A Method for the Transfer of Tumorigenicity Between Strains of Agrobacterium tumefaciens in Carrot Root Disks

C. H. Liao and G. T. Heberlein

Research Associate and Professor, respectively, Department of Biological Sciences, Bowling Green State University, Bowling Green, OH 43403. This investigation was supported by Public Health Service Research Grant Cal4516 and Ca20046 from the National Cancer Institute awarded to G. T. Heberlein.

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ABSTRACT

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Surface-sterilized carrot root disks were inoculated concurrently with 0.1 ml each of a virulent (donor) and an avirulent (recipient) strain of *Agrobacterium tumefaciens* cultures (1.2×10^9 colony-forming-units/ml). After 4-7 wk of contact between donor and recipient bacteria, two or three of 20 recipient colonies reisolated from tumor tissues became virulent. Transfer of tumorigenicity was not observed with recipient colonies reisolated from nontumor portions of tissue.

Kerr (7) demonstrated that a saprophytic species of *Agrobacterium*, after mixed infection with a pathogenic strain of *A. tumefaciens* in tomato plants, could be converted to a virulent derivative. He suggested that such conversion resulted from a genetic exchange between virulent and avirulent bacteria in the plant tissue. Similar observations of virulence transfer in planta were later confirmed in several other laboratories (4, 10, 11). The tomato plant now represents the only plant species which serves as the host environment for the transfer of bacterial virulence. We report here that the transmission of tumorigenicity also can be carried out in carrot root disks under aseptic conditions.

MATERIALS AND METHODS

The bacterial strains used were *A. tumefaciens* TT-133-1 and *A. tumefaciens* 38-7-5, which can be distinguished from each other by a number of characters (4). The virulent strain TT-133-1 (designated as donor) fails to grow in Stonier's medium (8), produces no ketolactosides in lactose agar medium (1), and tolerates 30 μg/ml of kanamycin sulfate. The avirulent strain 38-7-5 (designated as recipient), on the contrary, grows in Stonier's medium, produces ketolactosides in lactose agar medium, and tolerates 30 μg/ml of polymyxin B sulfate. Both strains were maintained at 4 C in nutrient agar containing Difco nutrient agar 2.3%, sucrose 0.5%, yeast extract 0.1%, and 30 μg/ml of the antibiotic listed. For the inoculation of carrot root disks, bacteria were grown in nutrient broth containing Difco nutrient broth 0.8%, sucrose 0.5%, and yeast extract 0.1% at 28C with shaking. Carrot roots were surface-sterilized by submerging them in 20% solution of Clorox (sodium hypochlorite, 5.25%) for 10 min, then rinsing twice in sterile distilled water. A sterile cork borer 1 cm in diameter was used to obtain a longitudinal cylinder of carrot tissue. A 2-cm portion from the top and bottom of each cylinder was discarded and the rest was cross-sectioned into 0.5 cm-thick disks. Five disks were transferred aseptically to a petri plate (100 × 20 mm) containing 40 ml of 1.5% water agar and placed with the surface proximal to the root apex in contact with the agar surface.

In the first experiment, a total of 10 petri plates containing 50 carrot root disks were inoculated by applying 0.1 ml of the donor (TT-133-1) culture (at 1.2×10^9 colony-forming=units/ml) over the surface of each disk. The recipient (38-7-5) culture was applied in the same manner 1 wk later. After a given period of contact between donor and recipient bacteria, ranging from 3 to 7 wk, recipients were reisolated weekly from the tumor or nontumor tissue. To do this, a piece of tumor or nontumor tissue (0.3 × 0.3 × 0.2 cm) was removed from one carrot root disk and ground in 9 ml of 0.9% saline in a sterile mortar. One-tenth ml portions of appropriate dilutions were plated on a selective medium containing nutrient agar plus 30 μg/ml of polymyxin B sulfate. From each sample, 20 recipient colonies were randomly selected and cloned three times before their pathogenicity was assayed.

RESULTS AND DISCUSSION

None of recipient colonies reisolated from nontumor portions of tissue was tumorigenic, whereas two or three of 20 recipients reisolated from tumor portions were virulent (Table1). We thought that the transfer of tumorigenicity to recipient bacteria might occur only in the tumor tissue. In the second experiment, the recipient was inoculated onto tumor tissue of various ages (2-6 wk) in the same manner as described for the first experiment. After 4 wk of contact between recipient and donor bacteria (or tumor tissue), 20 recipient colonies were reisolated from the tumor tissue of various ages. Two to four of 20 recipient colonies reisolated from tumor tissue of a given age were virulent (Table 2).

Twenty surface-sterilized carrot root disks which

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TABLE 1. Tumorigenicity transfer between *Agrobacterium tumefaciens* strain TT-133-1 (donor) and strain 38-7-5 (recipient) at various periods of contact in carrot tissue

	Duration of contact in carrot tissue (wk)									
	3		4		5		6		7	
Recipient colonies	T[a]	NT[a]	T	NT	T	NT	T	NT	T	NT
No. isolated	20	20	20	20	20	20	20	20	20	20
No. inducing tumors in tomato plants	0	0	2	0	3	0	2	0	3	0
No. inducing tumors in carrot root disks	0	0	2	0	3	0	2	0	3	0

[a] Portion of carrot tissue used for isolation; T = tumor, and NT = nontumor.

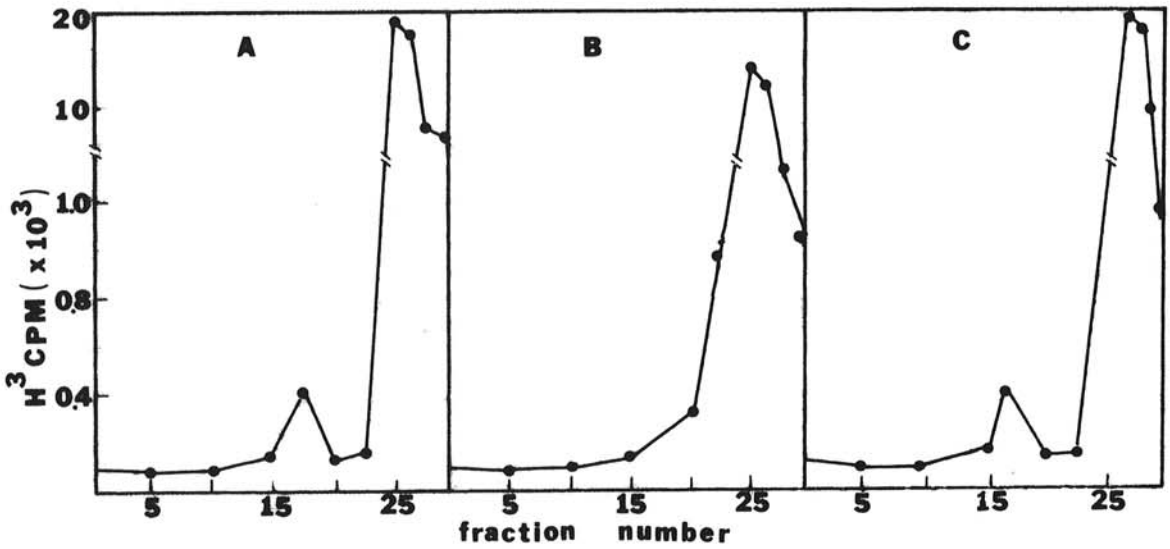

Fig. 1-(A to C). Alkaline sucrose gradient profiles of ^{3}H DNA from virulent, avirulent, and virulence-restored recipient strains of *Agrobacterium tumefaciens*. The method of Watson et al. (J. Bacteriol. 123:255-264) was used for the labeling, extraction, and separation of ^{3}H DNA. **A**) strain TT-133-1, **B**) strain 38-7-5, and **C**) strain 38-7-5 (TT VP).

TABLE 2. Transfer of tumorigenicity to the recipient strain of *Agrobacterium tumefaciens* after 4 wk of contact with carrot tumor tissue of various ages.

	Age of tumor tissue (wk)[a]				
Recipient colonies	2	3	4	5	6
No. isolated	20	20	20	20	20
No. inducing tumors in tomato plants	4	3	2	4	3
No. inducing tumors in carrot root disks	4	3	2	4	3

[a]Measured from time of inoculation.

received nutrient broth or recipient bacteria alone were kept at room temperature for 2 mo and used as controls. Forty colonies reisolated from carrot tissue which previously had been inoculated with recipient bacteria alone remained avirulent. Twenty surface-sterilized carrot root disks which had received nutrient broth were ground in 250 ml of fresh nutrient broth in a sterile blender and a 0.1-ml sample was removed and plated on nutrient agar. No bacterial or fungal growth was observed 5 days after the incubation.

All tumorigenic recipient isolates grew in Stonier's medium, produced ketolactosides, and were not resistant to 30 μg/ml kanamycin sulfate. They agglutinated with antisera against the strain 38-7-5, but not with antisera against the strain TT-133-1. These data indicate that tumorigenic recipient isolates obtained from the above two experiments cannot be the result of: (i) contamination with the donor strain TT-133-1, (ii) spontaneous mutation of the recipient strain 38-7-5, or (iii) outside contamination with other virulent agrobacteria.

The transfer of tumorigenicity between strains of *A. tumefaciens* in tomato plants (4, 10, 11) or in vitro (2, 3, 6, 9) has been shown to be associated with the transfer of a large plasmid. We labeled DNA of strain TT-133-1, strain 38-7-5, and virulence-restored strain 38-7-5 (38-7-5, TT VP) with ^{3}H and extracted ^{3}H DNA with the method of Watson et al. (11). The ^{3}H-DNA profiles of these three strains are compared in Fig. 1. The DNA of virulent strain

TT-133-1 contains a chromosome and a dense satellite peak. The dense satellite peak also is observed in DNA of the virulence-restored strain 38-7-5, but not in DNA of the avirulent strain 38-7-5. In addition, we found that one of the 38-7-5 (TT VP) isolates lost its tumorigenicity under conditions (5) which promoted the loss of the plasmid. These data suggest that the transfer of tumorigenicity between strains of agrobacteria in carrot root disks also is due to the incorporation of plasmid genes rather than a chromosome mutation or subsequent activation of episomes, although the activation of tumor-inducing chromosome genes by the presence of a plasmid is still a possiblity.

The precise mechanism of the transfer of tumorigenicity to recipient bacteria in planta has not been elucidated. Although we thought that the transfer of tumorigenicity occurs only in the tumor tissue, it is not clear whether oncogenic genes are transmitted directly from virulent bacteria or indirectly via a plant tumor cell. Since carrot root disk assays can be performed under aseptic conditions in a relatively short time, and occupy less laboratory space, the use of this system offers a number of advantages over the tomato plant system (7) for the future study of host-parasite genetic interactions.

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