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Mini review

Use of the genetically engineered *Agrobacterium* strain K1026 for biological control of crown gall*

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Abstract

Strain K84 of *Agrobacterium* (formerly called *A. radiobacter*) has been a successful biocontrol agent of crown gall disease for almost 30 years all over the world. In spite of its demonstrated efficiency, the most important risk of failure when using strain K84 is the possibility of transfer of plasmid pAgK84 to pathogenic *Agrobacterium* strains. pAgK84 codifies production of and immunity to agrocin 84, the main factor involved in crown gall biocontrol by strain K84. Then, a second generation of strain K84 was obtained and the genetically engineered strain was called K1026. It contains a deletion in the transfer region of pAgK84. To date, a considerable number of studies have been performed to compare both strains in its ability to control crown gall, plasmid transfer, antibiotic production, root colonization and survival in the rhizosphere. The aim of this review is to discuss all this comparative available information which advises that strain K1026 should be used as a biopesticide to safeguard biocontrol of crown gall wherever strain K84 is employed.

Introduction

Crown gall is a world-wide distributed plant disease caused by *Agrobacterium* species and is responsible for nursery and field losses among a large variety of plants. Successful biological control of crown gall using the nonpathogenic strain K84 of *Agrobacterium* has been practiced on a commercial scale for almost 30 years. Considering its world-wide use and the presence of only a few reports concerning serious failures or unexpected deleterious effects, the K84 biocontrol system is perhaps the most successful biocontrol product currently available for bacterial diseases of plants (Farrand, 1990). However, a second generation of strain 84 was obtained by genetic engineering to safeguard crown gall biocontrol (Jones et al., 1988). This strain was called K1026 and became the first living genetically engineered microorganism (GEM) registered to be released as a commercially available pesticide (Jones and Kerr, 1989). A considerable number of studies have been performed to compare strain K1026 with its parent strain K84 but, to date, no review has been written compiling all this available information. The aim of this review is to give a current overview of the use of strain K1026 for biological control of crown gall disease.

Crown gall disease

Crown gall disease caused by different species of the genus *Agrobacterium* (e.g. *A. tumefaciens*, *A. rhizogenes* and *A. vitis*, formerly called biovars 1, 2 and 3 of *Agrobacterium* respectively) affects a wide range of crops. More than six hundred host species have been described (DeCleene and DeLey,

*We dedicate this review to the memory of our crown gall colleague Larry W. Moore who tragically died in 2000 while this article was in press.

1976), but the disease has been reported as economically important only in some of the potential hosts, such as: fruit trees (almonds, apples, apricots, cherries, peaches, pears, plums, etc.), nut trees (walnuts, pecans, etc.), caneberries (raspberry, blueberry, etc.), grapes and a wide variety of dicotyledoneous plants including some ornamental species (*Euonymus*, chrysanthemums, roses, poplars, etc.) (El-Fiki and Giles, 1981; Braun, 1982; Cooksey and Moore, 1982; Kerr and Tate, 1984; López et al., 1987; 1989; Moore and Canfield, 1996; Martí et al., 1999).

Losses in production due to this disease have not been recently evaluated in Europe, but a survey made in the 1980s in Spain indicated that 70% of nurseries had some attacks of crown gall, even with disease incidences up to 90% of galled plants in some plots (López et al., 1983). The disease has significant effects on the quality of the infected material (Journal Officiel des Communautés Européennes No. L 250/4-8 (7/10/93)), and although the disease can damage growing crops, economic losses are incurred primarily in nurseries where galled plants must be culled and discarded.

Agrobacterium species are soil-inhabiting bacteria which infect only at wound sites or lenticels and induce plant cells to proliferate as a tumor. This is achieved by the transfer of a discrete fragment of bacterial DNA (called T-DNA) to the nuclei of plant cells, where it directs the overproduction of plant growth hormones (auxins and cytokinins) and the synthesis of novel compounds called opines. The T-DNA is localized on tumorigenic *Agrobacterium* strains in large plasmids, called tumor-inducing (Ti) plasmids. As summarized by Kerr (1991), crown gall induction involves several steps: (a) chemotaxis of bacteria, responding to the production of some phenolic compounds from wounded tissue; (b) attachment and anchoring of bacteria to plant cells; (c) induction of the virulence genes by plant phenolic compounds; (d) processing of T-DNA, which is transferred to a plant cell; (e) integration of T-DNA into the plant genome; (f) synthesis of T-DNA-encoded plant hormones; (g) rapid plant cell division to form galls; (h) synthesis of opines. This process is followed by the preferential growth of *Agrobacterium* because of opine utilization and the possible transfer of Ti plasmid to other bacterial cells, induced by opines. The mechanism of this interkingdom gene transfer has been studied in great detail over the last decades and several reviews have been published on this topic (Hooykaas and Schilperoort, 1984; Kado, 1991; Winnans, 1992; Hooykaas and Beijersbergen, 1994; Sheng and Citovsky, 1996).

Characteristics of strain K84

The discovery of strain K84 was the product of keen observational skills in the field when New and Kerr (1972) observed that the ratio of pathogenic to nonpathogenic agrobacteria was closely correlated with the incidence of crown gall on almond seedlings. They then attempted to determine whether by increasing the number of nonpathogens on the roots, these bacteria would inhibit tumor induction by the pathogen. Strain K84 was isolated in Australia from soil obtained from around a peach gall, and when inoculated did not cause galls. It was selected among other nonpathogen isolates because when co-inoculated with a pathogen in a ratio 1 : 1 on plant roots, it completely prevented crown gall formation (Kerr, 1972; New and Kerr, 1972). Strain K84 was classified as *A. radiobacter*, following the taxonomy used at that time, based on its lack of pathogenicity, and belonged to the biovar 2 of *Agrobacterium* (Kerr and Panagopoulos, 1977). According to the current taxonomic classification of the genus *Agrobacterium*, strain K84 should be called *A. rhizogenes* (Sawada et al., 1993; Bouzar, 1994), however, as this new nomenclature of *Agrobacterium* species has not been adopted yet by many authors, the classical nomenclature will be used in this review.

Strain K84 harbors three indigenous plasmids: pAgK434 (also called pAtK84a) (> 300 kb) codifying for agrocin 434 production (Donner et al., 1993); pNoc (also called pAtK84b) (173 kb) encoding catabolism of nopaline (Sciaky et al., 1978; Ellis et al., 1982) and pAgK84 (47.7 kb) which encodes production and immunity to agrocin 84 (Ellis et al., 1979; Ryder et al., 1987). As pNoc has large areas of homology (over 50%) with the Ti plasmid of *A. tumefaciens* strain C58, it was suggested that pNoc might be a deletion product of a pTiC58-type plasmid that has been disarmed in the T-DNA and *vir* regions leading to a loss of oncogenicity (Clare et al., 1990).

Biocontrol with strain K84

By far, the most successful method for preventing crown gall has been the use of strain K84, and several reports have shown the efficacy of K84 in controlling crown gall in different hosts and countries all over the world (Moore and Warren, 1979; López et al., 1987; 1989; Farrand, 1990; Tawfik, 1990; Jones et al., 1991; Moore and Canfield, 1996). The primary trait exhibited by this biocontrol strain was the

production of the highly specific anti-agrobacterial antibiotic agrocin 84 (Kerr and Htay, 1974). This antibiotic is a di-substituted, fraudulent adenine nucleoside analog, which is active against certain pathogenic strains of *Agrobacterium* species (Kerr and Htay, 1974; Murphy and Roberts, 1979; Tate et al., 1979; Hayman and Farrand, 1988). Strain K84 successfully controls strains that are susceptible to agrocin 84 and production of this antibiotic is required for an effective control (Kerr and Htay, 1974; Cooksey and Moore, 1982; López et al., 1989). However, under field conditions, strain K84 can also control pathogens that are resistant to agrocin 84 (Cooksey and Moore, 1980; López et al., 1987; 1989; Bouzar et al., 1991; Vicedo et al., 1993; Penyalver and López, 1999; R. Penyalver and M.M. López, unpubl.). The mechanisms used by strain K84 to control agrocin 84-resistant pathogens have not yet been completely determined, but the available data suggest that the biocontrol achieved by this agent is a complex phenomenon, with production of agrocin 84 being only one of the traits involved in the process (Farrand and Wang, 1992; Vicedo et al., 1993).

Strain K84 produces a second anti-agrobacterial substance (agrocin 434), which is less inhibitory than agrocin 84 *in vitro* (Donner et al., 1993). This agrocin, which most probably is a di-substituted cytidine nucleoside (Fajardo et al., 1995), inhibits only biovar 2 strains of *Agrobacterium* (now called *A. rhizogenes*). Then, agrocin 434 may play a role in biocontrol of agrocin 434-susceptible pathogens of biovar 2 (McClure et al., 1998). K84 also produces a third *A*ntibiotic-*L*ike *S*ubstance named ALS84, which *in vitro* inhibits many tumorigenic *Agrobacterium* strains (Peñalver et al., 1994). The inhibitory activity of ALS84 is correlated to the production of siderophores by K84 under iron-limiting conditions (Penyalver et al., 2000). The chemical structure and the possible role of ALS84 in crown gall biocontrol by strain K84 have not yet been determined.

Possible causes of failure in biocontrol with strain K84

In spite of the success of K84, some potential problems could be associated with its application (Farrand, 1990; Jones et al., 1991; Vicedo et al., 1993; 1996; Moore and Canfield, 1996). Most of the few reported failures have not been explained because there is no available information about the strains isolated from K84-treated galled plants. Failures could have arisen from the presence of high populations of agrocin 84-resistant pathogens, latent infections, presence of *Agrobacterium* as endophytic pathogens, formulations with low numbers of viable K84 cells, low concentration of K84 on the roots compared to the soil pathogen inoculum, inappropriate inoculation treatments, poor survival of K84 in the soil, presence of antagonists or competitors for root colonization, etc. Furthermore, it is necessary to remark that biological control experiments should mimic natural conditions of infection and treatments as being representative of the real efficacy of strain K84. Co-inoculation experiments treating stems or roots with a mixture of pathogen and biocontrol agent are not adequate because they do not reproduce conditions achieved by K84 when used in nature.

Another cause of failure related to plasmid transfer is discussed here in more detail. Panagopoulos et al. (1979) reported a failure of K84 in a field experiment where K84 and an agrocin 84-susceptible pathogen were co-inoculated on to almond roots. Pathogens resistant to agrocin 84 were subsequently isolated in the many galls which developed, and among them many isolates were also producers of agrocin 84. The inference from this data was that genes controlling agrocin 84 production and resistance were transferred from strain K84 to the pathogenic recipient, resulting in a breakdown in this biocontrol experiment because after such transfer the pathogenic recipient becomes resistant to agrocin 84. At that time pAgK84 was also transferred *in vitro* from K84 to recipient agrobacteria (Ellis and Kerr, 1979; Ellis et al., 1979). The occurrence of this transfer event in this biocontrol experiment was probably due partly to the high population densities of both the pathogen and of the control agent, which were each introduced artificially. Nevertheless, these results raise the question of whether such a breakdown could occur during the normal practice of biological control using K84 in the field. In this regard, we reported that the transfer of pAgK84 from K84 to *A. tumefaciens* could also be demonstrated in several biocontrol experiments, in which soil inoculation and plant treatments simulated closely the conditions found when biological control is performed in nurseries (Vicedo et al., 1993; Vicedo, 1995). Furthermore, Lu (1994) reported the recovery of several pathogenic *Agrobacterium* isolates containing pAgK84 from galls on plants given a pretreatment with K84 and grown in commercial nurseries in USA. Also, Stockwell et al. (1996) reported field detection of pathogenic transconjugants resulting from a co-inoculation experiment on cherry. All these reports show the relatively frequent

occurrence of pAgK84 transfer during practical use of strain K84. This could dramatically decrease its efficacy because transconjugants are agrocin 84 producers and resistant to the antibiotic. Moreover, biocontrol experiments using such transconjugants as challenge pathogens demonstrated that they were not controlled by K84 and suggested that they were threatening K84 efficacy in the short or medium term (Vicedo, 1995). To avoid this transfer and safeguard the biocontrol of crown gall against breakdown due to conjugal transfer of pAgK84 from K84 to pathogenic *Agrobacterium* strains, the GEM strain K1026, harboring a pAgK84 transfer deficient derivative (Tra$^-$), was developed (Jones et al., 1988).

The GEM strain K1026

Strain K1026 was obtained after successful cooperation between the Australian teams involved in the discovery and characterization of strain K84 and a North American team working on the biology of *Agrobacterium* spp. plasmids (Jones et al., 1988). Restriction maps of pAgK84 were produced and the conjugal transfer determinants (Tra region) defined by transposon mutagenesis (Figure 1) (Slota and Farrand, 1982; Farrand et al., 1985; Ryder et al., 1987). Derivatives of K84 containing transfer-deficient Tn*5*-insertion mutants of pAgK84 controlled crown gall as effectively as K84 (Shim et al., 1987). However, these mutants were not desirable replacements for pAgK84 since they could revert to Tra$^+$ and because Tn*5* carries three different antibiotic resistance genes (Jones et al., 1988). Using recombinant DNA techniques, a stable Tra$^-$ deletion mutant of pAgK84, called pAgK1026, was constructed which has neither of these undesirable properties. The new derivative of pAgK84 has a 5.9 kb deletion of *Eco*RI fragments D1 and H (Figure 1), which contains the transfer (Tra) region. pAgK1026 is incapable of conjugal transfer at a detectable frequency in the laboratory (Jones et al., 1988). Later, sequence analysis of 0.5 kb from the right side of the *Eco*RI fragment H has shown that it contains sequences with homology to genes of other conjugal transfer systems, while the next *Eco*RI fragment F contains open reading frames with homology to genes involved in plasmid stability as previously reported by mutagenesis analysis (R. Penyalver and S.K. Farrand, University of Illinois at Urbana-Champaign, Illinois, USA, unpubl.). This sequence analysis suggests that the Tra region of pAgK84 could extend further than previously described (Figure 1).

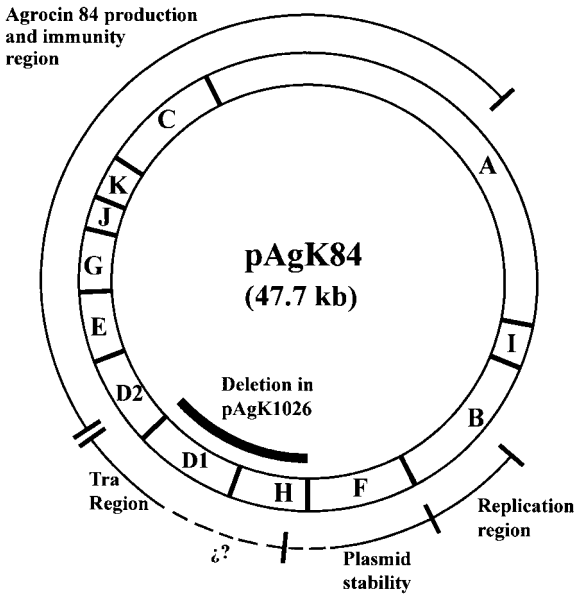

Figure 1. *Eco*RI restriction map of pAgK84 showing the transfer, plasmid stability, replication, and synthesis and immunity to agrocin 84 regions, according to Farrand et al. (1985, 1992). The Tra$^-$ pAgK1026 does not contain *Eco*RI fragments D1 and H (Jones et al., 1988).

The new derivative of strain K84 with pAgK1026 was named strain K1026 (Jones et al., 1988).

Biocontrol efficiency of strain K1026

The comparative *in vivo* efficacy of strains K84 and K1026 against *Agrobacterium* strains susceptible and resistant to agrocin 84 is summarized in Table 1. These experiments clearly demonstrated that strain K1026 is as efficient as K84 in controlling crown gall under different experimental conditions, hosts and countries. Both strains controlled crown gall even under high disease pressure. Both were also able to control disease caused by both agrocin 84-susceptible and -resistant pathogens, showing that strain K1026 was also able to use other mechanisms not related to agrocin 84 susceptibility in the same manner as strain K84. Due to restrictions to the use of a GEM in the open in many countries, the only available results about comparative biocontrol efficacy in open field conditions are those from Tunisia and USA, where both strains showed similar results. In addition, four major commercial nurseries in Australia have provided declarations on the efficacy of strain K1026 to control crown gall disease in a number of plant hosts. In some of these

Table 1. Comparitive biocontrol efficacy of strains K84 and K1026

Assay	Country	Host	Challenge pathogen	Susceptibilty to agrocin 84	Treatment	Number of analyzed plants	% of plants with galls	Index of biocontrol efficiency[a]	Source
1	Australia	Young almonds	K27	Susceptible	Untreated	12	100		Jones and Kerr, 1989
					K84	14	14	86	
					K1026	12	25	75	
		Old almonds	K27	Susceptible	Untreated	15	100		
					K84	15	20	80	
					K1026	15	27	73	
2	Jordan	Tomato	B4	Susceptible	Untreated	12	75		Fakhouri and Khalaif, 1996
					K84	12	0	100	
					K1026	12	0	100	
			8301	Susceptible	Untreated	12	83		
					K84	12	0	100	
					K1026	12	0	100	
			B1	Resistant	Untreated	12	67		
					K84	12	0	100	
					K1026	12	0	100	
3	Spain	Hybrid peach × almond GF677	804-42	Susceptible	Untreated	110	3		Vicedo et al., 1993
					K84	110	0	100	
					K1026	110	0	100	
			805-3	Susceptible	Untreated	110	13		
					K84	110	0	100	
					K1026	110	1	92	
		Hybrid peach × almond Adafuel	804-42	Susceptible	Untreated	110	21		
					K84	110	1	95	
					K1026	110	0	100	
			805-3	Susceptible	Untreated	110	71		
					K84	110	0	100	
					K1026	110	1	99	
		Hybrid peach × almond GF677	678-2	Resistant	Untreated	50	50		
					K84	50	17	66	
					K1026	50	0	100	
			436-3	Resistant	Untreated	50	19		
					K84	50	0	100	
					K1026	50	0	100	
4	Spain	Hybrid peach × almond GF677	325-4	Susceptible	Untreated	78	69		Vicedo et al., 1996
					K84	85	6	91	
					K1026	99	1	96	
5	Spain	Hybrid peach × almond GF677	325-4	Susceptible	Untreated	61	91		López-López et al., 1999
					K84	50	4	96	
					K1026	57	7	92	
6	Spain	Hybrid peach × almond GF677	B6	Resistant	Untreated	76	9		Penyalver and López, 1999
					K84	56	0	100	
					K1026	70	0	100	
			66R	Resistant	Untreated	66	18		
					K84	80	0	100	
					K1026	61	2	89	
7	Spain	Cherry ‘Camil’	678-2	Resistant	Untreated	41	66		R. Penyalver and M.M. López (unpubl.)
					K84	38	8	88	
					K1026	39	8	88	
		Cherry ‘Colt’	678-2	Resistant	Untreated	57	54		
					K84	57	7	87	
					K1026	57	5	90	
		Cherry ‘Damil’	678-2	Resistant	Untreated	52	8		
					K84	28	0	100	
					K1026	32	0	100	

Table 1. (*Continued*)

Assay	Country	Host	Challenge pathogen	Susceptibilty to agrocin 84	Treatment	Number of analyzed plants	% of plants with galls	Index of biocontrol efficiency[a]	Source
		Cherry 'F12.1'	678-2	Resistant	Untreated	39	85		
					K84	20	30	65	
					K1026	22	14	84	
		Cherry 'Inmil'	678-2	Resistant	Untreated	65	19		
					K84	46	4	77	
					K1026	39	3	86	
8	Tunisia	Sour almond	Natural infection		Untreated	47	100		A. Boubaker (unpubl.)
					K84	59	3	97	
					K1026	117	3	97	
9	USA	Apple	n.a.[b]	n.a.	Untreated	125	7		L.W. Moore (unpubl.)
					K84	125	2	71	
					K1026	125	0	100	
		Apricot/Peach	n.a.	n.a.	Untreated	125	17		
					K84	125	1	94	
					K1026	125	0	100	
		Pear	n.a.	n.a.	Untreated	125	10		
					K84	125	1	90	
					K1026	125	0	100	
		Prunus tomentosa	n.a.	n.a.	Untreated	125	9		
					K84	125	0	100	
					K1026	125	0	100	
		Walnut	n.a.	n.a.	Untreated	125	7		
					K84	125	0	100	
					K1026	125	0	100	

[a] Index of crown gall biocontrol = 100% − [(% of disease incidence in treatment × 100)/(% of disease incidence in corresponding untreated control)] according to Penyalver and López (1999).
[b] Not available.

nurseries, strain K1026 has been in use now for over 10 years (G.K. Bullard, Bio-Care Technology Pty. Ltd., Somersby, Australia, pers. comm.). We can infer from the reported data that strain K1026 would significantly control crown gall disease resulting from natural infections in nurseries or in the field, where the incidence of the disease is usually lower that those resulting from deliberate inoculations with the pathogen.

Agrocin 84-plasmid transfer

Comparative studies have been performed to evaluate the transfer frequency of the agrocin 84-plasmid from strains K84 and K1026 to *Agrobacterium* recipients in several biocontrol assays performed with different plant hosts (peach × almond hybrids Adafuel and GF677, osier and rose). In four out of 10 assays carried out recently, pAgK84 was transferred from K84 to the recipient pathogen in crown gall tissues from galled K84-treated plants, but in none of the assays was pAgK1026 transfer detected in galls from K1026-treated plants (Vicedo et al., 1993; Vicedo, 1995). Transfer of pAgK84 was detected in tumours from peach × almond GF677, osier and rose hosts. These data suggest a quite high frequency of pAgK84 transfer from K84, as discussed above, and show the absence of such transconjugants when using strain K1026 for biocontrol of crown gall disease in semi-natural conditions.

Antibiotic production by strain K1026: agrocins 84, 434 and ALS84

Strain K1026 produces agrocin 84, indicating that pAgK1026 retains the agrocin 84 biosynthetic locus of pAgK84 (Jones et al., 1988). Furthermore, the amounts of agrocin 84 produced by strain K1026 are similar to those produced by K84, suggesting that pAgK1026 retains the copy number of its pAgK84 progenitor and that no plasmid stability determinants were removed

(Figure 1). Strain K1026 also produces agrocin 434 to the same extent as K84, since production of this agrocin is encoded on pAgK434, which is present in both strains (Donner et al., 1993). ALS84 is also produced by strain K1026 in the same amount as by strain K84, since its production seems to be chromosomally encoded (Peñalver et al., 1994; Penyalver et al., 2000).

Root colonization and survival in the rhizosphere by strain K1026

A biocontrol agent should grow and persist on the surface of the plant it protects. Colonization and survival in the rhizosphere is widely believed to be essential for biocontrol of soilborne pathogens (Weller, 1988; Handelsman and Stabb, 1996). K84 has proved to be a good colonizer of the root systems of different hosts (Ellis et al., 1979; Shim et al., 1987; Macrae et al., 1988; Stockwell et al., 1993; Vicedo et al., 1993). It is reasonable to hypothesize that the capacity of strain K84 to colonize the roots of treated plants is an important factor required for the successful biocontrol of crown gall. In this regard, levels of root colonization of peach seedling by strains K84 and K1026 were similar, showing that K1026 was as good a colonizer as K84, at least during the 21-day study period (Vicedo et al., 1993).

Strain K1026 was also recovered from roots seven months after inoculation on almond seedlings (Jones and Kerr, 1989). Moreover, studies comparing the abilities of strains K84 and K1026 to persist in the rhizosphere have shown that both strains survived at levels of ca. 10^6 colony forming units (CFU) per gram of root eight months after they had been inoculated on to the roots of peach × almond GF677 plants (López-López et al., 1999; Penyalver and López, 1999).

In conclusion, deletion of *tra* from pAgK84 in strain K84 to produce K1026 does not affect its ability to colonize and survive in the rhizosphere.

Possible causes of failure in biocontrol with strain K1026

Crown gall biocontrol could also fail via the transfer of Ti plasmid from a pathogenic *Agrobacterium* donor to strain K84, which then becomes pathogenic while retaining agrocin 84 production and immunity. pNoc of strain K84 belongs to the same incompatibility group (Inc Rh1) as the nopaline-type Ti plasmids (Kerr and Ellis, 1982; Clare et al., 1990; Farrand, 1993) and its presence in strain K84 has been thought to safeguard biocontrol by preventing acquisition of Ti plasmid (Kerr and Ellis, 1982; Clare et al., 1990). Even though incompatible plasmids are unable to replicate in the same cell (Kerr and Ellis, 1982; Novick, 1987), such protection may not be as effective as was once thought. Stockwell et al. (1990) reported that a transposon-tagged Ti plasmid had transferred to strain K84 after co-inoculation of *A. tumefaciens* and K84 in tomato stems, but no characterization of the transconjugants was performed. Furthermore, we have described the spontaneous transfer of the Ti plasmid from *A. tumefaciens* to strain K84 in crown gall tissue. This transfer event was detected only once in one tumor in a biocontrol assay. Southern blot hybridization analysis suggested that recombination between Ti plasmid and pNoc could have occurred in K84, resulting in a new Ti plasmid (Vicedo et al., 1996). The frequency at which the Ti plasmid could be conjugatively transferred to strain K84 in the field and its repercussions on biocontrol effectiveness are not yet known. The behavior of the virulent transconjugant derived from K84 obtained in those studies was compared with the behavior of the wild-type *A. tumefaciens* donor of the Ti plasmid (López-López et al., 1999). Host range, ability to induce tumors in several fruit trees and stability of the pathogenic determinants in isolates from tumors did not differ between both strains. However, the transconjugant was not controlled by K84 and survived in the root system at larger population densities than the wild-type Ti-plasmid donor strain (López-López et al., 1999). Then, appearance and the possibility of persistence in soil of strains harboring a Ti plasmid in K84 background has been described (Vicedo et al., 1996), but it seems an infrequent event. Nevertheless, it would represent a risk to the biocontrol of crown gall using strain K1026, if this strain is also able to acquire a Ti plasmid and then become pathogenic. Transfer of Ti plasmids from different strains of *A. tumefaciens* to strain K1026 has never been detected in several biocontrol experiments (López-López et al., 1999).

Production, formulation and delivery

K84 is supplied commercially by several companies in many different preparations: a bacterial culture in agar plates, as freeze dried cells, in a formulation containing carboxymethyl cellulose, and in a finely ground peat preparation similar to the *Rhizobium* inoculants. Different carriers and production technologies have been tested for commercial formulations of K84, and those

currently available satisfy requirements for production, storage, cost and application considered desirable for an effective commercial biocontrol product (Moore and Warren, 1979; López et al., 1987; Presenti-Barili et al., 1991). Strain K1026 has the same properties as its parent strain K84 for commercial formulation and it is currently supplied as a peat preparation.

Safety

Toxicity studies with K84 and the evidence of its world-wide use for nearly 30 years indicate that it is harmless to plants, animals and humans (Moore and Warren, 1979). Strain K1026 is a GEM but evidence indicates that it is still harmless to these organisms and also to the environment (Jones and Kerr, 1989). It should be pointed out that: (1) strain K84, the progenitor of K1026, has been registered as a biopesticide and been used commercially in several countries where there have been no reports of harm; (2) strain K1026 is identical to strain K84 except that it lacks a 5.9 kb portion of the agrocin 84-plasmid, therefore preventing agrocin 84-plasmid transfer (Jones et al., 1988). No foreign DNA remains in GEM K1026 (Jones et al., 1988); (3) strain K1026 contains no Ti-plasmid-encoded genes involved in crown gall induction (Clare et al., 1990); (4) strain K1026 is a biovar 2-strain of *Agrobacterium* and cannot grow at 37 °C (human body temperature) (Kerr and Panagopoulos, 1977). *A. radiobacter*-like organisms are becoming an increasingly opportunistic human pathogens (Edmond et al., 1993), but those clinical strains showed a higher temperature tolerance and can grow at 35 °C, while strain K1026 does not; (5) the agrocin 84 produced by K1026 and K84 is specific for agrocinopine-catabolizing agrobacteria, most of which are plant pathogens; other organisms are unaffected (Engler et al., 1975). Therefore, just as no ecological damage has been reported from production of agrocins 84, 434 and ALS84 when using K84, none should result from production of these substances when using K1026.

Commercial availability

Strain K1026 was registered as a biopesticide in Australia at the end of 1988 and became the first, live GEM to be registered for commercial release to the public. The commercial product is sold as NOGALL™, by Bio-Care Technology Pty. Ltd., Somersby, Australia. It is produced as a peat-based formulation that contains a minimum of 10^9 bacteria per gram, and has an advertized shelf life of six months. Recently, NOGALL™ received official approval to be used as a biopesticide in the USA (G.K. Bullard, Bio-Care Technology Pty. Ltd., Somersby, Australia, pers. comm.).

Lanning (1991) pointed out that a 'model' GEM product is needed to convince both regulators and the public of the benefits and acceptability of GEMs. The described properties of strain K1026 qualify it as a model GEM; it is relatively noncontentious from a regulatory viewpoint since it involves a deletion, rather than the more controversial insertion of new DNA, and the deletion was done in a well-characterized organism.

The available information supports the registration of the GEM strain K1026 to be used as a biopesticide for biocontrol of crown gall disease because it is effective under different conditions, hosts, countries, and because its utilization should not represent a risk either for living organisms nor for the environment. Strain K1026 should be used wherever strain K84 is employed to safeguard biological control of crown gall disease.

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References

Bouzar H (1994) Letter to the editor: request for a judicial opinion concerning the type species of *Agrobacterium*. International Journal of Systematic Bacteriology 44: 373–374

Bouzar H, Daouzli N, Krimi Z, Alim A and Khemici E (1991) Crown gall incidence in plant nurseries of Algeria,

characteristics of *Agrobacterium tumefaciens* strains, and biological control of strains sensitive and resistant to agrocin 84. Agronomie 11: 901–908

Braun AC (1982) A history of the crown gall problem. In: Kahl G and Schell JS (eds) Molecular Biology of Plant Tumors. (pp 155–210) Academic Press, New York

Clare BG, Kerr A and Jones DA (1990) Characteristics of the nopaline catabolic plasmid in *Agrobacterium* strains K84 and K1026 used for biological control of crown gall disease. Plasmid 23: 126–137

Cooksey DA and Moore LW (1980) Biological control of crown gall with fungal and bacterial antagonists. Phytopathology 70: 506–509

Cooksey DA and Moore LW (1982) Biological control of crown gall with an agrocin mutant of *Agrobacterium radiobacter*. Phytopathology 72: 919–921

DeCleene M and DeLey J (1976) The host range of crown gall. Botanical Gazette 42: 389–466

Donner SC, Jones DA, McClure NC, Rosewarne GM, Tate ME, Kerr A, Fajardo NN and Clare BG (1993) Agrocin 434, a new plasmid encoded agrocin from the biocontrol *Agrobacterium* strains K84 and K1026, which inhibits biovar 2 agrobacteria. Physiological and Molecular Plant Pathology 42: 185–194

Edmond MB, Riddler SA, Baxter CM, Wicklund BM and Pasculle AW (1993) *Agrobacterium radiobacter*: a recently recognized opportunistic pathogen. Clinical Infectious Diseases 16: 388–391

El-Fiki F and Giles KL (1981) Agrobacterium tumefaciens in agriculture and research. In: Giles KL and Atherley AG (eds) Biology of the Rhizobiaceae (pp 47–58) International Review of Citology. Suppl 13. Academic Press, New York

Ellis JG and Kerr A (1979) Transfer of agrocin 84 production from strain 84 to pathogenic recipients: a comment on previous paper. In: Schippers B and Gams W (eds) Soil-borne pathogens (pp 579–583) Academic Press, New York

Ellis JG, Kerr A, Van Montagu M and Schell J (1979) *Agrobacterium* genetic studies on agrocin 84 production and the biological control of crown gall. Physiological Plant Pathology 15: 311–319

Ellis JG, Kerr A, Petit A and Tempe J (1982) Conjugal transfer of nopaline and agropine Ti-plasmids – the role of agrocinopines. Molecular and General Genetics 186: 269–274

Engler G, Holsters M, Van Montagu M, Schell J, Hernalsteens JP and Shilperoort R (1975) Agrocin 84 sensitivity: a plasmid determined property in *Agrobacterium tumefaciens*. Molecular General Genetics 138: 345–349

Fakhouri WD and Khalaif H (1996) Biocontrol of crown gall disease in Jordan. *Dirasat*, Agricultural Sciences 23: 17–22

Fajardo NN, Tate ME and Clare BG (1995) Agrocin 434: an additional biological control component for crown gall. In: Ryder MH, Stephens PM and Bowen GD (eds) Improving Plant Productivity with Rhizosphere Bacteria (pp 128–130) CSIRO Division of Soils, Adelaide, Australia

Farrand SK (1990) *Agrobacterium radiobacter* strain K84: a model control system. In: New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases (pp 679–691) Alan R. Liss, Inc

Farrand SK (1993) Conjugal transfer of *Agrobacterium* plasmids. In: Clewell DB (ed.) Bacterial Conjugation (pp 255–291) Plenum Press, New York

Farrand SK and Wang C (1992) Do we really understand crown gall control by *Agrobacterium radiobacter* strain K84? In: Tjamos ES et al. (ed.) Biological Control of Plant Diseases (pp 287–293) Plenum Press, New York

Farrand SK, Slota JE, Shim JS and Kerr A (1985) Tn5 insertion in the agrocin 84 plasmid: the conjugal nature of pAgK84 and the location of determinants for transfer and agrocin 84 production. Plasmid 13: 106–117

Farrand SK, Wang C, Hong S, O'Morchoe SB and Slota JE (1992) Deletion derivatives of pAgK84 and their use in the analysis of *Agrobacterium* plasmid functions. Plasmid 28: 201–212

Handelsman J and Stabb EV (1996) Biocontrol of soilborne plant pathogens. Plant Cell 8: 1855–1869

Hayman GT and Farrand SK (1988) Characterization and mapping of the agrocinopine-agrocin 84 locus on the nopaline Ti plasmid pTiC58. Journal of Bacteriology 170: 1759

Hooykaas JJ and Beijersbergen GM (1994) The virulence system of *Agrobacterium tumefaciens*. Annual Review of Phytopathology 32: 157–179

Hooykaas JJ and Schilperoort RA (1984) The molecular genetics of crown gall tumorigenesis. Annual Review of Genetics 22: 209–283

Jones DA and Kerr A (1989) *Agrobacterium radiobacter* K1026 a genetically engineered derivative of strain K84, for biological control of crown gall. Plant Disease 73: 15–18.

Jones DA, Ryder MH, Clare BG, Farrand SK and Kerr A (1988) Construction of a Tra^- deletion mutant of pAgK84 to safeguard the biological control of crown gall. Molecular General Genetics 212: 207–214

Jones DA, Ryder MH, Clare BG, Farrand SK and Kerr A (1991) Biological control of crown gall using *Agrobacterium* strains K84 and K1026. In: Komada H, Kiritani K and Bay-Petersen J (eds) The biological control of plant diseases (pp 161–170) FFTC Book Series no. 42. Food and Fertilizer Technology Center for the Asian and Pacific Region, Taipei, Taiwan

Kado CI (1991) Molecular mechanisms of crown gall tumorigenesis. Critical Reviews in Plant Sciences 10: 1–32

Kerr A (1972) Biological control of crown gall: seed inoculation. Journal of Applied Bacteriology 35: 493–497

Kerr A (1991) The Genus *Agrobacterium*. In: Balows A, Trüper HG, Dworkin M, Harder W and Schleifer KH (eds) The Prokaryotes 2nd ed. Vol. III (pp 2214–2235) Springer-Verlag, New York, Inc

Kerr A and Ellis JG (1982) Conjugation and transfer of Ti plasmid in *Agrobacterium tumefaciens*. In: Kahl G and Schell JS (eds) Molecular Biology of Plant Tumors (pp 321–344) Academic Press, New York

Kerr A and Htay K (1974) Biological control of crown gall through bacteriocin production. Physiological Plant Pathology 4: 37–44

Kerr A and Panagopoulos CG (1977) Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. Phytopathologische Zeitschrift 90: 172–179.

Kerr A and Tate ME (1984) Agrocins and the biological control of crown gall. Microbiological Sciences 1: 1–4

Lanning S (1991) Bright future for GEMs? Bio/Technology 9: 915

López MM, Salcedo CI, Orive RJ and Temprano FJ (1983) Características de aislados españoles de *Agrobacterium radiobacter* pv. *tumefaciens*. Anales Instituto Nacional de Investigaciones Agrarias, Serie Agrícola 24: 239–249

López MM, Gorris MT, Temprano FJ and Orive RJ (1987) Results of seven years of biological control of *Agrobacterium tumefaciens* in Spain. Bulletin EPPO/OEPP Bulletin 17: 273–280

López MM, Gorris MT, Salcedo CI, Montojo AM and Miró M (1989) Evidence of biological control of *Agrobacterium tumefaciens* strains sensitive and resistant to agrocin 84 by different *Agrobacterium radiobacter* strains on stone fruit trees. Applied and Environmental Microbiology 55: 741–746

López-López MJ, Vicedo B, Orellana N, Piquer J and López MM (1999) Behavior of a virulent strain derived from *Agrobacterium radiobacter* strain K84 after spontaneous Ti plasmid acquisition. Phytopathology 89: 286–292

Lu SF (1994) Isolation of putative pAgK84 transconjugants from commercial cherry and raspberry plants treated with *Agrobacterium radiobacter* strain K84. MS Thesis. Oregon State University, University of Missouri, Colombia, USA

Macrae S, Thomson JA and Van Staden J (1988) Colonization of tomato plants by two agrocin-producing strains of *Agrobacterium tumefaciens*. Applied and Environmental Microbiology 54: 3133–3137

Marti R, Cubero J, Daza A, Piquer J, Salcedo CI, Morente C and López MM (1999) Evidence of migration and endophytic presence of *Agrobacterium tumefaciens* in rose plants. European Journal of Plant Pathology 105: 39–50

McClure NC, Ahmadi AR and Clare BG (1998) Construction of a range of derivatives of the biological control strain *Agrobacterium rhizogenes* K84: a study of factors involved in biological control of crown gall disease. Applied and Environmental Microbiology 64: 3977–3982

Moore LW and Canfield M (1996) Biology of *Agrobacterium* and management of crown gall disease. In: Hall R (ed) Principles and Practice of Managing Soilborne Plant Pathogens (pp 151–191)

Moore LW and Warren G (1979) *Agrobacterium radiobacter* strain 84 and biological control of crown gall. Annual Review of Phytopathology 17: 163–179

Murphy PJ and Roberts WP (1979) A basis for agrocin 84 sensitivity in *Agrobacterium radiobacter*. Journal of General Microbiology 114: 207–213

New PB and Kerr A (1972) Biological control of crown gall: field measurements and glasshouse experiments. Journal of Applied Bacteriology 35: 279–287

Novick RP (1987) Plasmid incompatibility. Microbiological Review 51: 381–395.

Panagopoulos CG, Psallidas PG and Alivizatos AS (1979) Evidence of a breakdown in the effectiveness of biological control of crown gall. In: Schippers B and Gams W (eds) Soil-Borne Plant Pathogens (pp 569–578) Academic Press, London, United Kingdom

Penyalver R and López MM (1999) Co-colonization of the rhizosphere by pathogenic *Agrobacterium strains* and non-pathogenic strains K84 and K1026, used for crown gall biocontrol. Applied and Environmental Microbiology 65: 1936–1940

Peñalver R, Vicedo B, Salcedo CI and López MM (1994) *Agrobacterium radiobacter* strain K84, K1026 and K84 Agr^- produce an antibiotic-like substance, active *in vitro* against *A. tumefaciens* and phytopathogenic *Erwinia* and *Pseudomonas*. Biocontrol Science and Technology 4: 259–267

Penyalver R, Oger P, López MM and Farrand SK (2000) Iron-binding compounds in *Agrobacterium* spp.: the biological control strain K84 produces a hydroxamate siderophore (Submitted for publication)

Presenti-Barili B, Ferdani E, Mosti M and Degli-Innocenti F (1991) Survival of *Agrobacterium radiobacter* K84 on various carriers for crown gall control. Applied and Environmental Microbiology 57: 2047–2051

Ryder MH, Slota JE, Scarim A and Farrand SK (1987) Genetic analysis of agrocin 84 production and immunity in *Agrobacterium* spp. Journal of Bacteriology 169: 4184–4189

Sawada H, Ieki H, Oyaizu H and Matsumoto S (1993) Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. International Journal of Systematic Bacteriology 43: 694–702

Sciaky D, Montoya AL and Chilton MD (1978) Fingerprints of *Agrobacteirum* Ti plasmids. Plasmid 1: 238–253

Sheng J and Citovsky V (1996) *Agrobacterium*-plant cell DNA transport: have virulence proteins, will travel. Plant Cell 8: 1699–1710

Shim JS, Farrand SK and Kerr A (1987) Biological control of crown gall: construction and testing of new biocontrol agents. Phytopathology 77: 463–466

Slota JE and Farrand SK (1982) Genetic isolation and physical characterization of pAgK84, the plasmid responsible for agrocin 84 production. Plasmid 8: 175–186

Stockwell VO, Kawalek MD, Moore LW and Loper JE (1990) Plasmid transfer between *Agrobacterium radiobacter* K84 and *A. tumefaciens* in crown gall tissue. Phytopatology 80: 1001

Stockwell VO, Moore LW and Loper JE (1993) Fate of *Agrobacterium radiobacter* K84 in the environment. Applied and Environmental Microbiology 59: 2112–2120

Stockwell VO, Kawalek MD, Moore LW and Loper JE (1996) Transfer of pAgK84 from the biocontrol agent *Agrobacterium radiobacter* K84 to *A. tumefaciens* under field conditions. Phytopathology 86: 31–37

Tate ME, Murphy PJ, Roberts WP and Kerr A (1979) Adenine N^6-substituent of agrocin 84 determines its bacteriocin-like specificity. Nature (London) 280: 697–699

Tawfik AE (1990) Comparison of biological and chemical soil treatments for controlling crown gall in peach. Agricultural Research Review 68: 555–561

Vicedo B (1995) Plasmid transfer between *Agrobacterium tumefaciens* and *A. radiobacter* in biological control assays: characterization and behavior of the transconjugants. PhD Thesis. University of Valencia, Valencia, Spain

Vicedo B, Peñalver R, Asins MJ and Lopez MM (1993) Biological control of *Agrobacterium tumefaciens*, colonization, and pAgK84 transfer with *Agrobacterium radiobacter* K84 and the Tra-mutant strain K1026. Applied Environmental and Microbiology 59: 309–315

Vicedo B, López MJ, Asins MJ and López MM (1996) Spontaneous transfer of the Ti plasmid of *Agrobacterium tumefaciens* and the nopaline catabolism plasmid of *A. radiobacter* strain K84 in crown gall tissue. Phytopathology 86: 528–534

Weller DM (1988) Biological control of soilborne plant pathogenic in the rhizosphere with bacteria. Annual Review of Phytopathology 26: 379–407

Winnans SC (1992) Two-way chemical signaling in *Agrobacterium*-plant interactions. Microbiological Reviews 56: 12–31