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Correlation between binding of *Agrobacterium tumefaciens* by root cap cells and susceptibility of plants to crown gall

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Abstract

We compared the binding of Agrobacterium tumefaciens by freshly isolated root cap cells with susceptibility of plants to crown gall tumorigenesis. A high binding reaction was strongly correlated with susceptibility to tumorigenesis in a survey of the binding of strain B6 to cells from 48 species in 17 families. In reciprocal experiments with nine virulent A. tumefaciens strains, tumors developed in plant-bacteria combinations that gave a high binding response in the root cap cell assay. Binding was quantified by direct measurement of the number of bacteria bound to the periphery of individual cells. Root cap cells from six susceptible species bound significantly more bacteria than did cells from five resistant species.

Introduction

Binding of A. tumefaciens by host cells may be one of the first steps in infection. Lippincott and Lippincott (1980) proposed that virulent A. tumefaciens cells bind to specific sites in the cell walls of susceptible plants. Resistant plants are believed to lack such sites, so they cannot bind bacteria and consequently cannot be infected. Virulent agrobacteria bind to cells and tissues of susceptible plants (reviewed by Pueppke 1984), and there is genetic evidence consistent with the hypothesis that binding is a necessary component of bacterial virulence (Douglas et al. 1985). However, the hypothesis that susceptible and resistant plants vary in their abilities to bind A. tumefaciens has been controversial (Nester et al. 1984; Pueppke 1984).

The recent successful infection of asparagus and other monocot species previously thought to be resistant to crown gall underscores the importance of coordinated studies of binding and disease reaction in determining the relationship between binding and tumor formation (DeCleene 1985; Hernalsteens et al. 1984; Hooykaas-Van Slogteren et al. 1984). We developed a binding assay that uses root cap cells, which can be isolated nondestructively from many species (Hawes and Pueppke 1986). We have used the assay to address two questions: (1) Are there differences in the abilities of freshly isolated cells from different plants to bind A. tumefaciens? (2) If so, are these differences correlated with susceptibility and resistance of the plants to crown gall tumorigenesis?

Materials and Methods

Bacteria: All Agrobacterium strains except Ag63 and R1000 were described by Pueppke and Benny (1981). Ag63 isolates were gifts from R. Goodman, of this department, and from M. Thomashow, Washington State University, Pullman. A. rhizogenes R1000 was a gift from H. Flores, Louisiana State Univ., Baton Rouge. E. coli SM10/1011 was a gift from K.T. Shanmugam, Univ. of Florida, Gainesville. All bacteria were recovered from glycerol stocks kept at -70°C; cells were grown overnight on solidified medium, and maintained at 5°C for up to 3 weeks. All cultures except E. coli and Ag63, which were maintained and grown in nutrient broth (Difco), were cultured on gluconate-mannitol medium (Pueppke and Benny 1981). Strains B6 and ACH5 were grown in nutrient broth in some experiments to determine if growth conditions influenced binding. Strains used for binding assays were cultured overnight at room temperature in 25 ml of liquid medium with constant agitation at 125 rpm. Clumps of bacteria that formed occasionally were removed by filtering the cultures through Whatman #1 paper. The concentration of bacteria was estimated turbidimetrically.

Binding assays: All binding assays were conducted in a double blind manner. Root cap cells for the qualitative binding assay were adjusted to a density of 2×10^4/ml by dilution with water or concentration on a 10-µm mesh filter. Root cap cell samples (100 µl) were placed into wells of a 96-well polystyrene microtiter plate. Bacteria (100 µl at 2×10^7/ml) were added, and the mixture was incubated at room temperature. After 1 to 2 h, the suspension in each well was stirred with a micropipette and 20-µl samples were removed. Each droplet was placed into a cone made from a 10-µm mesh nylon filter, and the liquid was removed by touching the tip of the cone to a tissue. The liquid was replaced with 100 µl of water, and the procedure was repeated twice. Binding of bacteria to at least 200 cells in each of duplicate samples was evaluated microscopically, and each species or cultivar was tested two or three times. Thus, each value is based on observation of 800 to 1200 plant cells. Reactions were rated "high" when 90% or more of the plant cells had at least a few bacteria attached around the periphery. In "low" reactions, at least 90% of the cells had no more than one bound bacterium visible.

Binding was measured directly by microscopic observation in a quantitative assay of bacteria bound per unit of root cap cell perimeter length. Samples of root cap cells (100 µl at 2×10^4/ml) were placed into wells of a 96-well microtiter plate, and bacteria (100 µl at 2×10^7/ml) were added. The mixtures were incubated at room temperature, and washed as described for the

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qualitative binding assay. A 20-µl sample from each replicate was examined microscopically. Each cell was projected onto a video screen attached to an Olympus microscope outfitted with 400X phase-contrast optics, and the objective was adjusted so that the cell wall was in focus around the entire periphery of the cell (the water mount must not exceed 20 µl to permit such focusing). The number of bacteria visible at the edge of the cell was counted, and the perimeter of the cell was measured by tracing the cell outline with a map reader (Minerva). At least 35 randomly selected cells from each of duplicate or triplicate samples were compared, and experiments were repeated at least twice. Thus, values are means from measurements of 120 to 180 plant cells.

Host range surveys: Root cap cells from 10 to 200 seedlings--depending on the yield per root from each species--were harvested as described previously (Hawes and Pueppke 1986), and were pooled for qualitative binding assays. At least 10 of the seedlings then were inoculated by stabbing the root, crown, and stem with a dissecting needle dipped into inoculum (10^8 to 10^9 bacteria/ml). Plants were grown in the greenhouse and disease reactions were evaluated within 6 weeks. Each experiment was performed twice. A disease reaction was considered negative when all inoculation sites did not differ from controls inoculated with Ti plasmidless strain ACH5C3 or with IIBNV6. In several experiments, octopine assays were conducted by the method of Otten and Schilperoort (1978).

Results

Binding is selective: At least 90% of pea root cap cells accumulated a surface layer of A. tumefaciens within one h after addition of the bacteria (Fig. 1A). Root cap cells were aggregated into massive clumps after three to four h (Fig. 1B). Vigorous agitation caused the aggregates to dissociate, but bacteria remained attached to the plant cells (Fig. 1C). In contrast, virtually no bacteria attached to the surfaces of oat root cap cells or to the exudate surrounding the cells (Fig. 1D). The halo-like exudate did not exclude E. coli cells, whose response to oat root cap cells was indistinguishable from that of pea cells (Fig. 1E). Furthermore, individual A. tumefaciens cells were frequently observed swimming through the exudate. Thus, A. tumefaciens cells did not fail to bind to oat root cap cells simply because they were mechanically prevented from doing so. E. coli did not accumulate around root cap cells of either pea or oats (Fig. 1E), and no bacterial cells remained attached to the washed root cap cells.

Binding correlates with tumor formation: On the basis of experiments with pea and oat root cap cells, we designated two easily distinguishable categories for binding. A high reaction is one in which at least 90% of the cells have a visible accumulation of bacteria on the surface. In a low reaction, at least 90% of the washed cells have no more than one visible bound bacterium. We used these criteria in screening assays designed to determine if the observed differences between pea and oat root cap cell binding are representative of a more general phenomenon among resistant and susceptible species. Strain B6 was tested with 48 plant species in 17 families, including monocots, dicots, and gymnosperms (Table 1). Of the 31 dicots tested, 29 gave a high binding reaction, and all except soybean developed tumors on at least 80% of the inoculation sites. Soybean stems developed tumors only when the wound was kept moist within a piece of rubber tubing. Approximately 20% of soybean root inoculations (5/22) developed tumors. Amaranthus tricolor and Celosia cristata cells exhibited a low binding response, and inoculated plants did not form tumors. The three pine species gave a high binding response and also developed tumors following inoculation. Of the 14 tested monocots, chives, yucca, and all the gramineous species exhibited a low binding response, and they failed to develop tumors or swellings. Root cap cells from roots on bulbs of Muscari sp. and Narcissus sp. exhibited a strong binding response. Both species developed swellings on their stems within a week after inoculation with B6, but not with IIBNV6. The swellings did not increase in size over a 6-week period, however, and octopine assays of the tissue were negative (data not shown). Several cultivars of ten species were compared with one another (Table 1), but large intraspecies differences in binding reaction and disease response were not apparent.

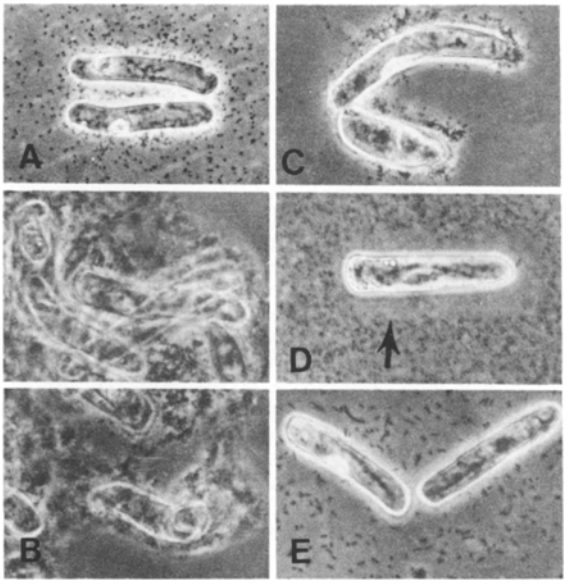

Fig. 1. Binding of A. tumefaciens strain B6 to isolated root cap cells of pea (Pisum sativum L. cv. Little Marvel) and oat (Avena sativa L. cv. Victorgrain). (A-B) Isolated pea cells after incubation for 1 h (A) or 4 h (B). (C) Pea root cap cells from (B) after agitation and washing over a 10- µm mesh screen. (D) Oat root cap cells after incubation for 24 h with A. tumefaciens. The arrow denotes halo-like exudate surrounding cells, where bacterial density remained low. (E) Isolated pea root cap cells after a 24-h incubation with E. coli. The initial plant and bacterial cell concentrations were 10^4 cells/ml and 6×10^7 cells/ml, respectively.

The above survey with a single Agrobacterium strain and 65 plants indicated that there is a correlation between binding response of root cap cells and susceptibility of plants to tumorigenesis. We used nine virulent Agrobacterium strains in reciprocal experiments to compare root cap cell binding with susceptibility of pea and sunflower, two species that are known to be capable of developing large tumors in response to some strains (Table 2). Two strains that lack a functional Ti plasmid were included as controls. Both A. tumefaciens IIBNV6 and A. radiobacter strain 4718 bound in high numbers to root cap cells of both species, but did not induce tumors. Seven of the Ti plasmid-containing strains bound to root cap cells of pea and sunflower, and also induced tumors on the plants. Ag63, which causes tumors on grape and binds to grape suspension culture cells and to grape xylem vessels (G. Cleveland and R. Goodman, personal communication), did not bind to root cap cells of pea or sunflower, and did not cause tumors. The results with A. rubi were

Table 1. Adsorption of Agrobacterium tumefaciens strain B6 by root cap cells

FAMILY/SPECIES	BINDING RESPONSE	SUSCEPTIBILITY
MONOCOTYLEDONS		
Avena sativa L. (3)[a]	L	-
Festuca sp. (3)	L	-
Hordeum vulgare L. (2)	L	-
Oryza sativa L. (2)	L	-
Panicum mileaceum L.	L	-
Phalaris canariensis L.	L	-
Secale cereale L.	L	-
Triticum aestivum L.	L	-
Triticale	L	-
Zea mays L. (3)	L	-
Allium schoenoprasum L.	L	-
Muscari sp.	H	+[b]
Narcissus sp.	H	+[b]
Yucca sp.	L	-
DICOTYLEDONS		
Amaranthus tricolor L.	L	-
Celosia cristata L.	L	-
Betula pendula Roth	H	+
Beta vulgaris L.	H	+
Chenopodium amaranticolor Coste and Reynier	H	+
Helianthus annuus L.	H	+
Tithonia rotundifolia (Mill.) S.F. Blake	H	+
Calonyction aculeatum House	H	+
Impomoea aquatica L.	H	+
Citrullus vulgaris Schrad.	H	+
Cucumis melo L. (3)	H	+
C. sativa L. (3)	H	+
Cucurbita maxima Duchesne (2)	H	+
C. pepo L.	H	+
C. pepo var. malopepo L. (2)	H	+
Ricinis communis L.	H	+
Canavalia ensiformis (L.) DC	H	+
Glycine max (L.) Merrill	H	+
Phaseolus vulgaris L. (3)	H	+
Sesbania exaltata (Raf.) V.L. Cory	H	+
Trifolium pratense L.	H	+
Vigna radiata (L.) R. Wilcz	H	+
V. unguiculata (L.) Walp.	H	+
Eschscholtzia californica Cham.	H	+
Fragaria vesca L.	H	+
Capsicum frutescens L.	H	+
Lycopersicon esculentum L.	H	+
Nicotiana tabacum L.	H	+
N. glutinosa L.	H	+
Petunia hybrida Vilm.-Andr.	H	+
Solanum melongena L.	H	+
Daucus carota L.	H	+
GYMNOSPERMS		
Pinus radiata D. Don.	H	+
P. sylvestris L.	H	+
P. pinaster Ait.	H	+

[a]Numbers in parentheses indicate the number of cultivars tested.

[b]Swellings appeared, but obvious tumors did not. Octopine was not detected.

different from those of any of the A. tumefaciens or A. radiobacter strains. In the presence of pea root cap cells, A. rubi gave a high binding response; it also induced tumors on pea seedlings. But the bacteria failed to induce observable tumors on sunflower seedlings, and exhibited a low binding response with sunflower root cap cells. Bacteria grown in nutrient broth exhibited the same binding responses as those grown in the standard growth medium (data not shown).

Table 2
Agrobacterium binding to root cap cells: strain X species specificity among dicots

BACTERIA[a]	SUNFLOWER[b] Binding	SUNFLOWER[b] Tumors	PEA[b] Binding	PEA[b] Tumors
A. tumefaciens				
B6	H	+	H	+
C58	H	+	H	+
Chry 5	H	+	H	+
Chry 8	H	+	H	+
ATCC 15955	H	+	H	+
Ag63	L	-	L	-
IIBNV6	H	-	H	-
A. rhizogenes				
ATCC 15834	H	+	H	+
R1000	H	+	H	+
A. rubi				
ATCC 13335	L	-	H	+
A. radiobacter				
ATCC 4718	H	-	H	-

[a]Bacteria are described in Pueppke and Benny (1981). Strains IIBNV6 and 4718 lack a functional Ti-plasmid, so are avirulent on all plants.
[b]The sunflower cv. was Mammoth, from W. Atlee Burpee Co., Warminster, PA. The pea cv. was Little Marvel from Royal Seeds, Kansas City, MO.

Binding differences can be quantified: Although all of the tested combinations fit into the designated "high" and "low" binding categories, there were variations within these classifications. Soybean cells in particular appeared to bind significantly fewer bacteria than those from pea and other dicots and conifers. Among the species with low binding reactions, C. cristata and maize root cap cell populations had fewer bacteria-free cells than did oat cell populations. In order to determine if these apparent differences were significant, and to more accurately define the high and low binding reactions, we measured binding directly using a microscopic assay based on counts of bacteria visible at the periphery of each cell. After a 1 h incubation with A. tumefaciens B6, root cap cells from susceptible dicots bound from 9 to 17 bacteria per cell perimeter, and the average perimeter length between bound bacteria varied from 14 to 27 μm (Table 3). Soybean cells bound five bacteria per perimeter, with 34 μm between bacteria. The number of bacteria per perimeter varied from 0.01 to 1 in species with low binding responses.

Discussion

Root cap cells are viable, developmentally similar cells that can be easily separated from one other for direct microscopic observation. Such cells can be isolated nondestructively, so that the same plant can be used to evaluate binding and to determine susceptibility to crown gall. Our results confirm previous reports (Douglas et al. 1982; Neff and Binns 1985) that some Ti plasmidless strains can bind to plant cells. However, binding of a given Ti plasmid-containing A. tumefaciens strain by root cap cells of a given plant was strongly correlated with a positive tumor response.

Table 3
Direct measurement of A. tumefaciens strain B6 binding to the periphery of isolated root cap cells from plants resistant or susceptible to crown gall[a]

SPECIES	MEAN PERIMETER /CELL (μm)	NO. BOUND BACTERIA/ PERIMETER
Susceptible		
Sunflower (Mammoth)	194 ± 5	14 ± 0.59
Pea (Little Marvel)	250 ± 6	17 ± 0.30
Tomato (Rutgers)	217 ± 8	10 ± 0.46
Cucumber (Straight Eight)	214 ± 7	9 ± 0.40
Pea (Laxton Progress #9)	260 ± 9	11 ± 0.45
Soybean (McCall)	170 ± 4	5 ± 0.28
Resistant		
Celosia cristata (Pampas Plume)	164 ± 6	1.00 ± 0.19
Corn (W64)	228 ± 4	0.80 ± 0.11
Wheat (Ward)	206 ± 8	0.02 ± 0.03
Sorghum (Colby)	145 ± 3	0.10 ± 0.01
Oats (Victorgrain)	174 ± 6	0.01 ± 0.01

[a]At least 35 cells were evaluated for each combination, and experiments were conducted 2 or 3 times.

Even if binding ability expressed by root cap cells is an important determinant of susceptibility to crown gall, it is only one of many steps that could be blocked prior to tumor development. Thus, it is to be expected that some plants with positive binding responses will not develop tumors. In no case, however, did we observe a binding deficient plant that developed clear-cut crown gall symptoms. The nondestructive nature of the root cap cell assay should make it possible to establish whether there is a genetic relationship between binding and susceptibility.

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