Tumor Initiation Complementation on Bean Leaves by Mixtures of Tumorigenic and Nontumorigenic Agrobacterium rhizogenes

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ABSTRACT

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The enhancement (as much as 50-fold) of tumor initiation (complementation) on bean leaves shown by certain mixtures of nontumorigenic and tumorigenic agrobacteria was investigated with tumorigenic strain ATCC 15834 and nontumorigenic strain TR7 of *Agrobacterium rhizogenes*. When either strain was inoculated with wounding at different times after the other, maximum tumor initiation was observed when the second strain was added 3 hr after the first. A 6-hr interval between inoculation of the two bacteria resulted in 40 to 60% as many tumors as obtained with a 3-hr interval but no enhancement occurred when the two strains were inoculated 24 hr apart. The optimum ratio of TR7 to ATCC 15834 for tumor initiation varied with the concentration of bacteria in the inoculum; the higher the concentration, the greater the proportion of TR7 necessary for maximum tumor initiation. The contribution of each strain to this effect when inoculated second was reduced by heat inactivated bacteria, suggesting that both strains undergo site attachment in order to participate in the complmentation process. New tumors were detected up to day 8 or 9 on leaves inoculated with two bacteria. This was similar to results obtained with virulent prototrophic strains of *Agrobacterium*, although both TR7 and ATCC 15834 are auxotrophs and the tumors initiated by ATCC 15834 alone, like those by other auxotrophs, are detected within 5 days after inoculation. A model consistent with these data, where both bacteria undergo site attachment independently at different wound sites, allowing transfer between sites of a product which is limiting for tumor initiation at one site, is suggested to account for these effects.

Certain pairs of *Agrobacterium* strains inoculated together initiate many more tumors on bean leaves than the total number of tumors obtained when each is inoculated separately (12). Based on the following criteria, these agrobacteria were designated as donors or receptors. All donor strains initiated tumors when inoculated separately, but failed to show synergistic effects on tumor initiation when inoculated together. Thus, their efficiency in establishing infection or "specific virulence" was not modified by the presence of a second tumorigenic strain. Most tumorigenic strains appear to belong to this category. Receptor strains, with one exception (a strain of very low specific virulence), failed to induce bean leaf tumors when inoculated separately and mixtures of two receptor strains inoculated on bean leaves were no more effective than when inoculated separately. When each of these nontumorigenic receptor strains was inoculated with a donor strain, however, the number of tumors obtained was much greater than that obtained with the donor strain alone. It was proposed that this increase in tumor initiation (complementation) resulted from a product either of the donor strain or of its interaction with a plant cell which permitted the receptor strain to initiate tumors.

Regardless of the actual nature of the complementation effects, the enhancement implies that at least two bacterial genes or gene products may be involved in the tumor initiation process. Characterization of this complementation thus may provide information about the nature, number, and sequence of events in crown-gall tumor initiation. This paper reports further evidence based on more detailed studies of a particular pair of complementing strains, that supports the proposed relation between donor and receptor strains. Complementation is shown to occur only during an early period in the tumor initiation process and to require site attachment by both donor and receptor strains. The ratio of the two bacteria in the inoculum which is optimum for tumor initiation is shown to vary with the absolute concentration of the inoculum.

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MATERIALS AND METHODS

Bacterial cultures.—*Agrobacterium rhizogenes* (strains ATCC 15834 and TR7) and *Agrobacterium tumefaciens* (strains B6 and IIBNV6) were grown to stationary phase (48 hr) in a nutrient-broth/yeast-extract medium, as previously described (12). Viable cell counts were determined by serial dilution and plating on nutrient-broth/yeast-extract medium containing 1.5% agar.

Preparation of bacteria for infectivity tests.—Bacterial cultures were centrifuged, washed, and resuspended in 0.1 M phosphate buffer, pH 7.0 (5). After being washed, the bacteria were kept in an ice-water bath for 0.1 to 3 hr until inoculation. The difference in time of storage at this temperature had no apparent effect on the results. Mixed inocula were prepared by combining equal volumes of the two strains in phosphate buffer a few minutes before

inoculation. For some experiments, bacteria were inactivated by heating for 20 min at 60 C (5).

Infectivity determinations.—Tumor initiating ability was determined by inoculating the samples on 7-day-old primary leaves of *Phaseolus vulgaris* L. 'Pinto' germinated and maintained under ambient greenhouse conditions as described by Lippincott and Heberlein (8). Each sample was inoculated by applying 0.1 ml of bacteria to each of 14 to 16 primary leaves with Carborundum wounding and the results reported are the mean number of tumors [± standard error (SE)] determined 7 days after inoculation. For experiments in which the two strains were added separately to the same leaves, the first strain was inoculated in the usual manner, the leaves were allowed to dry approximately 15 min, and the second strain was then applied by lightly spreading the bacterial suspension over the leaf with a glass rod, avoiding additional wounding. In other experiments of this type, where fresh wounds were desired for both strains, each strain was inoculated with addition of Carborundum in the usual manner. Tumor appearance rates were determined by daily tumor counts (9).

TABLE 1. Tumor initiation complementation between *Agrobacterium rhizogenes* strains ATCC 15834 and TR7 when applied to bean leaves in mixed or separate inocula

Strains and order of inoculation[a]		
First application	Applications app. 15 minutes later	No. of tumors/leaf Mean ± SE
15834	None	7.2 ± 1.5
TR7	None	0
15834 + TR7	None	38.6 ± 7.2
15834	TR7	40.4 ± 7.6
TR7	15834	58.8 ± 10.7
15834 + TR7	None	17.8 ± 3.7
15834	TR7	8.4 ± 1.9
TR7	15834	18.6 ± 6.6

[a]Viable cells per millilter of inocula: 15834 = 36×10^8; TR7 = 15×10^8 in the first five samples; in the last three samples, 15834 = 7×10^8; TR7 = 3×10^8.

RESULTS

Strains ATCC 15834 and TR7 of *A. rhizogenes* were selected for further study of tumor initiation complementation because this combination consistently gave a large increase in number of tumors and the low infectivity of ATCC 15834 permitted a wide variation in concentration of donor without the problem of a large number of tumors in the controls. Complementation occurs when the two strains are applied separately and the number of tumors initiated is typically as great or greater than when the strains were mixed prior to inoculation (Table 1). The tumors showed no morphological differences from those induced by ATCC 15834 alone or by virulent strains of *A. tumefaciens*. More tumors consistently were obtained when strain TR7 was applied first than when strain ATCC 15834 preceded TR7.

When the two bacteria were applied 3 hr apart (Experiment 1, Table 2), less than a third as many tumors were obtained, regardless of the order of addition. Only about 5% of the maximum number of tumors were formed when 6 hr or more elapsed between the addition of the two strains. As previously, application of strain TR7 first resulted in the greater number of tumors. Significant differences were not observed in the loss of the complementation effect with increasing time between application of the bacteria in the two orders of addition.

Because wound healing and/or drying can limit the effectiveness of the inoculum (7), similar experiments were conducted in which the second strain also was inoculated with Carborundum to provide a second set of

TABLE 2. Effect of separate application of *Agrobacterium rhizogenes* strains ATCC 15834 and TR7 at different times on tumor initiation complementation

Hours from addition of first strain to addition of second strain	Mean no. of tumors per leaf ± SE[a]		Ratio of tumors obtained by two modes of addition
	Strain 15834 first Strain TR7 second	Strain TR7 first Strain 15834 second	
Experiment 1: first strain applied with Carborundum wounding[b]			
0.2	37.1 ± 4.1 (100)	51.3 ± 6.0 (100)	1.4
3	6.9 ± 0.9 (19)	14.4 ± 2.9 (28)	2.1
6	2.4 ± 0.8 (6)	2.6 ± 0.9 (5)	1.1
24	1.4 ± 0.4	0.6 ± 0.3	
48	1.1 ± 0.4	0.0	
Experiment 2: each strain applied with Carborundum wounding[c]			
0.2	16.8 ± 5.1 (53)	37.1 ± 6.8 (70)	2.2
3	31.5 ± 5.7 (100)	52.9 ± 8.9 (100)	1.7
6	17.7 ± 3.5 (56)	18.2 ± 4.1 (34)	1.0
24	2.1 ± 0.6 (6)	0.6 ± 0.2 (1)	0.3
48	1.1 ± 0.3	0.8 ± 0.3	

[a]Tumor number expressed as percent maximum in parentheses.

[b]Strain 15834 inoculated at 3.5×10^9 viable cells/ml; strain TR7 at 3.6×10^9 viable cells/ml. Strain 15834 inoculated singly produced 1.0 ± 0.2 tumors/leaf; strain TR7 produced no tumors.

[c]Strain 15834 inoculated at 1.2×10^9 viable cells/ml; strain TR7 at 4.4×10^9 viable cells/ml. Strain 15834 inoculated singly produced 0.6 ± 0.2 tumors/leaf; strain TR7 produced no tumors.

wounds. This modification results in about a 3-hr shift in the time of optimal response, regardless of the order of addition (Experiment 2, Table 2). Application of strain TR7 first produced more tumors but, as shown by the ratio of tumors obtained by the receiprocal treatments, this difference was large at 0.2 hr, small at 3 hr, and there was no difference at 6 hr. Thus, in the absence of a second strain, the ability of strain TR7 inoculated leaves to show enhancement decreases more rapidly than that of strain ATCC 15834 inoculated leaves. A 6-hr period between inoculation of each strain resulted in fewer tumors than when the two were applied 3 hr apart and essentially no complementation was observed when they were applied 24 hr apart. These results are consistent with other estimates of the time of tumor initiation on bean leaves (9, 10).

At low and nearly constant total cell number the optimum ratio of the two bacteria for tumor initiation was about 5:1 (receptor: donor) (Fig. 1), based on either the absolute number of tumors initiated or the number of tumors per 10^8 bacteria per ml inoculum. A tenfold increase in tumor number was obtained at a total bacterial concentration of only 1.6×10^8 cells per ml. This is well within the linear range of the bioassay with highly virulent strain B6 (4, 8) where the assay follows the hypothesis of independent action, and higher bacterial concentrations are required before detectable competition for the same wound sites is observed (5).

When several different ratios of the bacteria at three concentrations were applied in the same experiment the number of tumors initiated was not directly related either to the absolute number of bacteria in the inoculum or to a particular ratio of the two bacteria (Table 3). Rather, the most effective ratio of the two strains varied with the total concentration of bacteria. At 6×10^8 cells per ml, a 1:5 ratio of receptor to donor produced the most tumors, but at 6×10^9 cells per ml a 5:1 ratio was most effective.

Two mixtures of strain TR7 with ATCC 15834, one approximating a 1:1 ratio and the second a 1:5 ratio, were inoculated at several different dilutions (Table 4). At the lower concentrations, the inocula with the higher proportion of donor strain induced the most tumors, but at higher bacterial concentrations, inocula with the lower proportion of the donor strain induced more tumors. Enhanced tumor formation results, therefore, from an interaction between strains in which the ratio of donor to receptor bacteria for optimum tumor initiation decreases as the total concentration of bacteria in the inoculum increases.

Complementary tumor initiation was reduced when either TR7 or ATCC 15834 was inoculated first and the second strain added with nonviable bacteria which compete for attachment sites (Table 5). Apparently, for complementation to occur, both strains must attach to host wound sites.

The time of tumor appearance on leaves inoculated with a mixture of ATCC 15834 and TR7 was compared with that of leaves inoculated with only ATCC 15834 or with strain B6 (Fig. 2). All tumors initiated by strain ATCC 15834 were detectable within 5 days after inoculation, as is typical of the pattern of tumor appearance obtained with auxotrophic strains of *Agrobacterium* (4, 11). The pattern of tumor appearance

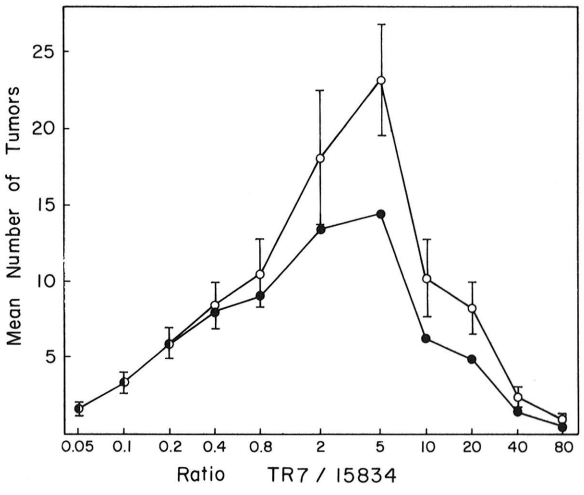

Fig. 1. Effectiveness of mixtures of *Agrobacterium rhizogenes* strains TR7 (nontumorigenic) and 15834 (tumorigenic) in different proportions in inocula used to initiate bean leaf tumors. Open circles, mean number of tumors per leaf; solid circles, mean number of tumors per leaf per 10^8 viable cells in the inoculum. The ratios on the abscissa are on a log scale, the concentration of strain TR7 varied from left to right on the abscissa from 0.44 to 17.6×10^7 viable cells/ml and for strain 15834 from 8.8 to 0.22×10^7 viable cells/ml.

TABLE 3. Dependence of tumor initiation complementation at different total cell concentrations on the ratio of *Agrobacterium rhizogenes* strains ATCC 15834 and TR7

Ratio TR7 : 15834	Mean no. of tumors per leaf ± SE[a]		
	6×10^8 cells/ml	2×10^9 cells/ml	6×10^9 cells/ml
20:1	8.2 ± 2.1	23.6 ± 4.6	16.7 ± 3.9
5:1	27.6 ± 6.6	40.2 ± 8.0	52.9 ± 6.3
1:1	21.6 ± 6.4	51.6 ± 8.1	48.4 ± 7.2
1:5	32.8 ± 8.0	18.8 ± 4.1	33.9 ± 6.2
1:20	8.1 ± 1.4	12.5 ± 1.2	12.6 ± 2.1

[a]The two cultures were adjusted to contain equal numbers of viable cells and the various mixtures prepared so that the total number of cells was constant in each of the three series at the value indicated.

TABLE 4. Titration of tumor initiation by mixtures of *Agrobacterium rhizogenes* strains TR7 and ATCC 15834 which differ 5-fold in the proportion of ATCC 15834

Relative concentration[a]	Mean no. of tumors per leaf ± SE[b]	
	15834:TR7 = 0.9	15834:TR7 = 4.5
1.0	67.1 ± 8.6	53.6 ± 7.4
0.3	116.1 ± 14.6	65.7 ± 12.3
0.1	52.9 ± 6.2	51.2 ± 7.5
0.03	17.1 ± 2.7	42.3 ± 7.7
0.01	5.9 ± 1.0	10.2 ± 2.0

[a]Concentration of TR7 = 2.2×10^9 in each mixture at a value of 1; concentration of ATCC 15834 = 2×10^9 and 10^{10}, respectively, at ratios of 0.9 and 4.5.

[b]Strain ATCC 15834 inoculated separately at 2×10^{10} cells per ml initiated 1.4 ± 0.2 tumors per leaf.

TABLE 5. Inhibition of tumor initiation complementation by challenging either *Agrobacterium rhizogenes* strain TR7 or ATCC 15834 with heat-inactivated bacteria

Strains and order of inoculation[a]		No. of heat-inactivated cells/ml ($\times 10^8$)	No. of tumors/leaf mean ± SE
First application	Applications app. 15 min later		
Experiment 1:			
15834	None		2.4 ± 0.7
15834	TR7		10.6 ± 2.4
15834	TR7 + TR7	22	5.4 ± 0.9
15834	TR7 + IIBNV6	800	4.0 ± 0.6
Experiment 2:			
15834	None		0.4 ± 0.2
15834	TR7		13.1 ± 4.4
15834	TR7 + TR7	26	8.3 ± 1.4
Experiment 3:			
TR7	15834		6.6 ± 1.1
TR7	15834 + 15834	1.3	5.6 ± 0.6
TR7	15834 + 15834	2.7	5.2 ± 0.8
TR7	15834 + 15834	13	4.2 ± 0.7
TR7	15834 + 15834	27	2.0 ± 0.5
TR7	15834 + 15834	130	0.4 ± 0.2

[a]Underlined strains heat inactivated 20 min at 60 C. Concentrations (viable cells/ml): Expt. 1, 15834 = 85×10^8; TR7 = 4.5×10^8; Expt. 2, 15834 = 7.4×10^8; TR7 = 10.4×10^8; Expt. 3, TR7 = 4×10^8; 15834 = 2.7×10^8.

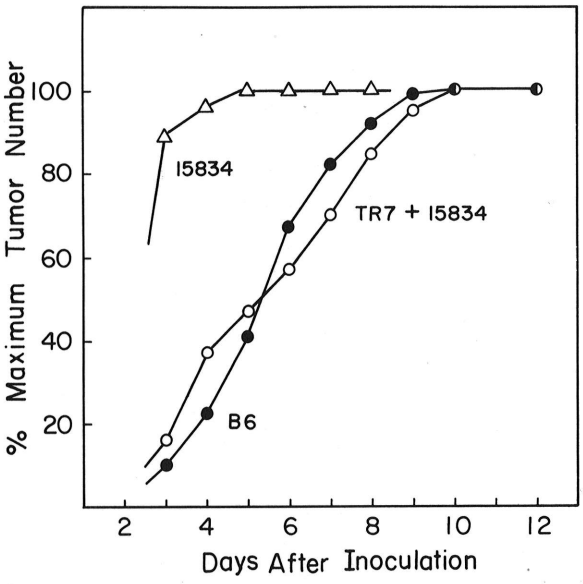

Fig. 2. Rate of tumor appearance in bean leaves inoculated with strain B6 of *Agrobacterium tumefaciens*, with *A. rhizogenes* strain 15834 (tumorigenic), or with a mixed inoculum of *A. rhizogenes* strains TR7 (nontumorigenic) and 15834. Data plotted are percentages of the mean number of tumors at day 12. The values for 100% were: strain 15834 = 4.8 ± 0.9; strain B6 = 46.7 ± 5.7; and strains TR7 + 15834 = 61.9 ± 9.2. Inocula concentrations (viable cells/ml): strain B6 = 2.3×10^8; strain 15834 = 3.1×10^9; and strains TR7 + 15834 = $3.8 \times 10^9 + 3.1 \times 10^9$, respectively.

obtained with the mixture of TR7 plus ATCC 15834, however, is similar to that observed with prototrophic strains, such as B6 where tumors continue to appear as late as day 8 to 10 after inoculation (11). That portion of the tumor initiation process sensitive to bacterial nutritional defects is apparently circumvented by the complementing strains, as both TR7 and ATCC 15834 are auxotrophic and have identical requirements for glutamate and biotin (11, 15). Tumor initiation was not promoted, however, by hourly applications (12 times) of these substances (glutamate, 1 mg/ml; biotin, 10 μg/ml) by wetting the uppersurface of leaves inoculated with either TR7 or ATCC 15834 with a ball of cotton soaked in the nutrient solution (4, 11). Similar treatment of leaves inoculated with other auxotrophs promotes tumor initiation (4, 11).

DISCUSSION

Strain TR7 utilizes octopine (6) and is tumorigenic on carrot root disks (11), two traits determined in *Agrobacterium* by a large virulence plasmid (14, 16). Strain TR7 carries a large covalently closed circular DNA plasmid (2) which suggests that TR7 should initiate tumors on bean leaves. Some feature of the wound environment on these leaves apparently limits the ability of this strain to initiate tumors and this limitation cannot be overcome by treatment with plant hormones (12) or by the nutritional factors which are growth-limiting for this strain. When strain TR7 is inoculated on bean leaves with highly tumorigenic agrobacteria (12), or with a weakly tumorigenic strain, as shown here, many more tumors are initiated than when the tumorigenic strains are inoculated separately.

The possibility that direct interaction between the two complementing strains of bacteria may account for these results appears improbable for the following reasons. Complementation was observed when the two strains were applied as much as 6 hr apart and was greatly increased at this time when the second strain was applied with formation of new wounds. There was no single ratio of the two strains which was optimal for tumor formation, as would be expected if there were direct interaction, and complementation occurred at bacterial concentrations at which the number of bacteria reaching wound attachment sites was noncompetitive (5). In experiments in which each complementing strain was independently challenged by heat-killed site-competing bacteria, the number of tumors was reduced. Thus, site attachment by both strains is apparently essential for complementation. Because the tumor initiation process follows the hypothesis of independent action (8) and the concentration of bacteria in many of these experiments was below the level where site competition is observed (5), these sites are apparently in separate wounds.

Although strain TR7 has not been isolated from inoculated leaves to determine if a transfer of tumor-initiating ability has occurred, studies of that kind have been made with another nontumorigenic receptor strain (IIBNV6) and no change in virulence was detected in 900 colonies tested (Lippincott and Lippincott, *unpublished*). Also, even though strain NT1 (a plasmid-free strain

obtained from tumorigenic strain C58) regains virulence when the plasmid is restored in a host plant (1), it fails to complement (12). Apparent plasmid transfer in host plants as shown by acquisition of virulence by nonpathogenic agrobacteria is not detected until weeks after inoculation of virulent and avirulent bacteria (3), well after tumor initiation is complete on bean leaves (9). Plasmid transfer between bacteria, therefore, does not appear to be a feasible mechanism to account for the complementation phenomenon observed on bean leaves.

In a previous study (12) the receptor strains were proposed to be capable of initiating tumors when supplied by a factor produced or induced by virulent strains. The data presented here are consistent with this model and show that this apparently occurs by a transfer of one or more products between bacteria or bacterium-host complexes at separate wound sites. The change in ratio of the two strains which is necessary for maximum complementation as the total concentration of bacteria is varied suggests that there is some limiting distance over which this promotion can take place. The higher the bacterial concentration, the greater was the proportion of TR7 in the inoculum necessary for maximum effect, as would be expected if TR7 were inducing tumors. A constant increase in absolute number of tumors was obtained when strain TR7 was inoculated with several different tumorigenic strains (12), the latter at different total cell concentrations and inducing different numbers of tumors when inoculated separately at the same concentration. This proportional relation between increase in number of tumors and TR7 concentration suggested that TR7 is capable of tumor initiation on bean leaves when mixed inocula are used and more tumors were consistently produced when TR7 was applied before ATCC 15834. Although the current data cannot rule out the opposite alternative, promotion of ATCC 15834 tumor initiation by TR7, in either case the latter strain apparently contributes one or more products essential for tumor initiation which it cannot make in the absence of ATCC 15834 or which is limiting for tumor initiation by ATCC 15834.

Evidence from experiments in which the two complementing strains were inoculated separately, each with wounding, indicates that both strains are maximally effective when inoculated about 3 hr after each other. The actual time at which the critical interaction is maximal, therefore, would appear to require at least a 3-hr "adjustment" on the part of each strain and thus when host plants are inoculated with mixtures of the bacteria the reactions which account for enhancement would be maximal between 3 and 6 hr after inoculation. The rate at which leaves inoculated with strain TR7 lose their ability to enhance tumor number in the absence of strain ATCC 15834 is somewhat faster than leaves inoculated with strain ATCC 15834 when both strains are inoculated with wounding. Because the difference is not great, the interacting components in this enhancement must be closely correlated in time to be effective.

The time of appearance of tumors initiated by mixtures of ATCC 15834 and TR7 suggests that the complementation effect circumvents nutritional restrictions which limit tumor initiation by auxotrophs to large wounds only (4, 11). Thus, the bacterial nutrition phase necessary for crown-gall initiation (13) may be critical for the synthesis of only one of the products which is essential for tumor initiation and responsible for these complementation effects. This relationship would be consistent with the hypothesis that the complementation factor is either a precursor of the tumor-inducing principle (TIP), a cocarcinogen, or an essential element of the transfer and/or functioning of the TIP. In all but the first case, an additional component(s) essential for tumor induction must either preexist or require only limited metabolism for its formation in the bacteria which induce tumors as a result of the complementation. Resolution of these possibilities, the nature of the complementation product(s) and its relation to the *Agrobacterium* virulence plasmid will be essential to a description of the crown-gall initiation process.

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