STUDIES ON AGROBACTERIUM TUMEFACIENS

IV. NONREPLICATION OF THE BACTERIAL DNA IN MUNG BEAN (PHASEOLUS AUREUS)

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Summary

DNA·DNA filter hybridization and DNA solution enrichment reassociation experiments showed that no Agrobacterium tumefaciens DNA was replicated in mung bean seedlings under the conditions specified in published reports for the uptake, integration, and replication of bacterial DNA in higher plants. Residual presumptive DNA hybrids that formed in a few instances were characterized by thermal chromatography on hydroxylapatite. The Tm and melting profiles of these hybrids from DNA-treated plants were the same as those from untreated control plants. The sensitivities of these procedures were sufficient to detect A. tumefaciens DNA in the order of 0.005% to 0.01% of the plant genome. These results do not concur with previous reports that large pieces of DNA (at least 30%) of the plant genome of bacterial-DNA-treated-plants is made up of bacterial donor DNA.

Introduction

Considerable interests have been engendered by the reports of uptake, integration, and replication of foreign bacterial DNA in eukaryotic cells (1-5). Of special interest has been the fate of Agrobacterium tumefaciens DNA in higher plants because non-self-limiting crown gall tumors are initiated through infection of plants by this bacterium (6) and because uptake, integration and replication of this bacterial DNA have been reported by Stroun et al. (7), and Anker and Stroun (8). These latter processes imply that A. tumefaciens DNA sequences persist and form increasing numbers of copies as the plant cells reproduce. This assumption appeared to be supported by the observation of a "hybrid" molecule composed of about equal amounts of bacterial DNA and plant DNA that was apparently synthesized in DNA-treated plants (as judged from density gradient centrifugation analysis data) (7,8). This would constitute ample

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quantities of foreign DNA molecules to afford very easy detection by current DNA reassociation techniques.

Since Stroun et al. (7) and Anker and Stroun (8) investigated normal plants and not crown gall tumors, it remains uncertain whether or not the reported bacterial DNA replication in host plants is relevant in the initiation of crown gall tumors. In fact, A. tumefaciens DNA sequences were not detected in crown gall tumors (9, 10) contrary to earlier observations (11-14). Nevertheless, since previous crown gall tumor-DNA experiments employed established tumor lines (9-14), it remains to be shown whether or not A. tumefaciens DNA replicates, even to a limited extent, during the early phases of tumorigenesis, i.e., a short time after the bacterial DNA has gained access into host cells.

We now have examined the fate of A. tumefaciens DNA in mung bean plants and present evidence that this bacterial DNA does not replicate under conditions which have been shown previously to be conducive for the uptake, integration and replication of foreign bacterial DNA in plants (3, 8; Ledoux, personal communication).

Materials and Methods

Preparation of DNA. A. tumefaciens DNA from strain 1D135 (ATCC 27912) was isolated and purified by using a modified procedure of Saito and Miura (15). Cells were grown in medium 523 as described previously (16). In the modified procedure, cells (7.5 g wet-packed weight) were washed twice with saline-EDTA buffer (0.15 M NaCl-0.1 M Na_2 EDTA, pH 8.0) and digested with lysozyme (250 µg/ml) in the same buffer at 37° for 30 min. The suspension was diluted to 100 ml with borate-EDTA (0.05 M Na borate-0.1 M Na_2 EDTA, pH 9.0). The cells were lysed by the addition of 10 ml of 20% Na dodecyl sulfate and 1 g Na tri-isopropylnaphthalene disulfonate (Eastman). The mixture was then shaken vigorously for 60 min with an equal volume of liquified phenol (adjusted with 1 M Tris to pH 9.0), centrifuged for 10 min at 12,000 x g, 4°, and the aqueous phase collected. The DNA was spooled onto a glass rod as the DNA was precipitated with 2 vol. of 95% ice-cold ethanol and then dissolved in 0.1 X SSC (SSC = 0.15 M NaCl - 0.015 M Na_3 citrate, pH 7.0). The DNA solution was made to 1 X SSC and treated with pancreatic ribonuclease I (50 µg/ml, deoxyribonuclease-free) for 30 min at 37°. The solution was then re-extracted with an equal volume of phenol for 10 min with shaking. DNA was collected on a glass rod from the aqueous phase as before and dissolved in 100 ml of 0.1 X SSC. Then 11 ml of 3 M Na acetate-1 mM Na_2 EDTA buffer, pH 7.0, was added and the DNA re-precipitated with 0.6 vol of ice-cold isopropanol, washed successively with 70%, 80%, and 95% ethanol. The DNA was dissolved in 0.1 X SSC, a few drops of chloroform were added, and the DNA solution was shaken vigorously at room temperature to free it of any living microorganisms. This was checked periodically by plating samples of the DNA solution on agar medium 523. The molecular weight of A. tumefaciens DNA used was 24 x 10^6 daltons as determined by viscometry (17).

Radioactive A. tumefaciens DNA was prepared by the same procedure except that the labelling was done by growing cells in medium 132 (18) containing [^{3}H]-6-thymidine (0.2 mCi/ml of medium, sp. act. = 15 Ci/mM, C.E.N./S.C.K., Belgium).

Bacillus subtilis DNA from strain 168 was purified as described by Saito and Miura (15).

Mung bean (Phaseolus aureus) DNA was isolated and purified as described by Lurquin et al. (19) with the following modifications: grinding of plant material was performed in borate-EDTA buffer, pH 9.0, according to Kado and Yin (20) and the DNA in 2 M NaCl from the agarose chromatography fractions was directly adsorbed on 2 ml hydroxylapatite columns (BioRad) which were then washed with 5 ml of 0.1 M Na phosphate (equimolar buffer). The DNA was eluted with 5 ml of 0.5 M Na phosphate and dialyzed against 0.1 X SSC.

DNA treatment of plants. Mung bean seeds were surface-sterilized by immersing them in a solution of 1% $HgCl_2$ for 5 min. The seeds were rinsed extensively with sterile water and dried at room temperature in a sterile flask. For DNA uptake studies, these seeds were individually treated with DNA by allowing them to germinate at 23° in 0.1 ml A. tumefaciens DNA (335 µg/ml; MW = 24 x 10^6) followed by the addition of 0.1 M [^{3}H]-6-thymidine (1 m Ci/ml; sp. act. = 15 Ci/mM) after 48 hrs. After an additional 24 hrs, 0.1 ml of A. tumefaciens DNA was again added followed by [^{3}H]-6-thymidine treatment. Each time the DNA was used, sterility checks were made as above. Seeds for the control set were treated in identical fashion except that 0.01 M sterile NaCl was used in place of DNA. After this series of treatments carried out under constant lighting (5700 lux, 55 cm distance of mazda and fluorescent lamps), each seed was transferred on sterile 0.8% agar in covered beakers. They were allowed to grow under the same conditions described by Anker and Stroun (8). Experimental lots of 10 seeds were used. After 7 days growth, the plants were harvested and their leaves, stems, cotyledons and roots were separated and stored at -15°. The necessary parameters that provide conditions near optimal for foreign bacterial DNA uptake were followed as described by Tshitenge and Ledoux (personal communication).

DNA·DNA filter hybridization. Analyses were performed as described previously (9). All high molecular weight and sheared DNA preparations were further treated with 0.1 M NaOH for 4-6 hrs at 37° and dialyzed against 0.1 X SSC until neutral. DNA filters were prepared as described before (9). Sheared DNA was obtained by sonication (described below). Hybridization reactions were carried out at 46.2° in a final vol. of 0.4 ml containing 50% formamide, 5 X SSC, denatured-sheared radioactive DNA and 5 mm nitrocellulose membrane filters (Sartorius, 0.2 µ pore size) loaded with either A. tumefaciens DNA (8.6 µg/filter) or B. subtilis DNA (4.5 µg/filter). The amount of DNA immobilized on the filters was measured chemically as before (9). The reactions were stopped after 24 hrs incubation by removing the reacting solution and immediately washing the filters 7 times with 5 X SSC in 50% formamide. The filters were placed individually in vials containing 7.5 ml of scintillation fluid (C.E.N./S.C.K., Belgium) and the radioactivity measured in a Packard liquid scintillation spectrometer, model 2425.

Thermal chromatographic analyses of rapidly renatured DNA. Radioactive leaf DNA from DNA-treated plants (3.78 µg/ml) or from untreated plants (5.50 µg/ml) was mixed with unlabelled A. tumefaciens DNA (225 µg/ml) in 0.12 M Na phosphate (equimolar) and then sonicated for 10 min with a MSE Mullard ultronic power unit to a molecular weight of 300,000 daltons (M. Janowski, personal communication). The DNA solutions were then heated for 15 min in boiling water and immediately transferred to a water bath set at 69°. Renaturation was allowed to proceed to Cot* = 200 for A. tumefaciens DNA. Under these conditions, Cot

*Cot = DNA concentration (moles nucleotides/liter) X seconds (21).

values of 3.3 and 4.9 were obtained for the DNA of DNA-treated and untreated plants, respectively. A control sample consisted of a mixture of sonicated and denatured A. tumefaciens radioactive DNA (1.9 µg/ml, 98650 cpm/µg) and unlabelled A. tumefaciens DNA (225 µg/ml) in 0.12 M Na phosphate (equimolar). This mixture was renatured along with the above leaf DNA samples under identical set of conditions. After renaturation, the double stranded DNA was obtained by hydroxylapatite chromatography at 69° (21). The double stranded DNA thus obtained was characterized by thermal chromatographic procedures (22).

Infectivity. P. aureus seedlings were inoculated with A. tumefaciens strain 1D135, grown under fluorescent lights on 0.8% agar, and examined after 10 days for tumors. Tumors were observed at the sites of inoculation.

Results

Mung bean seeds were germinated and allowed to grow in a solution of A. tumefaciens DNA and radioactive thymidine. Thus, if any A. tumefaciens DNA is replicated during DNA synthesis in mung bean, it would be easily detected by filter hybridization assays performed as follows: Highly labelled DNA from leaves, stems, cotyledons and roots from treated and untreated plants were hybridized at 46.2° (20° below Tm in 5 X SSC - 50% formamide) to A. tumefaciens DNA immobilized on nitrocellulose filters. Included in the hybridization solution were filters containing B. subtilis DNA and blank filters, both of which would detect non-homologous and non-specific bindings. The limits of detecting A. tumefaciens DNA were estimated by generating a calibration curve from measured amounts of the bacterial DNA. From the calibration plot, which took into account the input cpm and specific radioactivities of each DNA preparation, the limit of detection ranged between 0.005% and 0.01% apparent homology. This and the results of the hybridization assay of the DNA samples are summarized in Table 1. The data show the absence of radioactive A. tumefaciens DNA sequences in the DNA-treated plants with the possible exception of leaf DNA of these plants. This possibility was examined further by DNA sequence enrichment and characterization of the enriched DNA.

Radioactive leaf DNA from DNA-treated and untreated plants was allowed to reanneal under conditions that permitted enrichment of bacterial DNA sequences, i.e., in the presence of excess unlabelled A. tumefaciens DNA set at Cot = 200 for the bacterial DNA (see Materials and Methods). These conditions permitted 95% of the total bacterial DNA to renature whereas 11.7% of the total

Table 1. DNA·DNA filter hybridization of mung bean (P. aureus) DNA derived from A. tumefaciens DNA-treated and untreated plants

Plant part	Specific radioactivity (cpm/μg) DNA-treated	control	Input cpm used DNA-treated	control	Cpm hybridized per μg A. tumefaciens DNA[1] DNA-treated	control	Apparent homology[2] (%)
Cotyledons	44,114	70,972	70,930	85,490	0	0	0.000
Leaves	71,912	45,408	171,710	198,110	12.8	6.3	0.024
Roots	264,906	201,245	59,080	148,660	11.4	7.3	0.003
Stems	194,221	133,448	231,530	231,630	0	22.9	0.000

[1]Represents cpm remaining after nonspecific and non-homologous backgrounds were subtracted. Results of hybridization using the input cpm indicated at 46.2° for 24 hrs in 5 X SSC in 50% formamide.

[2]Under the same conditions of hybridization, 0.1 μg of A. tumefaciens [^{3}H]-DNA (sp. act. = 98,650 cpm/μg) yielded 2,047 cpm bound per μg unlabelled A. tumefaciens DNA. Based on these values and assuming equivalent synthesis of the bacterial putative DNA, the lower limits of detection in % apparent homology were 0.01% for cotyledon DNA, 0.005% for leaf DNA, 0.01% for root DNA and less than 0.01% for stem DNA.

leaf DNA from untreated plants renatured and similarly 9.7% of the total leaf DNA from DNA-treated plants renatured.

The properties of these double-stranded DNAs that resulted from solution enrichment were examined by thermochromatography on hydroxylapatite. Fig. 1A shows the derivative melting curves of these renatured DNA preparations. It can be seen that the melting curve of the enriched DNA from treated plants was virtually identical to that from untreated plants, and that the two curves were very distinct from that of the reassociated A. tumefaciens DNA. In Fig. 1B, the two reassociated DNAs have close Tm values (± 1°; with the Tm of the reassociated DNA from DNA-treated plants showing the lowest Tm) and possess no discontinuities in the region expected if A. tumefaciens DNA sequences were present.

Discussion

Stroun et al. (7) and Anker and Stroun (8) proposed that A. tumefaciens

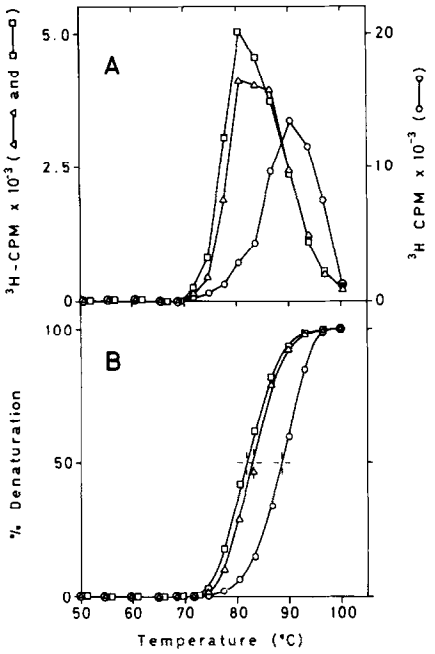

Fig. 1. Derivative and cummulative melting plots. (A) Derivative melting on hydroxylapatite in 0.08 M Na phosphate of [^{3}H]-DNA from A. tumefaciens (○——○); [^{3}H]-DNA from leaves of A. tumefaciens DNA-treated plants (□ — □) and [^{3}H]-DNA from leaves of control plants (△ —△). These DNA samples contained rapidly renatured DNA in the presence of excess unlabelled A. tumefaciens DNA (see text). (B) Cummulative melting curves (normalized to %) based on the radioactivity values shown above. Tm of A. tumefaciens DNA (○——○) renatured to Cot = 200 was 89.5° and that of rapidly renatured P. aureus DNAs was 82° for DNA-treated plants (□ — □) and 83° for untreated plants (△ —△).

DNA migrates into cell nuclei and replicates along with recipient plant nuclear DNA. In spite of the close adherence to optimal conditions conducive for foreign DNA replication in plants reported by them, our experiments showed that no A. tumefaciens DNA was replicated in mung beans that were pretreated with A. tumefaciens DNA. Thus, it is clearly evident that these findings do not support the previously reported observation of Stroun et al. (7) and Anker and Stroun (8). Our experiments employed filter hybridization and solution enrichment of DNA, techniques which we showed were extraordinarily sufficient to detect, at the least, 0.1% A. tumefaciens DNA in total plant DNA. This degree of sensitivity was more than adequate to detect that amount of bacterial DNA reported by Stroun et al. (7) to shift the buoyant densities of host nuclear DNA in CsCl density gradients to a position between the bacterial donor DNA and

the recipient plant DNA, i.e., in order to have such density shifts, at least 30% of the recipient plant DNA has to be bacterial DNA (23). From published data (7) it can be estimated (24) that the CsCl buoyant density of the replicated "hybrid" DNA molecules corresponded to DNA species composed of 50% plant DNA and 50% A. tumefaciens DNA. Hence, our analyses, which were 3,000-6,000-fold more sensitive than density-shift analysis, would have clearly detected the proported 50% foreign bacterial donor DNA in plant DNA.

Although our experiments employed P. aureus instead of a solanaceous host such as tomato or tobacco, we have recently obtained identical results with tobacco (Kado and Lurquin, in preparation), Arabidopsis (Lurquin, unpublished) and similar results have been obtained with tomato in another laboratory (Kleinhofs, personal communication).

Apparent uptake, integration, or replication of foreign bacterial DNA also have been reported from the laboratory of L. Ledoux in a wide variety of plants such as barley (Hordeum) (3, 25), broadbean (Vicia faba) (26), Arabidopsis thaliana (3), Synapis alba (27) and tomato (Lycopersicum esculentum) (28). All of these reports also have been based on buoyant densities shifts in CsCl density gradients alone. Although it would be of interest to re-examine each of these plants, the DNA "hybrid" band apparently observed in these density gradients may be due to bacterial contamination or artifacts due to the crude DNA used (Kleinhofs et al., in preparation).

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