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Biological Control of Crown Gall Using *Agrobacterium* Strains K84 and K1026*

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Abstract

Crown gall has been successfully controlled on a commercial scale for over 15 years by the use of *Agrobacterium radiobacter* strain K84. The continued success of strain K84 has been jeoparised by the possibility of transfer of the agrocin plasmid, pAgK84, to pathogenic agrobacteria, making them resistant to control. The construction of a transfer-deficient deletion mutant of pAgK84 has resulted in a strain (K1026) from which the agrocin plasmid (pAgK1026) can no longer be transferred to other agrobacteria. Strain K1026 controls crown gall as effectively as strain K84 and may be used to ensure that the danger of breakdown of biological control is minimised.

Introduction

Crown gall is caused by the soil-inhabiting bacterium *Agrobacterium tumefaciens* (Smith & Townsend 1907) Conn 1942. The disease is especially important in stone fruits (*Prunus* spp.), rose (*Rosa* sp.) and grape (*Vitis vinifera* L.), but a wide variety of dicotyledonous plants is susceptible (DeCleene and DeLey 1976). Losses in production due to the disease in the USA were estimated by Kennedy and Alcorn (1980), who listed *A. tumefaciens* as the third most important prokaryotic plant pathogen.

The majority of the genes responsible for crown gall induction by pathogenic agrobacteria are plasmid-borne. In the case of crown gall, a part of the tumour-inducing (Ti) plasmid, the T-DNA, is transferred to the plant chromosomal DNA where it is expressed and stably maintained. The expression of T-DNA genes coding for enzymes involved in the metabolism of plant growth substances leads to the tumorous growth habit. This subject has been reviewed extensively (see for example Bevan and Chilton 1982; Kahl and Schell 1982; Nester *et al.* 1984).

Other T-DNA genes code for enzymes that produce compounds called opines. Opines are low molecular weight metabolites that are not present in normal plant tissue and act as a nutrient source for the crown gall-inducing bacteria. The genes for use of opines are located on the Ti plasmid. Ti plasmids are classified according to the type of opine(s) present in the tumours they induce. Two of the Ti plasmid types are respectively nopaline/agrocinopine A (Ellis and Murphy 1981) and octopine/agropine (Firmin and Fenwick 1978).

Strains of *Agrobacterium* are classified into three biovars on the basis of carbon source utilisation and other biochemical tests (Kerr and Panagopoulos 1977). The biovars correspond to different chromosomal forms of the bacterium. The relative importance of the different

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biovars as causal agents of crown gall varies with location and plant host. For example, in Australia the main pathogenic forms on almond (*Prunus amygdali* L.) and stone fruit are nopaline/agrocinopine A strains of biovar 2. In Spain, biovar 2 strains commonly affect stone fruits whereas biovar 1 strains are important pathogens on rose (López *et al.* 1987). Biovar 3 strains are the most important pathogens of grape (Kerr and Panagopoulos 1977).

Biological Control of Crown Gall using Strain K84

A successful biological control for crown gall has been developed by Kerr and associates (New and Kerr 1972; Htay and Kerr 1974; Kerr and Htay 1974; Kerr 1980). The method involves the inoculation of sowing or planting material with strain K84 of *Agrobacterium radiobacter* (Beijerinck & van Delden 1902) Conn 1942. Kerr and others have amply demonstrated the usefulness of strain K84.

Agrobacterium radiobacter strain K84 was originally isolated from soil around a crown gall-affected peach tree near Adelaide, South Australia (New and Kerr 1972). It is non-pathogenic and belongs to the biovar 2 subgroup of the genus.

There are numerous publications on the experimental use of strain K84 to control crown gall. Moore (1979) tabulated accounts of the use of strain K84 against crown gall on different host plants, both experimentally and in nurseries. His list included plant species from many families including Rosaceae, Juglandaceae, Compositae and Salicaceae. The degree of control achieved varied, but strain K84 was very effective in many cases, often giving 100% control in naturally infested soil. In Australia, strain K84 is used commercially mainly on stone fruits, almond and rose.

The control method involves dipping the planting material (either seeds or roots of seedlings) in a tapwater suspension of strain K84 at 10^7 to 10^8 cells per mL immediately before sowing or planting. The widespread commerical use of the method is a testimony to its practical nature and usefulness. The success of this strain is remarkable, considering the range of conditions in which it has been applied: these include its use on many different kinds of horticultural and ornamental plants on at least four continents (i.e. Asia, Australia, Europe and North America).

Crown gall has been controlled by commercially available antibiotics (Schroth *et al.* 1971; Moore 1977), but biological control using strain K84 is in many situations used instead of chemical control because it is both more effective and less expensive. Moore (1977) showed that strain K84 performed better than commercial chemical treatments in preventing crown gall on cherry seedlings.

Strain K84, like most other methods of crown gall control, can only be used as a preventative measure. Strain K84 cannot control crown gall in all situations; for example octopine strains of biovar 1, and also the biovar 3 strains that cause crown gall in grape are not controlled (Kerr and Tate 1984; Kerr and Panagopoulos 1977). Searches for new biological agents to control crown gall where K84 is not successful have yielded strains that have been effective on an experimental scale but these have not yet been developed for commercial use. Most research programs have concentrated on organisms closely related to the pathogen.

Other Effective Strains

Hendson *et al.* (1983) reported that *A. tumefaciens* strain D286 (biovar 1), which was isolated from *Eucalyptus* in South Africa, produces an antibiotic that inhibits octopine strains. Although it was originally pathogenic, strain D286 has now lost its pathogenicity and is a possible biocontrol agent for all biovars of *Agrobacterium*. Webster *et al.* (1986) isolated *A. tumefaciens* strain J73 (biovar 2) from a gall on *Prunus* in South Africa. Strain J73 produces an antibiotic and has now been cured of its Ti plasmid. Preliminary tests using strain J73 showed that it can decrease the severity of crown gall on grape. In China, *A. radiobacter* strain HLB-2 (biovar 1) which was isolated from crown gall on hops (*Humulus*

lupulus), controlled crown gall (biovar 3) on grapevine shoots (Chen and Xiang 1986). Strain HLB-2 produces an antibiotic inhibitory to biovar 3 strains. Fungal isolates also have been tested for their ability to control gall (Cooksey and Moore 1980), but have not been developed further, even though some were effective.

The biological control of crown gall using a variety of *Agrobacterium* strains has been reviewed recently (Thomson 1987). This and other relatively recent reviews (Moore and Warren 1978; Kerr 1980; Garrett 1988) have covered the biological control of crown gall in general and this article will therefore concentrate on the recent modification of strain K84 which has led to the development of the biocontrol strain K1026.

Mechanisms in the Control of Crown Gall by Strain K84

Production of the antibiotic agrocin 84 is the major, but not the only, reason for the success of strain K84 (Kerr and Tate 1984). Survival of the biocontrol bacteria in the soil and colonisation of the root surface also play important roles (Shim *et al.* 1987; Macrae *et al.* 1988).

Agrocin 84 Production and Sensitivity to Agrocin

Agrocin 84, produced by strain K84, is specifically toxic to agrobacteria carrying a nopaline/agrocinopine A type Ti plasmid, and strain K84 is normally only effective against such strains (Kerr and Htay 1974; Roberts and Kerr 1974). These nopaline/agrocinopine A strains are responsible for most crown gall damage in orchards and nurseries (Kerr and Tate 1984). Kerr (1980) summarised the evidence that production of agrocin 84 is important in the control of crown gall. Two key points are that (a) only agrocin-sensitive strains are controlled reproducibly by strain K84, and (b) the transfer of a plasmid that codes for agrocin production by strain K84 to other agrobacteria gives the recipient strain the ability to synthesise agrocin and also to control crown gall (Ellis and Kerr 1978).

Agrocin 84 is an adenine nucleotide analogue with two substituent groups (Roberts *et al.* 1977), and is specifically taken up by nopaline/agrocinopine A strains via a permease for

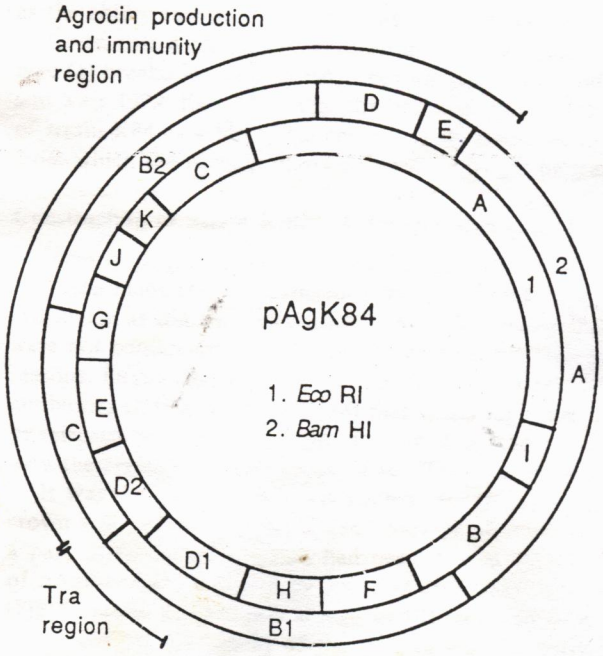

Fig. 1. *Bam*HI and *Eco*RI restriction map of pAgK84 showing the regions coding for plasmid transfer, agrocin 84 synthesis and immunity to agrocin (from Jones *et al.* 1988).

the opine agrocinopine A (Ellis and Murphy 1981; Hayman and Farrand 1988). Part of the agrocin 84 molecule mimics the structure of agrocinopine A, the usual substrate recognised by the permease. The origin of the name 'agrocinopine' lies in the discovery of this interaction (Ellis and Murphy 1981).

The Agrocin Plasmid

Production of agrocin 84 by strain K84 is coded for by a plasmid, designated pAgK84. pAgK84 is 48 kilobases in length, i.e. about one quarter the size of many Ti plasmids. The plasmid pAgK84 has been characterised by Farrand and co-workers, and regions coding for agrocin production, immunity to agrocin, and plasmid transfer have been located (Slota and Farrand 1982; Farrand *et al.* 1985; Ryder *et al.* 1987). These regions are shown on a physical map of pAgK84 (Fig. 1).

The agrocin plasmid can be transferred to other agrobacteria at the same time as pNOC, the nopaline catabolic plasmid of strain K84 (Ellis *et al.* 1979), but pAgK84 also has its own transfer functions (Farrand *et al.* 1985). The frequency of transfer of pAgK84 is highest (a) in the presence of pNOC, and (b) in the presence of nopaline which induces the conjugal transfer of pNOC.

The transfer (Tra) region of pAgK84 was mapped by mutagenesis using the transposon Tn*5* (Farrand *et al.* 1985, Fig. 1) and is 3·5 kb long. Derivatives of pAgK84 carrying Tn*5* insertions in the Tra region were not transferred at a measurable frequency to other agrobacteria in mating experiments.

Root Colonisation

The chromosomal background (biovar) in which pAgK84 is present has an influence on the ability of the strain to control disease. Thus, not all strains carrying pAgK84 are effective control agents. When the ability to produce agrocin was transferred from strain K84 (biovar 2) to strains of biovar 1, the new strains did not control disease as effectively as K84 (Ellis *et al.* 1979; Shim *et al.* 1987). Biovar 2 strains containing pAgK84 were more efficient than biovar 1 strains in colonising the roots of almond seedlings (Shim *et al.* 1987); initially colonisation was similar, but 3 or more months after inoculation the populations of biovar 2 strains on the root surface were consistently three to four times higher than those of biovar 1 strains.

Macrae *et al.* (1988) found that strain K84 was superior to the potential biocontrol strain J73 (also a biovar 2 isolate) in its ability to colonise the roots of tomato plants grown in both sterile and non-sterile soil. The reasons for this difference are unknown.

Possible Reasons for Breakdown of Biological Control of Crown Gall

Biological control using strain K84 could break down for several reasons. The most important, listed here, are all related to the production of agrocin 84.

(*a*) Panagopoulos *et al.* (1979) reported that in a field experiment where strain K84 and a pathogenic biovar 1 strain were coinoculated onto almond seedlings in a 1:1 ratio, pathogens resistant to agrocin 84 were subsequently isolated. This transfer was undoubtedly due partly to the high population densities of both the pathogen and the control agent, which were each introduced artificially. Nevertheless, the report caused concern that such a breakdown could occur during the normal practice of biological control using strain K84.

Breakdown in control was due to the transfer of the agrocin plasmid (pAgK84) from strain K84 to the pathogenic agrobacteria. Such a transfer, in which the pathogenic bacteria became resistant to agrocin 84 and also produced the antibiotic, was also demonstrated *in vitro* (Ellis *et al.* 1979). The frequency of transfer of pAgK84 *in vitro* is markedly increased in the presence of nopaline (Ellis and Kerr 1979). The transfer frequency presumably would be higher in the vicinity of a gall containing nopaline than in the soil or on the root at some distance from a gall.

(*b*) The second way in which biological control could break down is by the transfer of a Ti plasmid from a pathogenic *Agrobacterium* into strain K84. The control agent would then become pathogenic while retaining agrocin 84 production and immunity. There are no data available on the frequency of Ti plasmid transfer into strain K84. Such an event may be less common than the frequency of transfer of pAgK84 into a pathogen described in (*a*) above. The reason behind this assertion is that in addition to pAgK84, strain K84 also carries pNOC, a Ti-like plasmid which codes for the catabolism of nopaline but does not confer virulence on strain K84 (Sciaky *et al.* 1978; Clare *et al.* 1990). In most cases where strain K84 is successfully used, pNOC and the Ti plasmid of the pathogenic strains present in the soil are incompatible, i.e. the two plasmids cannot coexist in the same cell. Strain K84 would first (or simultaneously) need to lose pNOC in order to accept an incoming Ti plasmid. This requirement would probably result in the transfer event being less frequent than that described in (*a*) above.

(*c*) Laboratory studies (Süle and Kado 1980; Cooksey and Moore 1982) have shown that strains of *Agrobacterium* sensitive to agrocin 84 can mutate at a relatively high frequency to become resistant to the antibiotic. These resistant mutants arise in the zone of inhibition around a colony of K84 on solidified agar medium that has been overlaid with a sensitive strain. Cooksey and Moore (1982) showed that some of the resistant mutants retained their pathogenicity while others appeared to have lost their Ti plasmid. The genetic locus for sensitivity to agrocin 84 is located on the Ti plasmid (Engler *et al.* 1975; Hayman and Farrand 1988), and this is presumably where the mutation to resistance occurs, either through loss of part of the plasmid, or through a point mutation.

Mutation to agrocin resistance could also occur *in vivo* and give rise to strains of *Agrobacterium* not controlled by strain K84. At present we do not know whether such resistant mutant strains arise in the soil after treatment with strain K84. Such agrocin-resistant mutants would be difficult to combat. Mixed inoculation using strain K84 and a strain producing a different agrocin might give some protection against spontaneous agrocin 84-resistant mutants of the pathogen.

There have been no reports that other characteristics important in biological control, such as the ability to colonise plant roots, are unstable in strain K84.

Given that a type (*c*) breakdown in control would be difficult to overcome and that a type (*a*) breakdown had already been observed in a field experiment, it was reasoned (Ellis and Kerr 1979; Kerr 1980) that the best and most practical way to extend the effectiveness of strain K84 as a biological control agent would be to produce a derivative of the strain from which the agrocin plasmid could no longer be transferred to pathogenic strains.

Construction of Strain K1026, a Transfer-deficient (Tra$^-$) Deletion Mutant of Strain K84

Shim *et al.* (1987) demonstrated that two strains of biovar 2, containing pAgK84 which had been made transfer-deficient (Tra$^-$) by insertion of Tn*5*, controlled crown gall just as effectively as did the unmodified strain K84. However, the Tn*5*-induced Tra$^-$ derivatives were not considered suitable for the commercial biological control of crown gall for two reasons. Firstly, the insertion of Tn*5* confers on strain K84 the genes for resistance to three antibiotics (Genilloud *et al.* 1984) that strain K84 does not have, and therefore should not be released for commercial use; and secondly, there is the possibility that Tn*5* can be lost, with the resultant reappearance of the Tra$^+$ parent strain.

It was decided that the best strategy for guarding against breakdown in biocontrol of crown gall was to generate a Tra$^-$ variant of strain K84 containing pAgK84 from which a part of the transfer region had been deleted. The construction of a deletion derivative of pAgK84 was described by Jones *et al.* (1988). Using the restriction endonuclease map (Fig. 1), it was decided that at least *Eco*RI fragment D1 should be removed, since this would delete 2·8 kb (i.e. 80%) of the 3·5 kb transfer region. To make the deletion, *Bam*HI fragment B1 of pAgK84 (Fig. 1) was first cloned into a cloning vector, plasmid pBR325 of

Escherichia coli. Both *Eco*RI fragments D1 and H were then removed using recombinant DNA techniques. A total of 5·9 kb of pAgK84 DNA had been deleted. The removal of *Eco*RI fragment H as well as fragment D1 did not affect the replication or stability of pAgK84 in *Agrobacterium*.

After the two *Eco*RI fragments had been deleted, the cloned *Bam*HI B1 fragment was extended at one end by adding the part of *Bam*HI fragment C which normally adjoins fragment B1 on pAgK84 (Fig. 1). The deletion site was now flanked at each end by enough pAgK84 DNA to allow a reasonable frequency of homologous recombination between this plasmid and pAgK84.

The cloned segment of pAgK84 containing the deletion was then transferred to pAgK84 by a deletion-marker exchange.

(*a*) A mating was performed between the *E. coli* strain with pBR325, which carried the segment of pAgK84 with the deletion, and an *Agrobacterium* strain containing pAgK84 marked with the transposon Tn*5*. The Tn*5* insertion in pAgK84 was just outside the transfer region but still inside the area covered by the deletion. The selection in this mating was for an *Agrobacterium* strain that had both the chloramphenicol resistance of pBR325 and the kanamycin resistance of Tn*5*. Because the *E. coli* plasmid pBR325 does not survive by itself in strains of *Agrobacterium*, it could only be recovered as a combination of both plasmids (a cointegrate plasmid) following homologous recombination with pAgK84.

(*b*) The cointegrate plasmid was then transferred to a derivative of strain K84 which had spontaneously lost pAgK84. It was important to have the Tra$^-$ derivative of pAgK84 in the same chromosomal background as the original pAgK84 because (a) strain K84 possesses a good ability to colonise the rhizosphere and root surface, and (b) when pAgK84 was transferred to other strains of *Agrobacterium*, the resulting strain was usually not as effective as strain K84 in controlling crown gall (Shim *et al.* 1987).

(*c*) The cointegrate plasmid was still transfer-proficient (Tra$^+$), because the Tn*5* insertion in the pAgK84 part of the plasmid was outside the Tra region. It was therefore relatively easy to transfer the cointegrate by conjugation to the derivative of K84 which had lost pAgK84.

(*d*) The deletion-marker exchange to form the Tra$^-$ derivative of pAgK84 was now completed by growing the strain containing the cointegrate for many generations in a medium lacking antibiotics and then screening for a colony that had lost the kanamycin resistance coded for by Tn*5*. A kanamycin-sensitive colony would arise as a result of a homologous recombination on the side of the deletion opposite that on which the earlier recombination had occurred during the formation of the cointegrate. Cells of this strain would contain pAgK84 lacking *Eco*RI fragments D1 and H. A colony of this type was recovered as a single occurrence amongst approximately 7000 replica-plated colonies. This spontaneous kanamycin-sensitive derivative was designated K1026.

Properties of Strain K1026

Strain K1026 was tested in laboratory experiments to ensure that it had the desired properties (Jones *et al.* 1988). There was no difference between strains K1026 and K84 in their abilities to produce agrocin 84 *in vitro*.

The agrocinogenic plasmids of strain K1026 and strain K84 were compared for size, restriction fragment patterns, plasmid transfer frequency and plasmid stability. Plasmids were isolated from strains K1026 and K84 and when the plasmid sizes were compared by electrophoretic separation in an agarose gel, the smallest plasmid in strain K1026, the agrocinogenic pAgK1026, was clearly smaller than the corresponding agrocinogenic plasmid (pAgK84) from strain K84 because of the deletion. When the plasmids were digested with the restriction endonuclease *Eco*RI, pAgK1026 did not contain fragments D1 or H. In addition, no DNA

fragments from the *E. coli* vector were detected, confirming that no foreign DNA remained in strain K1026. Both strains also contained a large cryptic plasmid and the slightly smaller pNOC, which codes for nopaline catabolism.

To determine plasmid stability, a strain containing either pAgK1026 or pAgK84 marked with resistance to chloramphenicol was grown without antibiotic selection and subcultured 10 times consecutively. There was no loss of pAgK1026 or pAgK84, indicating that both are stable plasmids.

Plasmid transfer frequency was determined from experiments in which strains harbouring antibiotic resistance-marked derivatives of pAgK1026 and pAgK84 were mated with a strain of *Agrobacterium* biovar 1. There were no transconjugants in the cross with the pAgK1026 derivative, whereas many transconjugants were observed from the mating with the pAgK84 derivative. pAgK1026 was clearly a Tra^- mutant of pAgK84.

Control of Crown Gall by Strain K1026

After appropriate permission had been granted by Australian authorities, strain K1026 was tested for its ability to control crown gall on almond seedlings in an open air pot experiment (Jones and Kerr 1989). Younger (2 month) and older (10 month) seedlings were dipped in a suspension of either strain K1026, strain K84 or water, and planted in soil infested with a pathogenic *Agrobacterium* biovar 2, and damage due to disease was assessed after 7 months. There was no significant difference in the incidence and weight of galls on plants treated with strain K1026 or K84 (Table 1) as judged by the Kruskal–Wallis test ($0{\cdot}5 < P < 0{\cdot}7$). Strain K1026 was as effective as strain K84 in controlling crown gall.

Table 1. 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 the pathogenic *A. tumefaciens* strain K27 (biovar 2)

Fifteen plants per treatment were inoculated and then grown outdoors in pots for 7 months (from Jones and Kerr 1989)

Plant age (months)	Treatment	Number of plants surviving	Percentage of plants galled	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

Registration and Commercial Use of Strain K1026

Because strain K1026 is as effective as strain K84 in limiting damage due to crown gall, and because its agrocin plasmid cannot be transferred to other agrobacteria, it should be used in preference to strain K84. In order to make strain K1026 commercially available, it was registered as a pesticide in New South Wales. The product can be sold to growers in any state and has been available since late 1988. The strain is sold as NoGall, a peat-based formulation containing 10^9 bacteria per gram, with an advertised shelf life of over 6 months.

The evidence that strain K1026 is harmless to humans, animals, plants and the environment was presented by Jones and Kerr (1989). As this bacterial strain is the first genetically manipulated microbe to be sold as a commercial product, it will be worthwhile to repeat this information here. Strain K1026 is regarded as safe because: (1) strain K84, the progenitor of strain K1026, has been registered as a pesticide and used commercially in many countries for over 15 years with 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, thereby preventing plasmid transfer

(Jones *et al.* 1988); (3) no foreign DNA remains in strain K1026; (4) strain K1026 contains no Ti-plasmid-encoded genes involved in crown gall induction (Clare *et al.* 1990); (5) strain K1026 is a biovar 2 strain of *Agrobacterium* and cannot grow at 37°C (human body temperature; Kerr and Panagopoulos 1977); (6) agrocin 84 is specifically toxic to agrocinopine-catabolising agrobacteria, most of which are crown gall pathogens (Engler *et al.* 1975).

Depending on the crop and the country involved, the commercial use of strain K1026 may require further tests of its efficacy before registration requirements can be completed.

Conclusion

The biological control of crown gall with strain K84 can now be extended in its usefulness by replacing it with strain K1026, a Tra$^-$ derivative of K84 from which the agrocin plasmid can no longer be transferred to other agrobacteria.

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