The Persistence of Engineered *Agrobacterium tumefaciens* in Agroinfected Plants

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Several plant species, including tomato (*Lycopersicon esculentum), Gynura aurantiaca,* avocado (*Persea americana*), and grapefruit (Citrus paradisi) grafted on Troyer citrange (*Poncirus trifoliata* × *C. sinensis*) were "agroinfected" with *Agrobacterium tumefaciens* strain LBA4404, carrying a mini-Ti plasmid with a dimeric cDNA of citrus exocortis viroid (CEVd). Extracts prepared from tissues of the agroinfected plants 38–90 days after inoculation were plated on selective media and found to contain large amounts of the engineered bacteria. These observations suggest the need for more stringent quarantine measures when handling *A. tumefaciens* cells harboring constructs for "agroinoculation" with plant viruses or viroids.

DNA copies of the genomes of DNA or RNA plant viruses or of viroids have been inserted into the Ti plasmid carried by *Agrobacterium tumefaciens*. Infection of plants by *A. tumefaciens* carrying the engineered plasmids enables the transmission of plant viruses and viroids to the respective host plants. The method, termed "agroinfection" or agroinoculation has been found convenient for transmission of viroids and viruses, especially members of virus groups that depend mainly on insect transmission such as geminiviruses and luteoviruses (Matthews 1991; Leiser *et al.* 1992; Donson *et al.* 1988; Grimsley *et al.* 1987; Hayes *et al.* 1988).

A. tumefaciens vectors engineered for agroinfection have been used for several years for a variety of experimental purposes (Grimsley *et al.* 1986; reviewed by Grimsley and Bisaro 1987). However, no results have been published on the survival of the engineered bacteria in the inoculated hosts, and the danger of their possible escape, as a new type of self-vectored pathogen, has not been sufficiently discussed. The present paper describes the results of such analyses for several plants experimentally agroinfected with an engineered Ti plasmid harboring a dimeric cDNA of the citrus exocortis viroid (CEVd) genome (Gross *et al.* 1982; Semancik 1980). A dimeric cDNA of CEVd was extracted from a 1% agarose gel and ligated into the *Sma*I site of pBI121 (Jefferson *et al.* 1987). All enzyme reactions were performed according to supplier instructions. Transformation of *E. coli* strain JM101, DNA preparation, and manipulations were done according to Sambrook *et al.* (1989). Tri-parental mating for introducing the CEVd dimer into the disarmed *A. tumefaciens* strain LBA4404 (Clontech), was according to Draper *et al.* (1988). For agroinfection, *A. tumefaciens* cells were grown in 100 ml of LB medium containing 100 mg/ml rifampicin and 100 mg/ml kanamycin for 48 hr at 28° C. The suspension was centrifuged at 8,000 *g* for 15 min, and the pellet was resuspended in 5 ml of sterile water.

Grapefruit (cv. Star Ruby) scions grafted on Troyer citrange rootstock, West Indian avocado seedlings, Gynura, and tomato plants (*Lycopersicon esculentum* 'Rutgers') were used for agroinfection. The woody plants were slash-inoculated at five stem sites 10 cm apart, starting at about 5 cm aboveground. The inoculum was applied by dripping approximately 300-μl cell suspension at each wound site. Inoculation of the herbaceous plants was done by puncturing the plant stems and apices with a syringe. The wounds were sealed with Parafilm and the plants were kept in a growth chamber under natural light conditions at 27–31° C.

Bacteria were recovered from treated plants by grinding the stem pieces from wound sites in saline (1 g/ 5 ml). The slurry was agitated at room temperature for an hour, diluted, and plated on nutrient agar medium plates (Difco) supplemented with 0.01% yeast extract (NAY), 100 mg/ml rifampicin, 100 mg/ml kanamycin, and 250 mg/ml cycloheximide at 28° C for at least 48 hr.

The authentic nature of the isolated bacteria was confirmed by immunofluorescence staining procedure (Schaad 1978), using antibodies prepared against *A. tumefaciens* strain biovar 1 and by polymerase chain reaction (PCR) analysis (Ashulin *et al.* 1992) using CEVd primer oligonucleotides, 5'CGGGGATCCCTGAAGGACTT3' (antisense 78–98) and 5'GGGAAACCTGGAGGAAGTCG3' (sense 98–128) based on Gross *et al.* 1982.

Table 1 summarizes the bacterial counts in extracts from the rootstock and scion parts of the grafted grapefruit and avocado plants. High concentration levels of viable cells persisted for 3 mo after inoculation (the longest time examined). Similar recovery tests with extracts of agroinfected tomato and *G. aurantiaca* plants exhibiting CEVd symptoms showed that the engineered *A. tumefaciens* cells remained viable for 90 days postinoculation (the longest

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time examined). PCR amplification of randomly sampled colonies, which were recovered from each of the plant species tested showed the presence of a 371-bp DNA band corresponding to the size expected from the CEVd genome (Fig. 1).

The results indicate that engineered *A. tumefaciens* remains viable in agroinfected plants for prolonged periods and point to the possibility of serious epidemiological consequences when handling agroinfected plants under conditions unsuitable for pathogen spread in the absence of its natural vectors. Moreover, newly formed combinations of persistently transmitted viruses and the opportunistic and systemically moving *Agrobacterium* vector (Hill 1928; Tarbah *et al.* 1986) infectious to a wide host range (including Gymnosperms and dicotyledonous Angiosperms), might eventually cause infection and damage to crop plants or natural vegetation not presently visited by the traditional (insect) vectors of the virus disease. The combination of *A. tumefaciens* with viroids (Diener 1979; Sanger 1982; Symons 1990), normally not transmitted by insect vectors, poses an even more serious problem.

The history of horticulture contains several severe epidemics caused by the introduction of viruses, or their vectors, to previously disease-free areas (Bar-Joseph *et al.* 1989). The research advantages of agroinfection might eventually prove harmful to practical farming. It is therefore suggested that we control the future use of this technique and use experimental conditions that will minimize possible spread.

Table 1. Bacterial counts in extracts from agroinoculated plants[a]

Plant	TPI	Cfu/g
Troyer citrange	2 hr	4.8×10^9
	38 days	2.2×10^4
	90 days	1.4×10^5
Grapefruit	2 hr	1.1×10^{10}
	38 days	2.0×10^5
Gynura	38 days	6.4×10^5
Tomato	80 days	2.3×10^4
Avocado	38 days	5.3×10^4

[a] TPI, Time postinoculation; Cfu, Colony-forming unit.

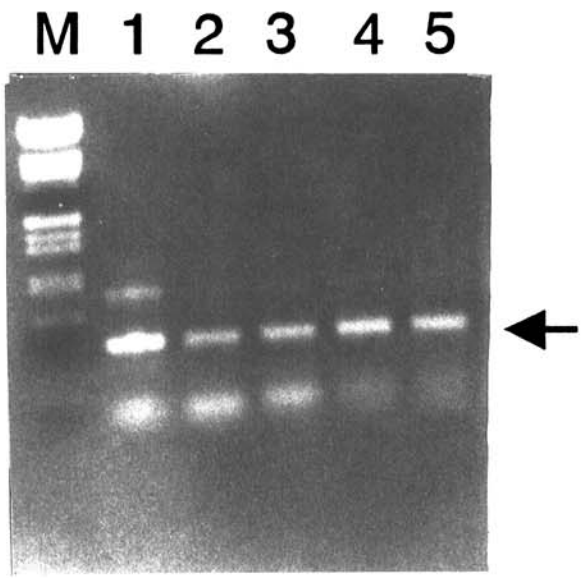

Fig. 1. PCR amplification products, separated on a 1% agarose gel from randomly sampled *Agrobacterium tumefaciens* colonies, recovered 38 days after agroinfection from different plant species. M; DNA size markers were cut from 1 with *Eco*RI and *Hin*dIII. 1, Troyer citrange; 2, grapefruit; 3, gynura; 4, tomato; 5, avocado. Arrow indicates the predicted 371-bp PCR amplification product which was obtained by using the CEVd primers described in the text.

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