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"Agroinfection," an alternative route for viral infection of plants by using the Ti plasmid

(*Agrobacterium tumefaciens*/cauliflower mosaic virus)

NIGEL GRIMSLEY*, BARBARA HOHN*, THOMAS HOHN*, AND RICHARD WALDEN†

*Friedrich Miescher-Institut, P.O. Box 2543, CH4002 Basel, Switzerland; and †Leicester Biocentre, Medical Sciences Building, University of Leicester, University Road, Leicester, LE1 7RH, United Kingdom

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ABSTRACT **Most plant viruses are transmitted by insect vectors. We present an alternative method for the introduction of infectious viral DNA that uses the ability of *Agrobacterium* to transfer DNA from bacterial cells to plants. Cauliflower mosaic virus was chosen to develop this method because it is the best characterized plant DNA virus and can be introduced into plants via aphids, virus particles, viral DNA, or suitably treated cloned DNA. We show that systemic infection of turnips results from wounding and inoculation with strains of *Agrobacterium tumefaciens* in which more than one genome of cauliflower mosaic virus have been placed tandemly in the T-DNA of the tumor-inducing plasmid. Thus such constructions allow escape of the viral genome from the T-DNA once inside the plants. The combined use of the tumor-inducing plasmid and viral DNA opens the way to molecular biological approaches that are not possible with either system alone.**

While some cloned viral DNAs are infectious following mechanical inoculation of leaves, others are not. The natural route for viral infection of plants (1, 2) is through insects that have fed on virus-infected plants, which, for our purposes, is inconvenient. Stocks of insects must be maintained under strictly contained conditions, and the completely controlled introduction of insects to the plants is difficult. Although it is possible to clone and study a variety of types of viral genomes—for example, single-stranded DNA viruses by cloning double-stranded replicative-intermediate forms (3)—no way had been found of reintroducing these genes into plants. Thus the use of these viruses for *in vitro* mutagenesis or other applications of recombinant DNA technology was precluded.

The T-DNA of the crown gall bacterium *Agrobacterium tumefaciens* can be transferred into plant cells; thus we have used this system to develop a method for the transformation of dicotyledons (4) with cloned viral DNA from bacteria to plants via the tumor-inducing (Ti)-plasmid using cauliflower mosaic virus (CaMV) as a well-characterized model system. This is potentially a very flexible technique, because there is some other work that suggests that at least some monocotyledonous plants may also be transformed by this bacterium (5, 6).

The CaMV genome is an 8-kilobase pair (kbp) double-stranded DNA molecule that can be used as a vector to introduce a limited amount of foreign DNA into plant cells. In the plant cell, the virus replicates many times, systemically infects the plant, and expresses the foreign DNA (7). Cloned CaMV DNA is not infectious when inoculated onto test plants, but viral DNA is infectious if it is excised from the bacterial vector at the cloning site, either as a monomer (8, 9) or as subgenomic fragments (10, 11), showing that *in vivo* ligation within the plant cell can generate circular infectious viral DNA. The insertion of one genome of CaMV in the T-DNA would, therefore, not be a suitable way to test transfer of infectious virus to plants, as no mechanism for specific excision of the virus is likely to exist. On the other hand, it has been found that bacterial plasmids containing tandemly duplicated CaMV genomes are infectious (11, 12). Infection arises either as a result of intramolecular recombination or via production of a 35S transcript. This transcript, which spans the entire CaMV genome and bears a 180-base long terminal direct repeat, has been implicated as an intermediate in replication (refs. 13–15; for a review see ref. 16). Thus, vectors with tandem repeated sequences of CaMV in the T-DNA might allow the virus to escape giving rise to systemic infection when transferred to plants (Fig. 1).

MATERIALS AND METHODS

Plasmid Constructions. pCa305 was constructed by replacing the small *Eco*RI–*Cla* I fragment of pHC79 (17) with a fragment encoding spectinomycin/streptomycin resistance from pMON30 (a precursor of pMON120, ref. 18) and replacing the 2.5-kilobase (kb) *Sal* I–*Bst*EII fragment of the resulting plasmid (pV118) with the 3.3-kb *Sal* I–*Bst*EII fragment of CaMV S (19) to produce pCa292. A complete CaMV 4-184 genome (20) was then inserted in the remaining *Sal* I site (Fig. 2). pGV96414D was constructed by inserting the two CaMV genomes from the clone pLW414 (8) into the *Sal* I site of pGV96 (a plasmid with the kanamycin-resistance encoding sequence of Tn*903*, ref. 21, inserted in the *Eco*RI site of pBR325, ref. 22). As a control the broad host range plasmid pGV1106 (23) was cut with *Eco*RI and introduced into the *Sph* I site of pCa305 to produce pEA1, which can replicate in *A. tumefaciens*. The presence of these plasmids in *A. tumefaciens* either integrated into the Ti plasmid or replicating independently was confirmed by Southern blotting. Details of plasmids and strains used are given in Table 1.

Strains. Bacterial host strains were *Escherichia coli* DH1 (nalidixic acid resistant, ref. 26) and *A. tumefaciens* C58 (rifampicin resistant, GV3101, ref. 27). Plasmids were introduced into *A. tumefaciens* by (*i*) conjugation or transformation into *E. coli* GJ23 (pGJ28, pR64*drd11*) (24) and (*ii*) introduction into C58 (pGV3850) (25). Clones of *A. tumefaciens* containing the plasmid of interest either integrated in the T-DNA or, in the case of pEA1, freely replicating could then be selected.

Inoculation of Plants. *Plasmid DNA and bacteria.* Three-week-old turnip plants (*Brassica rapa* var. Just Right) were inoculated either on two leaves wounded by rubbing with celite or at the crown by making a hole through the cluster of petioles near the surface of the soil with a sterile toothpick. Leaves were inoculated with 2.5 μg of plasmid DNA in 10 μl

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Abbreviations: CaMV, cauliflower mosaic virus; Ti, tumor-inducing.

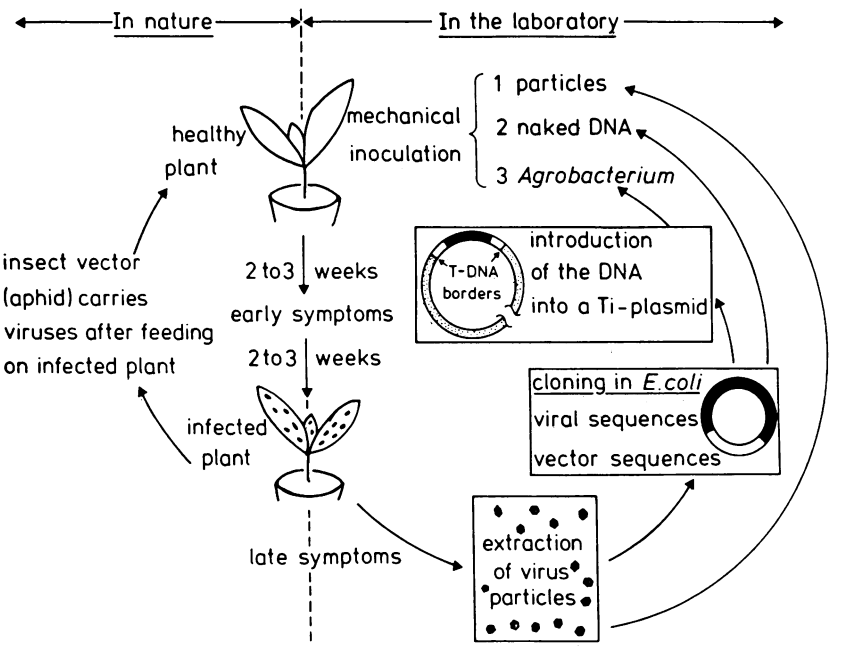

FIG. 1. (Not to scale.) Routes of viral infection. Normal life cycle of CaMV (left side). In turnip, characteristic vein clearing develops 2–3 weeks after the inoculation, and, after a total of 6–10 weeks, stunting and premature senescence of leaves occurs. In the laboratory (right side) crushed leaf tissue from infected plants or naked viral DNA suitably cloned in a bacterial vector can give rise to infection following mechanical inoculation of leaves (routes **1** and **2**). We propose an alternative method of inoculation, "agroinfection" (route **3**).

of 0.1× SSC (1× SSC = 15 mM NaCl and 1.5 mM sodium citrate, pH 7.0) or with 10 μl of a bacterial suspension prepared by growing the bacteria for 40 hr in YEB (Bacto beef extract at 5 g/liter, Bacto yeast extract at 1 g/liter, peptone at 5 g/liter, sucrose at 5 g/liter, 2 mM $MgSO_4$, pH 7.2), collecting them by centrifugation, and resuspending them in 0.01 vol of YEB. Crowns were inoculated with 5 μg of plasmid DNA in 20 μl 0.1× SSC or 20 μl of bacterial suspension.

Total bacterial DNA. (*i*) C58 (pGV3850::pCa305). For inoculations of 1.5 mg of DNA per plant, six leaves of each 4-week-old turnip plant were wounded with celite, and 250 μl of total bacterial DNA at 1 mg/ml (28) in 10 mM Tris·HCl, 1 mM EDTA, pH 8, was applied to each leaf. Three-week-old turnip plants were used for inoculations of 100 μg of DNA per plant and were each inoculated on two leaves with 50 μl per leaf of the bacterial DNA solution above. (*ii*) C58 (pGV3850::pGV96414D). Three-week-old plants were inoculated with either about 5 μg of DNA in 20 μl 0.1× SSC on each of two leaves or 10 μg of DNA in 40 μl 0.1× SSC at the crown.

RESULTS

Plasmids. The introduction of CaMV into plants has been tested by using a variety of constructs. pCa305 and pGV96414D contain 1.4 and 2 tandem genomes of CaMV, respectively, (Fig. 2) and can produce the large transcript,

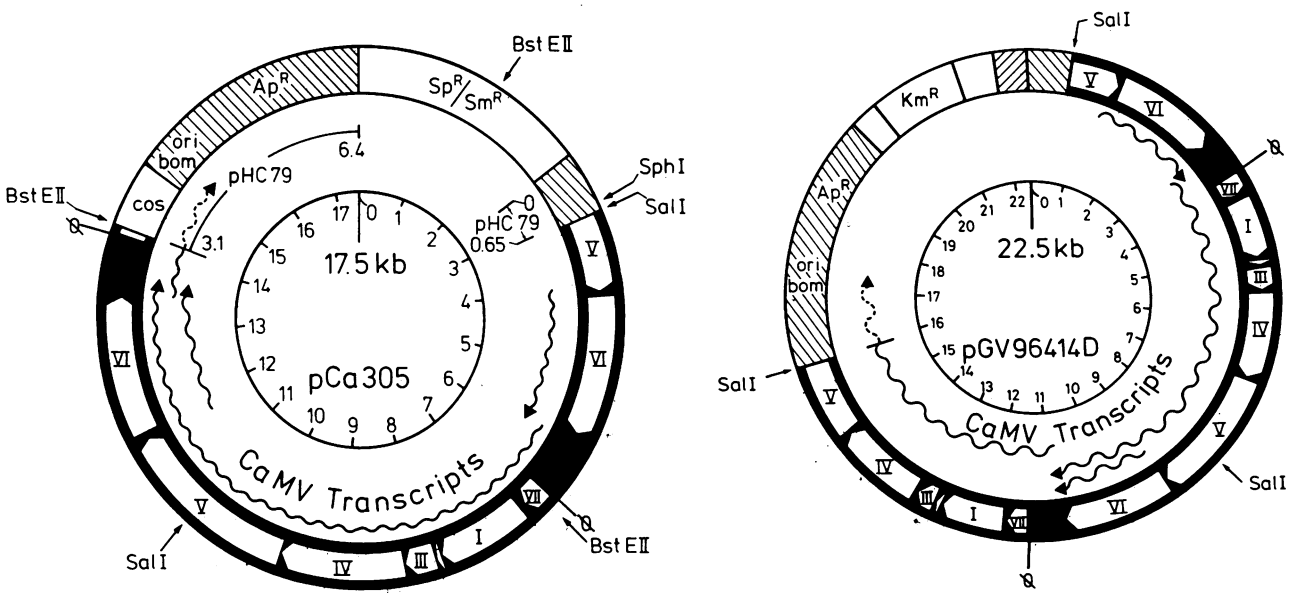

FIG. 2. Vectors used for inoculation of plants either as naked DNA or in bacterial cells. CaMV sequences are shown as a black background with white open reading frames containing roman numerals, and regions of homology to pBR322 are shown hatched. "Ø" indicates the position of base pair 1 on a standard CaMV genome map (19, 20).

Table 1. Plasmids

Name	Bacterial host	Antibiotic resistances	Reference
pGV96	DH1	Km, Ap, Tc	pBR325 with Km^R of Tn*903* in the *Eco*RI site (refs. 21 and 22)
pCa305	DH1	Ap, Sm/Sp	Fig. 2
pGJ28	DH1	Km/Nm	Ref. 24
pR64*drd*11	DH1	Sm, Tc	Ref. 24
pGV3850	C58	Cb	Ref. 25
pGV3850::pCa305	C58	Cb, Sm/Sp	Fig. 2
pGV3850::pGV96414D	C58	Cb, Km	Fig. 2
pGV1106	DH1 or C58	Km, Sm/Sp	Ref. 23
pEA1	DH1 or C58	Ap, Km, Sm/Sp	Table 2

Resistances: Ap, ampicillin; Cb, carbenicillin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

including terminal repeats. When suitably complemented by functions provided by other plasmids (24), the *bom* site of pCa305 or pGV96414D allows conjugal transfer from *E. coli* to *A. tumefaciens*; since replication in *A. tumefaciens* is not possible, they can only be rescued if a suitable target site for integration is available. The nononcogenic Ti plasmid vector pGV3850 contains pBR322 sequences between the T-DNA borders and, under appropriate selective conditions, allows rescue of plasmids by homologous recombination into the pBR322 region (25). Integration of pCa305 or pGV96414D into the T-DNA was verified by probing Southern transfers of digested total *A. tumefaciens* DNA (unpublished data). The dimeric construction was found to be stable in *A. tumefaciens* for at least 80 generations (R.W., unpublished data).

The ability of strains of *A. tumefaciens* produced in this way to give rise to viral infection would not be sufficient by itself to conclude that the T-DNA is instrumental in the transfer process, since pCa305 naked DNA should be infectious following mechanical inoculation. It had to be ruled out that pCa305 DNA from lysed *A. tumefaciens* cells could lead to systemic infection. Therefore, pGV1106, a broad host range plasmid that can replicate in *A. tumefaciens* (23) but does not contain T-DNA borders, was placed in the unique *Sph* I site of pCa305 (Fig. 2) to produce a plasmid subsequently referred to as pEA1. pEA1 was introduced to *A. tumefaciens*, where it could replicate independently. To ensure that pEA1 was maintained in the bacterium, selection was applied during growth prior to plant inoculation (see Table 1 for details of strains).

Infection of Plants. Since naked CaMV DNA is usually inoculated onto wounded leaves of turnip plants, and *A. tumefaciens* is inoculated by wounding at the crown, both routes of mechanical inoculation were tested with naked plasmid DNA, plasmids in *A. tumefaciens*, and total agrobacterial DNA containing the Ti plasmid construct. Results of these experiments are shown in Table 2. Naked DNA of pCa305, pGV96414D, and pEA1 was found to be infectious when rubbed on leaves but not when applied to wounded crowns. Inoculation with C58 (pGV3850::pCa305) or C58 (pGV3850::pGV96414D) bacteria rapidly led to viral infection following either leaf or crown inoculation, whereas total bacterial DNA from these strains was only infectious when applied to the leaves in very large amounts. C58 (pGV3850::pCa305) total DNA (1.5 mg) contains about 1.5 μg of CaMV DNA. Inocula of this size gave rise to infection, whereas 10 μg of C58 (pGV3850::pGV96414D) DNA (equivalent to the maximum amount of DNA that could be released upon lysis of all of the cells in the standard bacterial inoculum used in other tests) contains only about 10 ng of CaMV DNA and was insufficient to produce systemic infection. These observations agree with infectivity tests of pCa305 or pGV96414D, where infection rarely results when a plant is inoculated with less than 1 μg of naked plasmid DNA (unpublished data). Inoculation with C58 (pEA1) bacteria did not give rise to symptoms. We, therefore, conclude that the Ti plasmid has promoted the transfer of the infectious cloned viral DNA to the recipient plants. Inoculation with C58 (pGV3850::pCa305) and C58 (pGV3850::pGV96414D) gave rise to systemic infection more rapidly through the leaves than through the crown (unpublished data) and also gave rise to symptoms on the host plants mustard, rape, and radish.

It is difficult to compare the efficiency of infection of *A. tumefaciens* as a donor of viral DNA with naked DNA,

Table 2. Appearance of symptoms on turnips after inoculation

DNA or bacterial strain used	Whole plants showing symptoms/total plants inoculated, no./no.	
	Leaf inoculated	Crown inoculated
Plasmid DNA		
pCa305	8/12	0/4
pEA1	5/12	0/4
A. tumefaciens		
C58	0/12	0/8
C58 (pGV3850::pCa305)	8/8	14/14
C58 (pGV3850::pGV96414D)	8/8	8/8
C58 (pEA1)	0/24	0/16
Total bacterial DNA		
C58 (pGV3850::pCa305) (1.5 mg DNA per plant)	8/10	Not tested
C58 (pGV3850::pCa305) (100 μg DNA per plant)	0/10	Not tested
C58 (pGV3850::pGV96414D) (10 μg DNA per plant)	0/4	0/4

Symptoms were assessed 6 weeks after inoculation.

because the possibility of growth of bacterial cells following inoculation cannot be excluded, but we have observed that a 10^{-6} dilution of the bacterial cell suspension routinely used for inoculation has about 10^4 cells in a volume of 20 μl, and has reliably given rise to systemic infection through leaf inoculation.

DISCUSSION

We propose the term "agroinfection" for this method of inoculating plants with viral genomes by using *A. tumefaciens*. CaMV was chosen for this study, because it has been well characterized and because naked CaMV DNA is infectious. Through an appropriate control, we showed that the infection of plants does not result from viral DNA released from lysed *A. tumefaciens*.

Since two different isolates of CaMV, which have slightly different sequences and restriction maps, were used to make the vector pCa305, deductions can be made about the mechanism of viral escape from the T-DNA upon restriction mapping and sequencing the viral progeny (29). It may be possible to use "agroinfection" for many viruses that can infect either as a circular DNA molecule, where systemic infection could arise from homologous recombination within tandemly duplicated viral DNA or as an RNA intermediate.

"Agroinfection" is also convenient to use in tissue culture, where the small leaves of plants in culture vessels are difficult to abrade to inoculate them with DNA. Synchronous viral infection of a large population of cells should be possible by cocultivation of regenerating isolated protoplasts with suitable strains of *Agrobacterium*.

Although the investigation of viral-gene expression in nonhost plants has been limited (see, for example, refs. 30 and 31), the available evidence indicates that accurate recognition of viral expression signals occurs. Work has shown clearly that a graminaceous monocot can transcribe a hybrid gene that contains expression signals from CaMV, which normally infects dicotyledonous plants (32). Plasmid constructs containing oligomeric CaMV genomes described in this communication have been used to transform tobacco, and preliminary results indicate that CaMV sequences are expressed (R.W., unpublished data).

Information about the stage at which CaMV might escape from the T-DNA is beyond the scope of the present study. Perhaps the majority of viruses arises after transport of the T-DNA to the nucleus, as transcription is a prerequisite for replication by reverse transcription. Since regeneration of transformed whole plants—either with *A. tumefaciens* (4) or by naked DNA of isolated protoplasts (33)—is now possible, viral genomes can be introduced into the nuclear DNA of every cell in a plant. Thus it can now be established whether CaMV can escape following integration into the nuclear DNA. A proportion of the offspring from such transformed plants are expected to carry integrated nuclear CaMV tandem genomes, and the presence of freely replicating CaMV would be evidence that escape following integration is possible. CaMV is not normally transmitted to offspring.

Further work on the transformation of whole plants with fragments of the viral genome will open the way to various approaches: (*i*) Superinfection of transformed plants may allow the development of better viral vectors, as expression of viral genes from nuclear DNA could permit introduction of foreign DNA into the superinfecting virus. (*ii*) The scope for research on host–parasite relationships could be widened. (*iii*) Immunity of plants to viral infection may result from transformation with fragments of viral genomes containing certain control sequences or encoding antisense mRNA.

Very little is known about the mechanism of T-DNA transfer from bacterium to plants (34). Systemic viral infection in a recipient plant is a sensitive nononcogenic assay for the transfer of DNA from bacterium to plant cell, which may not require integration into the host genome. "Agroinfection" could, therefore, be an invaluable tool for studying this transfer process. In the case of monocotyledonous plants "agroinfection" may provide a more suitable assay for T-DNA transfer than oncogenicity genes or opine production, because the expression of viral genes in a particular host plant is established *a priori*. If a viral origin of replication is present in the DNA that is being transferred from bacterium to plant, it might be possible to rescue intermediates.

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