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A Semiquantitative Bioassay for Relative Virulence of *Agrobacterium tumefaciens* Strains on *Bryophyllum daigremontiana*

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An assay to determine the relative virulence of wild-type and mutant strains of *Agrobacterium tumefaciens* on leaves of *Bryophyllum daigremontiana* has been developed. This assay is reproducible, is easy to learn, is not time consuming, and requires little space. The relative virulence of cellulose-minus mutants of *A. tumefaciens* was investigated with this assay. Some of these mutants were unaltered in virulence, while others showed a marked reduction in virulence.

Agrobacterium tumefaciens induces crown gall tumors in wound sites on dicotyledonous plants. Various assays have been used to study tumor formation and to determine the relative virulence of mutant strains with respect to that of the wild-type bacterium. These assays include Anand and Heberlein's potato disc assay (1), Lippincott and Heberlein's pinto bean leaf assay (4), the pea root assay described by Hawes et al. (2), and a carrot root assay originally described by Klein and Tenenbaum (3) and modified by Peralta and Ream (7). Our goal was to develop a convenient assay for relative virulence of wild-type and mutant *A. tumefaciens* strains which did not occupy much space and which did not rely on the availability of uniform susceptible materials at local supermarkets. For these reasons we chose *Bryophyllum daigremontiana*, also called *Kalanchöe daigremontiana*. This plant reproduces by forming new plantlets at the leaf margins, so one has a constant supply of genetically identical plants. *B. daigremontiana* can be grown in a small space in the greenhouse year-round, and assays of relative virulence on leaves of this plant require only a few (four to eight) plants.

In nature, infection by *A. tumefaciens* occurs through wounds in the roots or crown of the plant, not through leaf wounds. Although this assay may not reflect the natural mode of plant infection, leaf wounds are much more convenient to perform and do not lessen the usefulness of this assay for virulence comparison.

We chose to develop this assay by testing two wild-type strains, C58 and A6, and a set of well-characterized mutants of the A6 wild type. These strains, Cel-1, Cel-3, Cel-4, Cel-11, and Cel-12, are cellulose-minus Tn*5* transposon mutants which retain their virulence. The reversion rate of each of these mutants is less than 1 in 10^9 cells (5). These strains are listed and described in Table 1.

For the virulence assays, cultures of *A. tumefaciens* A6, C58, and cellulose-minus mutants were grown overnight in Luria broth to early stationary phase (approximately 10^9 cells per ml). The use of cultures which contained less than 10^8 bacteria per ml gave results which differed significantly from those obtained with stationary-phase cultures. The cultures were diluted in Luria broth (in a range of 1/2 to 1/1,000) for inoculation of *B. daigremontiana* leaves. Dilutions in Luria broth gave more uniform results than dilutions in salt solutions such as phosphate-buffered saline. The range of dilutions was chosen so that the final 5-μl inoculum would contain between 10^3 and 10^7 cells. A viable cell count was done for each culture.

Just prior to leaf inoculation, *B. daigremontiana* leaves were wounded with a device created for this assay. A pair of kitchen tongs was fitted with a solid rubber stopper on one side and a one-hole stopper on the other. An Eppendorf 1-ml disposable pipette blue tip was inserted into the second stopper so that the tip protruded 2 to 3 mm. This device, when clamped on a leaf, cushioned the leaf on the underside while making a shallow wound on the upper side which would not puncture through the leaf. This allowed a measured amount of inoculum to be introduced to each wound.

Young *B. daigremontiana* plants, 5 to 8 in. (ca. 10 to 20 cm) in height, were used for this assay. The youngest two pairs of leaves which were at least 4 cm in length were inoculated. Older leaves on the plant were less sensitive to tumor formation. Eight wounds were made on each leaf, four in a row down each side. The leaf wounds were then inoculated with 5 μl of serial dilutions of bacterial culture, using a 200-μl pipette tip. The dilution series of the wild-type *A. tumefaciens* was inoculated on one side of the leaf, and the bacterial strain to be tested was inoculated on the other side. A minimum of four leaves on separate plants was tested. Testing a larger number of leaves decreased the variance in the results. Comparison of the tested strain with the wild type allowed the results from an occasional unresponsive leaf to be discarded. Inoculated plants were grown at room temperature; temperatures above 30°C or below 20°C adversely affected tumor formation. Humidity was uncontrolled and ranged between 30 and 70% relative humidity. No effect of changes in relative humidity within this range was observed.

The wound sites were scored as positive or negative for tumor formation once a week for 6 weeks. Tumors began to form on the plants after 1 or 2 weeks; new tumors ceased to form after 4 to 6 weeks.

We found that the best way to quantitate bacterial virulence in this assay was to determine the number of bacteria needed to form tumors at 50% of the inoculated sites. Other parameters, such as tumor weight, diameter, and length of time to form tumors, were explored but were not found to give a linear response as the number of bacteria inoculated increased.

Figure 1 shows the effect of changes in the number of

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TABLE 1. *A. tumefaciens* strains used in this assay

Bacterial strain	Relevant characteristics	Source or reference
Wild-type strains		
C58	Nopaline pTi	A. C. Braun
A6 (parent strain)	Octopine pTi	A. C. Braun
Mutants		
Cel-1	A6::Tn*5* cellulose minus	5
Cel-3	A6::Tn*5* cellulose minus	5
Cel-4	A6::Tn*5* cellulose minus	5
Cel-11	A6::Tn*5* cellulose minus	5
Cel-12	A6::Tn*5* cellulose minus	5

viable bacteria inoculated per wound site on the percentage of wound sites forming tumors for the parent strain A6 and the cellulose-minus mutant Cel-12. Determined from the point of 50% tumor induction, the virulence of the mutant Cel-12 was approximately 1/10 that of the parent strain.

Similar graphs were drawn for the other cellulose-minus strains and the wild-type strain C58. Table 2 presents the relative virulences of these strains in comparison with A6. C58 proved to be slightly more virulent than A6, in agreement with the general impression of most researchers. This assay showed Cel-1 and Cel-3 to be unaltered in virulence. Cel-4 exhibited a slight increase in virulence and was observed to grow faster than its wild-type parent A6 in Luria broth. An increased growth rate in the leaf wound may account for the small increase in virulence observed. Moderately decreased virulence was observed for Cel-12, while

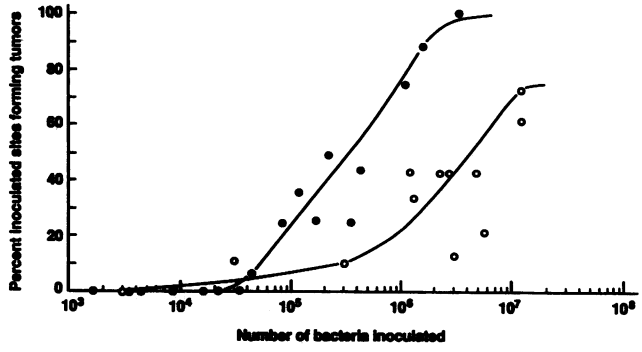

FIG. 1. Percentage of wound sites forming tumors as a function of the number of viable bacteria in the 5-μl inoculum placed in each site. Symbols: ●, wild-type strain A6; ○, mutant strain Cel-12. The standard deviation of the percent inoculated sites forming tumors was about 10% for a given bacterial number in different trials. The number of bacteria required for 50% of the inoculated sites to form tumors was about 2×10^5 for A6 and about 4×10^6 for Cel-12, suggesting that the virulence of Cel-12 is reduced about 10-fold compared with the wild-type parent.

TABLE 2. Relative virulence of *A. tumefacien* wild-type and mutant strains

Bacterial strain	No. of bacteria required for 50% tumor formation	Relative virulence[a]
Wild-type strains		
A6	$(2–4) \times 10^5$	1.0
C58	$(0.7–1) \times 10^5$	2.0–5.7
Mutants		
Cel-1	$(2–3) \times 10^5$	0.5–1.5
Cel-3	$(3–5) \times 10^5$	0.4–1.3
Cel-4	$(0.7–1) \times 10^5$	2.0–5.7
Cel-11	$>2 \times 10^7$[b]	<0.01
Cel-12	4×10^6	0.1–0.2

[a] Virulence relative to the A6 strain was calculated as the number of bacteria required for 50% tumor induction for A6 divided by the number of bacteria required for the tested strain.

[b] Cel-11 formed no tumors when diluted, although tumors did form when this strain was applied to leaf wounds as a paste.

the virulence of Cel-11 was greatly reduced. The Tn*5* insertions in Cel-1, Cel-3, and Cel-4 belong to one operon, while the insertions in Cel-11 and Cel-12 are located in another operon (6). Expression of genes in both of these operons is required for bacterial cellulose synthesis, but the functions of these genes are not known.

The effects of mutations on the virulence of *A. tumefaciens* may be effectively analyzed through the use of this assay when comparing the virulence of a mutated strain with that of the parent strain. In addition, this assay is easy to learn, is not time consuming, and requires little attention after inoculation. These advantages make this bioassay a useful alternative to other virulence assays presently available.

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