Inhibitory Effects of a Pectin-Enriched Tomato Cell Wall Fraction on Agrobacterium tumefaciens Binding and Tumor Formation

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ABSTRACT

A pectin-enriched soluble cell wall fraction (CWF) prepared from suspension cultured tomato cells inhibits binding of Agrobacterium tumefaciens to these cells. It was hypothesized that the CWF contains the plant surface binding site for A. tumefaciens (NT Neff, AN Binns 1985 Plant Physiol 77: 35–42). Experiments described here demonstrate that tomato CWF inhibited tumor formation on potato slices and Agrobacterium binding to intact tomato cells in a dose-dependent fashion. Boiling the fraction reduced both its binding and tumor inhibitory activities. Tumor inhibitory activity was titrated out by increased concentrations of bacterial inocula with no inhibition apparent at 1 × 10⁶ bacteria per milliliter. These results indicate that a tomato CWF is enriched for a putative A. tumefaciens binding site which may also be involved in tumor formation in potato.

The interaction of virulent strains of Agrobacterium tumefaciens with a wide variety of dicotyledonous plants results in proliferation of host tissue to yield tumors. These tissues are distinguished from their normal counterparts by the presence of a portion of the bacterial Ti plasmid DNA (the T-DNA), which becomes integrated into the plant genome. The presence and expression of these bacterial genes result in altered host cell properties that include the production of one or more novel (to the plant) metabolites called opines and the ability to grow in culture without exogenously added hormones (for reviews see Refs. 2 and 5).

Although an understanding of the sequence of A. tumefaciens/plant cell interactions prior to the integration and expression of the T-DNA has not yet been gained, two separate events have been identified. Plant-derived phenolic compounds induce expression of a portion of the virulence region of the Ti plasmid and the formation of circular T-DNA intermediate molecules (3, 16, 17). With the exception of one that is constitutive, genes in the virulence region are not expressed in the absence of such induction, and it is hypothesized that once susceptible plant tissue is sensed by the pathogen, gene products transcribed from this region are necessary for transfer and integration of T-DNA (17). Bacterial binding is another event thought to be required for tumorigenesis, although it has not been directly shown in one system that inhibition of bacterial binding results in a subsequent inhibition of tumor formation. Most of what is known about the relationship between agrobacterial binding and tumorigenesis has come from studies in which inferences about bacterial bind-

1 Supported by a grant from Agrigenetics Research Associates.

2 Abbreviation: CWF, cell wall fraction extracted from cell walls isolated from suspension cultured tomato cells with 1,2-diaminocyclohexanediame N,N',N'-tetraacetic acid.

Tissue Culture. Callus and suspension cultures of LA 1221 red cherry tomato VFNT were grown as previously described (12). Suspension cultured cells for binding experiments were used 3 to 5 d after subcultivation. Tobacco suspension cultured cells derived from pith callus initiated from Nicotiana tabacum L. cv

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“Havana 425” were grown in basal medium (1) in the dark. Sugar cane callus cultures were obtained from Joe Lutz (Agrigenetics Research Corp., Madison, WI). Suspension cultures of sugar cane were grown in initiation and maintenance medium (6) in the dark.

**Growth and Labeling of Bacteria.** Strains A348 (pTi A6 in A136 chromosomal background, obtained from E. Nester) and A6 (pTi A6, wild isolate, obtained from A. Braun) of Agrobacterium tumefaciens were maintained on minimal medium (per liter: 5 g glucose, 1 g NH4Cl, 2 g KH2PO4, 0.2 g MgSO4, 1 g glutamic acid, 1 μg biotin, 5 μg H3BO3, 10 μg CaCO3, 10 μg CuSO4, 300 μg FeSO4, 1 μg KI, 15 μg MnSO4, 5 μg ZnSO4, 7H2O, and 14 g agar) at 4°C and were transferred once a month.

Liquid cultures were initiated weekly in minimal medium from 48 to 72 h streaks and were grown at 26°C in a shaking water bath. Stationary phase cultures were diluted 10-fold in fresh medium at 24 h intervals. Estimation of bacterial cell number by A at 666 nm, viable counts, and radioisotope-labeling of bacteria with 30 μCl(35S)methionine/ml (specific activity: 7.2 g Ci/mmol, Amersham) were counted in basal buffer. Stationary bacteria with 30 MCi(35S)methionine/ml (specific activity; 1.2121 Ci/mmol, Amersham) were used. Each experiment was done at least four times and similar results were obtained for each experiment. Reported values are expressed as the mean ± se n = 4 for each experiment.

**Tumor Formation Assay.** A quantitative analysis of tumor formation on potato tuber slices by A. tumefaciens strain A348 was carried out with an assay in which a linear relationship exists between the log of the number of cells/ml bacterial inoculum and the log of the number of resulting tumors (13). The procedure used was as described by Pueppke and Benny (13) with the following changes. Organically grown, red-skinned potato tubers were obtained from local markets. The A348 cells were used at stationary phase and were obtained from 24 h liquid cultures grown in minimal medium. Bacterial cell concentration was adjusted by measurement of A at 666 nm and later quantified by viable colony counts from dilutions onto L medium plates as previously described (12). Bacteria were incubated with the soluble tomato cell wall fraction in 20 μl of sterile phosphate-buffered saline (PBS; per liter: 0.43 g KH2PO4, 1.48 g NaH2PO4, 7.2 g NaCl in distilled H2O, pH 7.2) for 30 min at room temperature prior to dilution to 30 ml with PBS and addition of potato tuber slices. After a further 15-min incubation, potato slices were drained and placed on water agar plates as previously described (13). Depending on the inoculum of bacteria, tumors were counted 18 or 26 d later with a dissecting microscope. Background tumor controls (i.e. tuber slices with no added A. tumefaciens) were done for each experiment. Depending on the batch of potato tubers, a small number of tumor-like growths appeared. These were negative when tested for presence of oc-topine or nopaline, and no bacteria could be recovered from them. The absolute number of tumors produced varied depending on the lot of potatoes used. Each tumorigenesis assay was repeated at least five times and similar results were observed in each experiment. The data are expressed as the mean number of tumors ± se, where n = 27 potato tuber discs/point.

**Preparation of the Inhibitory Cell Wall Fraction.** Cell walls, prepared from 7 d cultures of actively growing LA 1221 tomato cells, were extracted for 4 h at 4°C with a Ca2+ chelating reagent (1.2-diaminocyclohexanedicarboxylic acid and EDTA; N,N',N'-tetraacetic acid) as previously described (12). The soluble fraction was dialyzed against H2O at 4°C, filtered through two layers of Whatman GFC filter paper, lyophilized to dryness, reconstituted in 80 μl H2O per mg dry weight, and stored frozen at −20°C. Concentrations of this soluble fraction were normalized to protein content with BSA as a standard (11). To conserve our limited supply of cell wall factor, and to keep conditions as constant as possible in two assays with very different volumes, bacteria were incubated with CWF in a small volume (20 μl) prior to addition of tomato cells (binding assay) or potato tuber slices (tumor assay). Reported CWF concentrations for binding and tumor assays were calculated from this initial incubation (e.g. 1 μg CWF protein/20 μl = 50 μg CWF protein/ml).

**RESULTS**

Previous work demonstrated that a soluble-pectin enriched CWF from tomato suspension cultured cells inhibits binding of Agrobacterium tumefaciens to these cells in a dose-dependent fashion. This suggests that this fraction contained a putative plant cell surface receptor site for these bacteria (12). This CWF fraction also caused a dose-dependent inhibition of tumor formation on potato tuber slices with 50% inhibitory concentrations similar to those required for inhibition of binding (Fig. 1). Although the 50% inhibitory concentrations varied depending

![Fig. 1. Effect of CWF concentration on binding and tumor formation. Binding assays, which contained 3 x 108 [3S]methionine radiolabeled bacteria (strain A6) and the indicated concentrations of tomato suspension cultured soluble cell wall fraction, were done as described under "Materials and Methods." Tumor assays, which contained 4.2 x 108 bacteria/ml (strain A348) and the indicated concentration of CWF, were done as described under "Materials and Methods." Tumors were counted on d 26. 100% tumors = 6.5 ± 0.8/disc. 100% binding = 8190 cpm/104 cell, 0.21 cpm/bacterium, 4 bacteria/cell.](https://plantphysiol.org)
on the preparation, they were usually between 20 and 50 μg CWF protein/ml for binding and 50 to 75 μg/ml for tumor inhibition. Maximum reduction of tumor formation occurred at CWF concentrations in excess of 100 μg CWF protein/ml, which resulted in an 80 to 95% inhibition, when compared to controls (no CWF). It was unlikely that the putative receptor site was narrowly species specific, since the tomato cell wall fraction also inhibited A. tumefaciens binding to suspension cultured sugar cane and tobacco cells (Table I) as well as tumor formation on potato slices.

The CWF inhibition of tumor formation was not due to toxic or growth inhibitory effects on A. tumefaciens. Bacteria were incubated for periods of time ranging from 15 min to 6 h and subsequently diluted and plated. Colony counts 48 h later revealed no statistically significant reduction in bacterial viability when compared to untreated bacteria.

If the tumor formation and bacterial binding were inhibited by a moiety in the CWF, then its inactivation should result in reduction or loss of both inhibitory activities. Prolonged boiling of the soluble CWF substantially inactivated both its binding and tumor inhibitory activities (Fig. 2). Data represented in Figure 3 demonstrate that binding inhibitory activity was relatively stable to 3 h at 100°C but was virtually eliminated by an additional hour at this temperature. The extent of inactivation of CWF inhibitory activity with respect to tumor formation and binding varied depending on the CWF preparation used but was usually >50% of the initial inhibitory activity.

At a constant bacterial inoculum, inhibition of tumor formation and bacterial binding was dependent on the concentration of CWF (Fig. 1). Conversely, if A. tumefaciens bound to or reacted with some moiety in the CWF, it should be possible to titrate out the CWF inhibitory activity by increasing the concentration of the bacterial inoculum. Tumor inhibitory activity of tomato CWF was, in fact, eliminated by increasing the concentration of bacteria (Fig. 4). At bacterial concentrations above 10^8/ml, no effect of CWF was observed, while maximal inhibition was obtained at bacterial concentrations between 1–5 x 10^9/ml.

**DISCUSSION**

A pectin-enriched soluble plant cell wall fraction inhibits both A. tumefaciens plant cell binding and tumor formation in a dose-dependent fashion with similar inhibitory concentrations. Other plant cell wall derived substances can inhibit either tumor formation or bacterial binding or both (4, 9, 10, 13, 14, 18). In a wounded pinto bean assay system, a reduction in the number of tumors formed relative to controls is observed when particulate cell wall fractions—pectin polygalacturonic acid and arabinogalactan—are added in conjunction with virulent A. tumefaciens

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**Table I. Effect of Cell Wall Factor on Bacterial Binding to Tobacco and Sugar Cane Suspension Cultured Cells**

Binding assays containing varying concentrations (see below) of [35S] methionine-labeled bacteria (strain A6), the indicated concentration of tomato cell wall factor protein, and 1 x 10^8 tobacco or sugar cane suspension cultured cells were done as described under “Materials and Methods.” Cell associated counts per minute (cpm) equal those radioactive counts on washed Nitex filters from assays containing only bacteria (12). Specific bacterial concentrations were as follows: sugar cane experiment 1, 2 x 10^7/ml, experiment 2, 1 x 10^7/ml; tobacco cell experiment 1, 2 x 10^8/ml, experiment 2, 3.6 x 10^9/ml.

<table>
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<th>Cell Type</th>
<th>Exp. No.</th>
<th>μg CWF</th>
<th>X ± SEM Cell Associated</th>
<th>% Inhibition of Binding</th>
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<td>Protein/ml</td>
<td>cpm</td>
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**Fig. 2.** Effects of boiling inhibitory activity of CWF. CWF in 0.025 M Mes (pH 6.0) (200 μg protein/ml) was sealed in tubes under reduced pressure and heated for 4 h at 100°C. Control CWF was treated identically except that it was kept at room temperature for 4 h. Binding assays contained 125 μg CWF protein/ml and 1 x 10^10 [35S]methionine radiolabeled strain A6 bacteria and tumor assays contained 125 μg CWF protein/ml and 1.5 x 10^2 strain A348 bacteria. Tumors were counted on d 28. 100% tumors = 4.5 ± 0.6/disc. 100% binding = 9432 cpm/10^4 cells, 0.053 cpm/bacterium, 18 bacteria/cell.

**Fig. 3.** Effect of time at 100°C on inhibitory activity of CWF. CWF was heated as described in the legend to Figure 2. At the indicated times, aliquots were withdrawn and tested for inhibitory activity in binding assays containing 1 x 10^10 [35S]methionine radiolabeled strain A6 bacteria and 100 μg CWF protein/ml. 100% binding = 2996/10^4 cells, 0.28 cpm/bacterium, 1.1 bacterium/cell.
(8). Removal of pectin from cell wall fractions results in a loss of their inhibitory effect on tumor formation (18). Furthermore, pectic substances with low methyl content are much more active in tumor formation than highly methylated pectin (18). Taken together, results of these studies suggest that the plant binding site for *A. tumefaciens* is pectic in nature and is either demethylated or not highly methylated. Results from similar studies, however, using a quantitative assay for tumor formation on potato tubers do not support this hypothesis (14). While pectin and polygalacturonic acid substantially reduce the numbers of tumors formed (210–540 μg galactouronan/ml for 50% inhibition), the extent of methylation of these compounds has no effect on their inhibitory activity (14). Furthermore, pectin and PGA actually increase above control values the number of radiolabeled *A. tumefaciens* that attach to potato tuber slices (15). Thus, the tumor inhibitory mechanism of these compounds is apparently not due to a competitive reduction of attached bacteria and remains unexplained. Although the chemical nature of the inhibitory activity in tomato CWF fraction is not known, it is enriched for pectic moieties (12). This fraction inhibits both binding and tumor formation and is effective at relatively low galactouronan concentrations (100–260 μg galacturonic acid equivalents/ml for 50% inhibition of tumors).

The relative stability of the cell wall fraction inhibitory activity to heat further suggests that the putative receptor moiety may be a carbohydrate. It was previously found that heating to dryness (100°C for 4 h) did not affect inhibitory activity with respect to binding (12), perhaps due to lack of hydrolytic activity in the absence of water. When CWF is heated in sealed tubes to prevent evaporation, however, the identical treatment substantially in-activates both tumor and binding inhibitory activities (Fig. 2), although at least 3 h at 100°C are required before any reduction in activity is observed.

The soluble tomato cell wall fraction described here reduces both binding and tumor formation at relatively low protein and uronic acid concentrations. In addition, this inhibitory activity lacks species specificity, toxicity to bacterial viability, and does not agglutinate *A. tumefaciens* (12). For these reasons, the soluble cell wall fraction extracted from tomato cells appears a good candidate for further purification of a plant surface receptor site for *A. tumefaciens*.

LITERATURE CITED

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