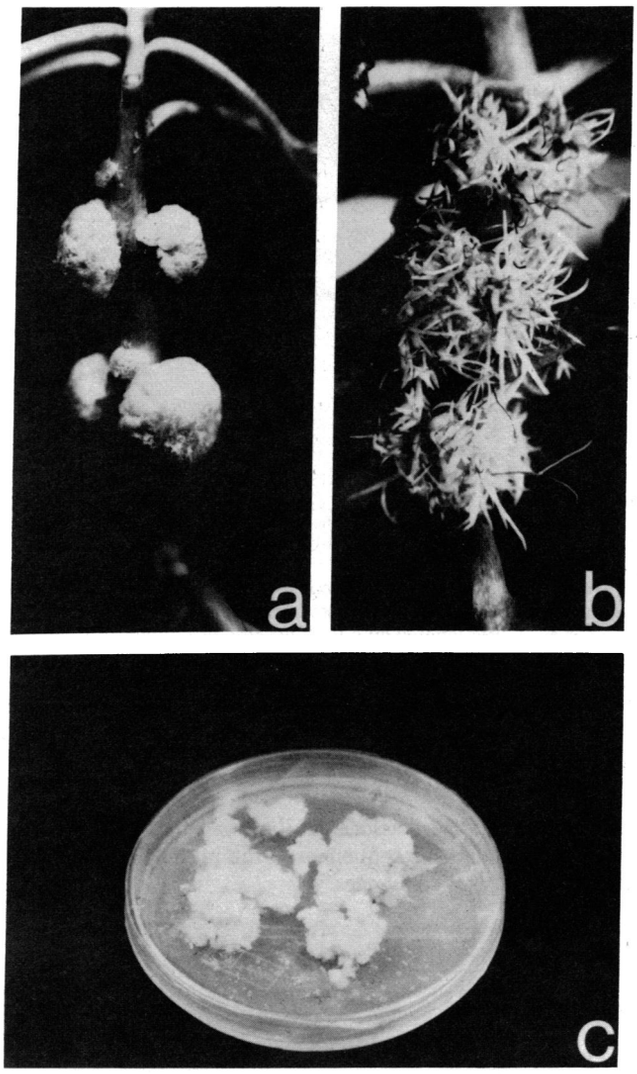

FIG. 1. Tumorigenicity of *A. rhizogenes* strain A4 inoculated on *N. glauca* (*a*) and *Kalanchöe diagremontiana* (*b*). Tumor tissue derived from *N. glauca* was cultured axenically on Murashige–Skoog medium (*c*).

probe to determine if this plasmid contained the same region of T-DNA as the parent plasmid. The plasmids from strain A4 were used to probe tumor DNA from tumor lines initiated by strains A4 and 15834. The plasmids of these two strains have nearly identical DNA sequences (9).

When radiolabeled pRiA4c was hybridized with tumor DNA that had been digested with restriction endonucleases and transferred to nitrocellulose, plasmid sequences were detected. Strong hybridization signals corresponding to fragments of 11.2 and 5.8 kb were observed when DNA from either 15834 or A4 tumors was digested with *Kpn* I (Fig. 3). When digested with *Bam*HI, the DNA from both tumors contained fragments of about 10.4 kb which hybridized to the plasmid DNA. The signals in the autoradiograph appeared as either a broad band or two closely spaced bands. (The conditions of electrophoresis in this experiment separated large fragments which differed in size by 0.1–0.2 kb.) When pRiA4b, the dissociation product of pRiA4c that codes for virulence functions, was used as the probe the results were similar. *Kpn* I fragments of 11.2 and 5.8 kb hybridized with pRiA4b (Fig. 4). *Bam*HI fragments of about 10.4 kb likewise hybridized to pRiA4b (Fig. 4). Each prominent signal in this experiment also represented at least two fragments

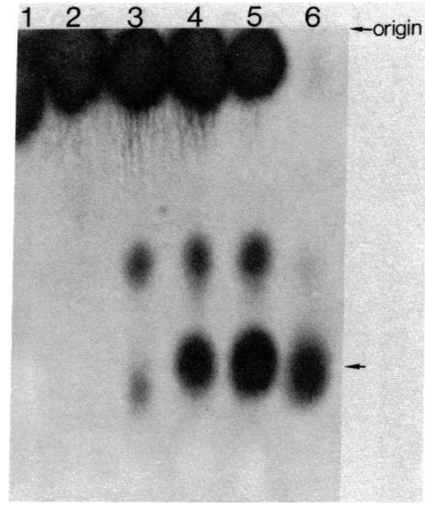

FIG. 2. Presence of agropine in axenic *N. glauca* tumors. Tumors were induced on *N. glauca* by *A. rhizogenes* strain A4 or 15834, grown on synthetic plant medium, and assayed for agropine. Tissue taken directly from uninfected *N. glauca* stems as well as two axenic tumors of *N. tabacum* that had been incited by *A. tumefaciens* strains T37 and B6806 were analyzed. Authentic agropine was included as a standard. Lanes: 1, BT37, a nopaline-producing tumor line of *N. tabacum* (strain T37); 2, uninfected *N. glauca* stem; 3, 15834-1a, a *N. glauca* tumor line (strain 15834); 4, A4-4b, a *N. glauca* tumor line (strain A4); 5, E9, an agropine-producing tumor line of *N. tabacum* (strain B6806); 6, agropine standard.

of approximately the same size, but the fragments were not separated under the electrophoretic conditions used. Thus, a portion of the Ri-plasmid that is contained within pRiA4c and pRiA4b was present in tumor DNA. These fragments were present in an average of three to five copies per diploid plant cell genome as determined from the one- and five-copy reconstruction experiments (Fig. 3).

Faint hybridization signals were observed for other tumor DNA fragments, and these appeared to be the same whether pRiA4c or pRiA4b was the probe. At least 10 such fragments, ranging in size from 12.5 to 3.0 kb, could be distinguished in the tumor DNA that was digested with *Bam*HI (Fig. 4). Two fragments that hybridized with less intensity were detected when tumor DNA was digested with *Kpn* I (Fig. 4). These fragments were estimated to be 15 and 6.0 kb, respectively. Other bands of lower intensity were evident in most experiments. In some cases, the fragments that hybridized with a low intensity aligned with Ri-plasmid fragments in the reconstruction experiments. These fragments may represent complete Ri-plasmid fragments that are present in fewer copies then the more prominent T-DNA fragments (e.g., the 4.2-kb *Bam*HI fragment in Fig. 4). In other cases these fragments were also detected in uninfected plant DNA and represented regions of homology between the Ri-plasmid and uninfected *N. glauca* DNA (e.g., the 3.3-kb *Bam*HI fragment in Fig. 3 and the 6.0-kb *Kpn* I fragment in Fig. 4). The fragments of the tumor DNA that hybridized with the Ri-plasmid and were not found in uninfected plant DNA may correspond to junction fragments of Ri-plasmid and plant DNA.

Presence of Ri-Plasmid Sequences in Normal Plant DNA. When DNA from uninfected plants was digested, transferred to nitrocellulose, and hybridized with radiolabeled Ri-plasmid, several fragments were detected. When the DNA was digested with *Bam*HI, fragments of approximately 12.5, 8.0, and 3.3 kb were readily observed (Fig. 3). Bands of lower intensity were present also. When *Kpn* I was used, a signal at 6.0 kb was de-

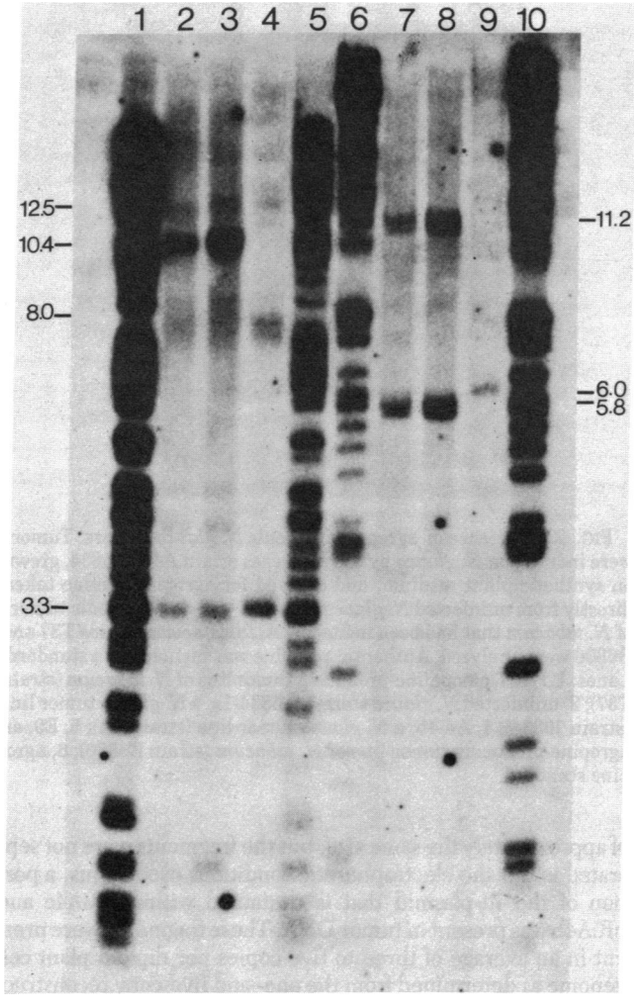

FIG. 3. Presence of Ri-plasmid sequences in tumor DNA. DNA extracted from plant tissues was digested with *Bam*HI (lanes 1–5) or *Kpn* I (lanes 6–10), fractionated by electrophoresis on a 0.7% agarose gel, and transferred to nitrocellulose. The filters were hybridized with ^{32}P-labeled pRiA4c, washed, and prepared for autoradiography. Lanes: 1, five-copy reconstruction; 2, tumor 15834-1b; 3, tumor A4-4b; 4, uninfected plant; 5, one-copy reconstruction; 6, one-copy reconstruction; 7, tumor 15834-1b; 8, tumor A4-4b; 9, uninfected plant; 10, five-copy reconstruction. Numbers at the sides are fragment sizes in kb.

tected (Fig. 4). Less-intense hybridization signals were detected which correspond to 12.5- and 10.0-kb fragments (Fig. 4). We have repeated the hybridizations with three independent uninfected plant DNA preparations and have obtained identical results in each experiment.

The position of the homologous plant and Ri-plasmid sequences with respect to T-DNA of the Ri-plasmid was defined further by cloning segments of the plasmid. The plasmid pRiA4b was digested partially with *Hin*dIII and ligated into the cosmid vector pHC79. One cosmid, pNW44, was isolated which hybridized to the same tumor DNA fragments as did pRiA4b (Fig. 5). The entire T-DNA region of the Ri-plasmid, in addition to the adjacent plasmid fragments, was contained within pNW44. This clone also hybridized to the same fragments of the uninfected plant DNA. In a *Hin*dIII-digest of uninfected plant DNA, a single fragment was detected (Fig. 5). The signal at the 6.0-kb fragment in the *Kpn* I digest of uninfected plant DNA was observed also, as well as the less-intense signals at the 12.5- and 10.0-kb *Kpn* I fragments (Fig. 5). The vector pHC79 showed no homology to the plant DNA (data not shown).

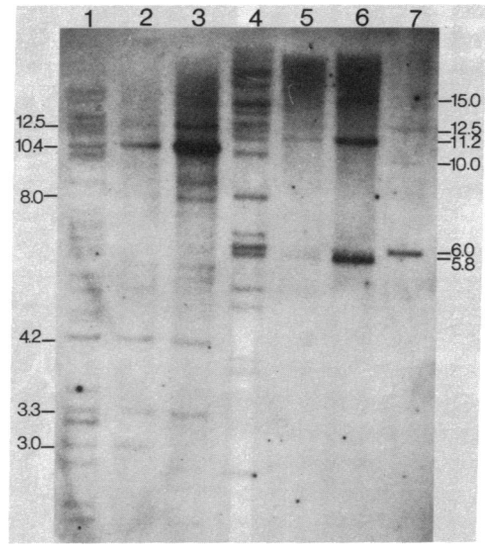

FIG. 4. Presence of pRiA4b sequences in tumor DNA. DNA was extracted from plant tissue, digested with *Bam*HI (lanes 1–3) or *Kpn* I (lanes 4–7), fractionated on a 0.7% agarose electrophoresis gel, and transferred to nitrocellulose. The filters were hybridized with ^{32}P-labeled pRiA4b and washed. Labeled fragments were detected by autoradiography. Lanes: 1, one-copy reconstruction; 2, tumor 15834-1b; 3, tumor A4-4b; 4, one-copy reconstruction; 5, tumor 15834-1b; 6, tumor A4-4b; 7, uninfected plant. Numbers at the sides are fragment sizes in kb.

Thus, there is a close association between the T-DNA region of Ri-plasmid and the sequences of the plasmid that have homology with plant sequences.

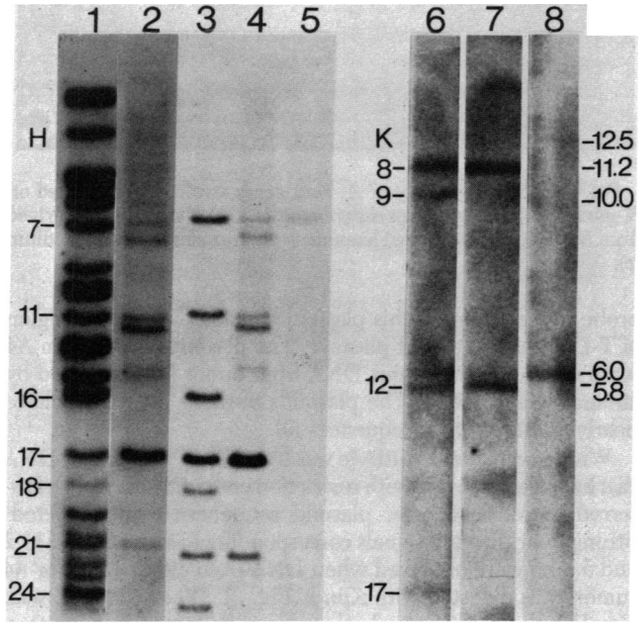

FIG. 5. Recombinant DNA plasmid containing the T-DNA region. Nitrocellulose filters prepared from plant DNA were hybridized with either ^{32}P-labeled pRiA4b (lanes 1 and 2) or ^{32}P-labeled pNW44 which contained the T-DNA of pRiA4b (lanes 3–8). (*Left*) *Hin*dIII-digested DNA (lanes 1–5). (*Right*) *Kpn* I-digested DNA (lanes 6–8). Lanes: 1, one-copy reconstruction; 2, tumor A4-4b; 3, one-copy reconstruction; 4, tumor A4-4b; 5, uninfected plant; 6, one-copy reconstruction; 7, tumor A4-4b; 8, uninfected plant. The numbers at the left designate the fragment number in the restriction digest of pRiA4b. The numbers at the far right are fragment sizes in kb for the *Kpn* I-digested DNA. H, *Hin*dIII; K, *Kpn* I.

DISCUSSION

The data presented clearly indicate that tumor induction by *A. rhizogenes* involves the transfer of Ri-plasmid sequences to the plant genome. Two independently established tumor lines originally incited with different strains both contained the same prominent T-DNA restriction fragments. These fragments often aligned with Ri-plasmid fragments in the reconstruction experiments and therefore probably represent internal fragments of the Ri-plasmid. Cloned fragments encompassing the T-DNA region of the Ri-plasmid contained the putative internal fragments and hybridized to all T-DNA fragments of the tumor. The simplest interpretation of our data is that each tumor consists of transformed cells which contain varying portions of the T-DNA region of the Ri-plasmid. The most prominent T-DNA fragments represent the core of the T-DNA region. The core fragments are present in an average of three to five copies per diploid genome. The 10.4-kb *Bam*HI fragment that is present in the tumor DNA suggests that the core region is near 10 kb in size. Other tumor fragments hybridized with the Ri-plasmid with lower signal intensities and represent either internal fragments of the Ri-plasmid T-DNA region present in fewer copies, junction fragments of Ri-plasmid and plant DNA, or regions of homology between the Ri-plasmid and normal plant DNA.

The transfer of Ri-plasmid DNA to the plant appears to be analogous to the phenomenon which until now has been studied exclusively in tumors transformed with the Ti-plasmid of *A. tumefaciens*. These latter tumors were incited by bacteria that harbored either nopaline- or octopine-type Ti-plasmids. In each case, a portion of the Ti-plasmid was transferred and integrated into the plant nuclear DNA (14, 30–32). In general, the T-DNA of the tumor was colinear with the T-DNA region of the Ti-plasmid (31, 33). Tumors initiated by strains carrying the same Ti-plasmid always contained a core portion of the T-DNA region (27) that codes for the maintenance of the tumorous state (34). Tumors incited by *A. rhizogenes* always contained at least one portion of the Ri-plasmid, and this region may represent the essential core T-DNA of hairy root tumors.

Nopaline- and wide host range octopine-type Ti-plasmids have extensive DNA sequence homology within their respective T-DNA regions. This fact implies that rather similar oncogenicity genes reside on both plasmids. In contrast, the Ri-plasmids have little homology with the core T-DNA region of nopaline- or octopine-type Ti-plasmids (9). Under relatively stringent conditions of hybridization (allowing approximately 12% base pair mismatch), no homology was detected to the core T-DNA region of pTiA6, an octopine-type Ti-plasmid; and only when the conditions were adjusted to allow 30% base pair mismatching was hybridization observed. Therefore, genes of the Ri-plasmid T-DNA either have diverged significantly from Ti-plasmid T-DNA genes or are novel transforming genes.

One intriguing finding of this study is the unexpected homology between the *N. glauca* genome and the Ri-plasmid. The regions of homology to normal plant DNA appear to be situated within or very near to the T-DNA region on the Ri-plasmid. Their presence raises many questions: Are these sequences present in other plant species? Do the homologous sequences play a role in the induction of the tumors? Does the Ri-plasmid T-DNA recombine into the homologous plant sequences; or do these sequences represent an endogenous plant oncogene?

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