Agrobacterium radiobacter Strain K1026, a Genetically Engineered Derivative of Strain K84, for Biological Control of Crown Gall

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ABSTRACT

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Agrobacterium radiobacter strain K1026 was tested in open-air pot trials, in comparison with strain K84, for its efficacy in controlling crown gall on almond seedlings. Seedlings aged 2 and 10 mo were dipped in water or a suspension of K1026 or K84, planted in soil infested with *Agrobacterium tumefaciens* strain K27, and grown for 7 mo. Galls were absent or rare on the roots of seedlings that had been treated with K1026 or K84. In contrast, the roots of seedlings treated with water had from 3 to 23 galls per plant for 2-mo-old seedlings and from 13 to 103 galls for 10-mo-old seedlings. There was no significant difference in gall incidence between plants treated with K1026 or K84, indicating that K1026 is as effective as K84 in controlling crown gall. Because K1026 is unable to transfer its agrocin-84 plasmid to pathogenic agrobacteria, it should be used as a replacement for K84 to prolong the effective biological control of crown gall. The registration of K1026 as a pesticide would seem to be justified and applications for such registration have been submitted.

Crown gall is caused by *Agrobacterium tumefaciens* (Smith & Townsend) Conn. This bacterium contains a tumor-inducing (Ti) plasmid (28,29) that directs the transfer of a segment of its own DNA (T-DNA) into plant cells (2). The T-DNA is integrated into the plant DNA (3,30) where it directs the synthesis of phytohormones, which cause tumerous cell growth (1,12,23), and opines, which act as novel nutrient sources catabolized almost exclusively by the inciting bacterium (11). One class of Ti plasmid directs the plant to synthesize, and the bacterium to catabolize, the opines nopaline and agrocinopine. Strains of *A. tumefaciens* carrying this type of Ti plasmid are subject to biological control by *A. radiobacter* (Beijerinck & Van Delden) Conn strain K84 (reviewed by Kerr and Tate [16]). Control by this strain is due largely to an antibiotic, agrocin 84 (14,16), whose synthesis is encoded by a 47 kb plasmid, pAgK84, carried by strain K84 (7,10). Agrocin 84 is taken up by a Ti-plasmid-encoded agrocinopine-permease (8) and acts by terminating DNA synthesis in the recipient bacterium (4,16,18).

Strain K84 has been used successfully throughout the world for more than 10 years to control crown gall. However, its continued success is threatened by the ability of pAgK84 to transfer conjugatively to pathogenic agrobacteria (5,10,20), which then become insensitive to agrocin 84 (7,22). Despite this threat, there have been no reports of agrocin-plasmid transfer following commercial use of strain K84, so the importance of agrocin-plasmid transfer in the field is not known. Because agrocin-plasmid transfer can be observed so readily in the laboratory, and because several naturally occurring pathogenic agrobacteria synthesize agrocin 84 and contain a plasmid very similar to pAgK84 (26, *unpublished*), it seems likely to be a natural phenomenon.

Agrocin-plasmid transfer is controlled by a defined region (Tra) on pAgK84 (10). Recombinant DNA techniques have been used to construct a new biological control strain, K1026, that is identical to K84 apart from a 5.9 kb deletion overlapping the Tra region of pAgK84 (3). K1026 is unable to transfer its mutant agrocin-84 plasmid, designated pAgK1026, to other agrobacteria, but remains inhibitory in vitro to strains sensitive to agrocin 84.

Because K1026 is a genetically engineered organism, we sought and obtained permission from the relevant Australian authorities to test K1026 in planta without biological containment. In this paper we report the results of an open-air pot trial testing the ability of K1026 to prevent crown gall on almond seedlings. To the best of our knowledge, this trial constitutes the first environmental release of a genetically engineered organism in Australia, and only the third in the world.

MATERIALS AND METHODS

Preparation of bacteria. *A. radiobacter* biovar-2 strains K84 and K1026 (13) were used to treat almond-seedling roots to prevent crown gall incited by *A. tumefaciens* biovar-2 strain K27 (14), which is sensitive to agrocin 84. Before the pot trial, a fresh culture of K27 was subcultured onto 90 10-ml yeast mannitol agar (YMA) slopes in McCartney bottles, and fresh cultures of K84 and K1026 were subcultured onto 40-ml YMA slopes in 200-ml medicine flats. All cultures were grown for 3 days at 25 C.

Preparation of almond seedlings. Fresh almond seeds (cultivar Challeston) were placed in peat moistened with distilled water containing 1 g/L of the fungicide captan and kept moist for 6 wk in the dark at 4 C to initiate germination. The seeds were then planted, one per pot, in 20-cm-diameter pots containing UC potting mix and kept moist. Seedlings appeared 3–4 wk later. One batch of seedlings was grown for 2 mo and another for 10 mo before the pot trial. Two days before the pot trial, seedling foliage was pruned severely to reduce stress due to transpirational water loss after replanting.

Preparation of soil. Ninety 25-cm-diameter pots were filled with a nonsterile sandy loam, 10 kg per pot, over a 2-cm layer of pine-bark chips. Two days before replanting the treated almond seedlings in this soil, the 90 3-day cultures of K27 were each suspended in 500 ml of nonchlorinated water and poured into the soil, one culture per pot. The suspensions were then mixed into the top 10 cm of soil and watered in. The suspensions of K27 were estimated by optical density measurements to contain about 2×10^7 cells per milliliter. So, assuming uniform dispersal in the soil, the resultant concentration would have been approximately 10^6 cells per gram. The actual distribution of K27 in the soil was not examined.

Treatment of almond seedlings. The 3-day cultures of K84 and K1026 were suspended in 5 L of nonchlorinated water. The suspensions were estimated by optical density measurements to contain about 10^7 cells per milliliter. The almond seedlings were removed from their pots, the soil was shaken gently from their roots, and the primary and lateral roots were trimmed to a length of approximately 20 cm. The plants were immersed for about 10 sec, to just above the crown, in either water or a suspension of K84 or K1026. They were then replanted, one per pot, in the soil infested previously with K27, and were watered. The distribution of K84 or K1026 on the

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roots was not examined.

The plants were grown outdoors for 7 mo and a soluble fertilizer was applied at 6-wk intervals, from June 1987 (early winter) to January 1988 (midsummer). They were then removed from their pots, the soil was shaken gently from their roots, and the roots were washed by repeated immersion in tap water. The number of galls on the roots was recorded for each plant.

Pot trial layout. The pot trial was set up with 15 replicates arranged in 15 rows of six plants. Each row was randomized with respect to the six combinations of the three treatments (water, K84, and K1026) and the two seedling ages (2 and 10 mo). There was no space between rows or between pots within rows. No precautions were taken to prevent pot-to-pot spread of the bacteria. This did not appear to be a problem.

Statistical analysis. Because the distribution of the data was skewed and not normal, even after transformation, parametric tests such as analysis of variance were inappropriate, so the data were analyzed nonparametrically using the Kruskal-Wallis test (25). The Kruskal-Wallis test was applied separately to the 2- and 10-mo-old seedlings and was used to compare all three treatments and to compare K84 and K1026 treatments.

Recovery of agrobacteria from roots and galls. Five grams of roots located within 10 cm of the crown were excised from each of five 10-mo-old seedlings treated with K84 and similarly for K1026. Each root mass was placed in sterile double-distilled water, shaken vigorously, placed at 4 C for 3 hr, and shaken vigorously once again. Dilutions of 10^{-1} and 10^{-2} in buffered saline (24) were prepared and 18 10-μl droplets of each dilution were placed onto New and Kerr medium (19) for isolation of *Agrobacterium* biovar-2. These plates were incubated for 4 days at 25 C.

Five "healthy" 1- to 2-g galls from each of five 10-mo-old seedlings treated with water were detached, immersed in 1.5% sodium hypochlorite for 2 min, and then rinsed three times in sterile double-distilled water. The surface-disinfected galls were then placed in 10 ml of sterile double-distilled water and macerated. The mixture was placed at 4 C for 3 hr and shaken vigorously. Two loops full of the supernatant were streaked onto New and Kerr medium and the plates were incubated as above. Similarly, eight "healthy" galls $\geq$0.75 g were sampled individually from seedlings treated with K84 or K1026.

All strains recovered were streaked on New and Kerr medium and pure cultures, isolated from the resultant single colonies, were maintained on YMA slopes for use in subsequent tests.

Testing recovered agrobacteria for agrocin 84 production or sensitivity. The method of Stonier (27), as modified by Kerr and Htay (14), was used for agrocin-84 bioassays. The recovered agrobacteria were tested for agrocin production by using them as producers in the agrocin bioassay with strain K198 used as the indicator. The latter is a biovar-1 strain of *A. tumefaciens* that harbors the same agrocin-sensitive Ti-plasmid as K27 (21). Strains that did not produce agrocin were then tested for agrocin sensitivity by using them as indicators in the agrocin bioassay with K1026 used as the producer.

Testing recovered agrobacteria for pathogenicity on tomato seedlings. The recovered agrobacteria were tested for tumorigenicity by multiple stab-inoculations of 6-wk-old tomato seedlings (cultivar Rouge de Marmande). Inocula were prepared from 3-day cultures on YMA slopes. Two loops full of bacteria were removed and suspended in 1 ml of sterile distilled water. A flame-sterilized needle was dipped into the suspensions and stabbed into the stems of the tomato plants, five times for each strain. The stems were assessed 6 wk later for gall formation.

Plasmid content of recovered agrobacteria. Minipreparations of plasmids from the recovered agrobacteria were performed as described by Farrand et al (10), and plasmid content was characterized by agarose gel electrophoresis as described by Maniatis et al (17).

Table 1. The effect of treating almond seedlings with water, a suspension of *Agrobacterium radiobacter* strain K84, or a suspension of *A. radiobacter* strain K1026 on crown gall induced by *A. tumefaciens* strain K27

Plant age (mo)	Treatment	Number of plants surviving	Percentage of plants with galls	Number of galls per plant		
				Mean	Median	Range
2	Water	12	100	9.33	7.5	3–23
	K84	14	14	0.21	0	0–2
	K1026	12	25	0.33	0	0–2
10	Water	15	100	46.33	41	13–103
	K84	15	20	0.20	0	0–1
	K1026	15	27	0.67	0	0–5

Table 2. Numbers of agrobacteria recovered and characterized for agrocin production, agrocin sensitivity, and tumorigenicity from the roots of 10-mo-old almond seedlings treated with K84 or K1026, and from galls on the roots of 10-mo-old almond seedlings treated with water, K84, or K1026

Source	Sample	Agrocin-producing, nontumorigenic (K84 or K1026)	Agrocin-sensitive, tumorigenic (K27)
Roots of plants treated with K84	1	0	9
	2	1	8
	3	5	7
	4	1	10
	5	3	10
Roots of plants treated with K1026	1	1	8
	2	0	9
	3	5	5
	4	10	0
	5	3	5
Galls on roots of plants treated with K84	1	1	9
	2	8	1
Galls on roots of plants treated with K1026	1	10	0
	2	10	0
	3	0	10
	4	10	0
	5	10	0
	6	6	4
Galls on roots of plants treated with water	1–5	0	15

RESULTS

Pot trial. Galls were rare or absent on the roots of seedlings treated with K84 or K1026, but frequent on those treated with water (Table 1). The difference is so clear as to obviate the need for statistical confirmation. Nevertheless, Kruskal-Wallis tests were used to compare the three treatments and were found to confirm a significant difference (H adjusted for ties = 29.00 and 34.02 for 2- and 10-mo-old seedlings, respectively, with $p < 0.001$ in both cases). The similarity of K84 and K1026 treatments is also so clear as to obviate the need for statistical confirmation. Again, Kruskal-Wallis tests were used to compare the two treatments and were found to confirm the similarity (H adjusted for ties = 0.41 and 0.35 for 2- and 10-mo-old seedlings, respectively, with $0.5 < p < 0.7$ in both cases).

Recovered agrobacteria. Only agrocin-producing, nontumorigenic agrobacteria and agrocin-sensitive, tumorigenic agrobacteria were recovered from the roots of K84- or K1026-treated plants (Table 2). All of the nontumorigenic

strains and a sample of the tumorigenic strains (five from each treatment) were analyzed for plasmid content. K84 and K1026 each contain three plasmids, a large cryptic plasmid (pAtK84a), a smaller nopaline catabolic plasmid (pAtK84b), and a still smaller agrocin-84 plasmid (pAgK84 and pAgK1026, respectively) (13), whereas K27 contains two plasmids, a cryptic plasmid (pAtK27), slightly larger than pAtK84a, and a Ti plasmid (pTiK27), intermediate in size to pAtK84a and pAtK84b. The plasmid analysis showed the nontumorigenic strains to correspond to K84, if recovered from K84-treated plants, or K1026, if recovered from K1026-treated plants and the tumorigenic strains to correspond to K27 (*data not shown*). There was great variation between plants in the population ratio of K84 or K1026 to K27, but clearly there was an overall excess of K27 (Table 2).

Similarly, only K84 or K1026, and K27 were recovered from the few galls that occurred on the roots of K84- or K1026-treated plants (Table 2). There appeared to be a bimodal polarization in the population ratio of K84 or K1026 to K27, with five of the eight galls containing almost all K84 or K1026 and two of the eight galls containing almost all K27. In contrast, only K27 was recovered from galls on the roots of water-treated plants.

DISCUSSION

It is clear from the results of the pot trial that K1026 is as efficient as K84 in controlling crown gall. It is also clear from the recovery data that relative to K84, K1026 has a similar ability to colonize and survive on roots as well as to colonize galls and displace inciting agrobacteria. Thus, K1026 appears to retain the ecological competence of its progenitor, K84.

It is interesting that there was an apparent bimodal polarization in the population ratios of K84 or K1026 to K27 in the few galls on K84- or K1026-treated plants. K84 and K1026 both use the opines nopaline and agrocinopine synthesized by K27-induced galls (6,22). So, consistent with the opine concept (11), those galls with an excess of K84 or K1026 presumably reflect K84 or K1026 colonization of these galls, with the concomitant displacement of K27, which, in the presence of agrocinopine, becomes more sensitive to agrocin 84 (8). The galls in which there was an excess of K27 presumably reflect opportunities for colonization that were missed.

It is also interesting that neither K84 nor K1026 prevented co-colonization of the roots of K84- or K1026-treated plants by K27, and in fact, there was an excess of K27 over K84 or K1026. Furthermore, very few galls were induced despite the excess of K27. This apparent anomaly, of inhibition of tumorigenesis without inhibition of root colonization and growth, is surprising and is perhaps worthy of further investigation.

However, since K1026 is clearly as efficient as K84 in controlling crown gall and because it is unable to transfer its agrocin-84 plasmid, it should be used as a replacement for K84 to prolong the effective biological control of crown gall. To enable K1026 to achieve widespread horticultural use, it is necessary to register it as a pesticide. Applications for registration of K1026 as a pesticide have been submitted in Australia and will soon be submitted in America and elsewhere.

Because strain K1026 may become the first genetically engineered organism to be released for general use by industry, it might be useful to summarize the evidence that it is harmless to man, animal, plant, and the environment. 1) K84, the progenitor of K1026, has been registered as a pesticide and used commercially in many countries, including Australia and America, for more than 10 years; there have been no reports of harm. 2) K1026 is identical to K84 except that it lacks a 5.9 kb portion of the agrocin-84 plasmid, therefore preventing agrocin-plasmid transfer (13). 3) No foreign DNA remains in K1026 (13). 4) K1026 contains no Ti-plasmid-encoded genes involved in crown gall induction (B. G. Clare, *unpublished data*). 5) K1026 is a biovar-2 strain of *Agrobacterium* and cannot grow at 37 C (human body temperature) (15). 6) Agrocin 84 is specific for agrocinopine-catabolizing agrobacteria, most of which are crown gall pathogens; other organisms are unaffected (9). So, just as no ecological damage has resulted from production of agrocin 84 by K84, none will result from production of agrocin 84 by K1026. For these reasons, we see no valid impediment to the registration and widespread use of K1026 in the biological control of crown gall.

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