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28. Components 1 through 6 are relatively simple organic compounds. Their structures were readily suggested by comparison with the available libraries of mass spectra or through interpretation of simple fragmentation patterns. Authentic chemicals were further analyzed to verify coincidence of both mass spectra and retention indices in high-resolution capillary gas chromatography. Although compounds 1, 4, and 6 are readily available from commercial sources, compounds 2, 3, and 5 were synthesized in our laboratory through simple procedures. A perfect match between mass spectra and chromatographic migration was obtained for all six components.
29. The six synthetic compounds were added to either urine from adrenalectomized females or water, corresponding to concentrations of 2.5, 0.28, 0.39, 0.50, 1.1, and 0.25 ppm, respectively.
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Transfer of *Agrobacterium* DNA to Plants Requires a T-DNA Border But Not the *vir*E Locus

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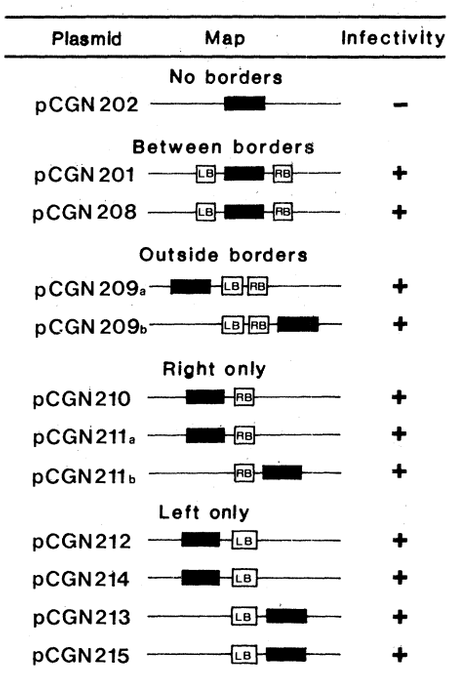
***Agrobacterium tumefaciens* induces tumors in plants by transferring and integrating oncogenes (T-DNA) into the chromosomes of host plant cells. *Agrobacterium* strains were used to transfer complementary DNA copies of a potato spindle tuber viroid (PSTV) to plant cells at a wound site on tomato plant stems. Subsequently, infectious viroid RNA was found in the leaves of these plants, indicating systemic PSTV infection. This process utilized the T-DNA transfer mechanisms of *Agrobacterium* since PSTV infection required most virulence genes (*vir*) as well as one of the DNA sequences that flank either side of the *Agrobacterium* T-DNA. However, transfer still occurred from *vir*E mutants of *Agrobacterium*, strains that fail to induce tumors even though a completely functional T-DNA is present. The *vir*E gene seems to be directly involved in the integration of foreign DNA into plant chromosomes.**

CROWN GALL DISEASE IS MEDIATED by the expression of T-DNA oncogenes introduced into the plant host by *Agrobacterium tumefaciens* (*1*). The T-DNA in the bacterial host resides on a Ti (tumor-inducing) plasmid that contains other genetic loci (*vir*) that are required for virulence (*2*). The boundaries of the portion of the plasmid constituting T-DNA are marked by DNA sequences termed border sequences (*3*). The requirement for border sequences and the *vir* genes has been established by assaying gall formation (*2, 4–8*). However, determining the functional roles of these elements in transformation requires an assay for intermediate steps in the process.

It has been shown (*9*) that *Agrobacterium* with multimeric complementary DNA (cDNA) copies of potato spindle tuber viroid (PSTV) (*10, 11*) in the T-DNA resulted in systemic infections by PSTV when the bacterial strain was inoculated onto tomato plants, a natural host of PSTV. Mock-inoculated tomato plants and tomato plants inoculated with *Agrobacterium* lacking viroid cDNA did not show the presence of viroid or viroid symptoms. Although other explanations are possible, these results suggested that the PSTV cDNA copies were transferred to the plant and subsequently transcribed into infectious RNA or that the T-DNA was transferred as an RNA intermediate containing infectious PSTV genomes. In either case, the infection process would not require T-DNA integration. The data described below indicate that the infection process does indeed require *Agrobacterium* T-DNA transfer and that this PSTV assay may separate the T-DNA transfer and integration events.



To examine the feasibility of this assay, we have used a binary vector system to introduce viroid cDNA into *Agrobacterium* (Fig. 1). Plasmid pCGN201 contains trimeric PSTV DNA located between the left and right borders of the T-DNA. This wide host range plasmid was conjugated into a virulent *A. tumefaciens* strain A722 containing the octopine-type Ti plasmid pTiA6NC (*6*), and into a nontumorigenic strain, PC2760-pAL4404 (*12*), which contains a Ti plasmid that has *vir* loci but no T-DNA. As expected, only the wild-type A722 strain caused tumors on tomato plants. However, all plants inoculated with either strain showed typical symptoms of systemic PSTV infection after 10 to 12 days. The consistent appearance of PSTV symptoms shows that this assay is a sensitive indicator of DNA transfer, since the transfer from *Agrobacterium* must be limited to a fairly small number of cells around the wound site. Infections were verified by hybridization analysis (Fig. 2).

To examine the role of the T-DNA borders in the process of infection, we inserted the PSTV-containing plasmid into wide host range plasmids that contained two bor-

Fig. 1. The role of border sequences in PSTV infection induced by strains of *Agrobacterium*. The structure of the wide host range plasmids containing PSTV trimeric DNA and combinations of the T-DNA borders is shown: the thin line refers to the pRK290 vector; the filled box is the pCGN163a containing the PSTV trimer; open boxes designate borders, drawn in the same orientation as they are present in the T-DNA. Each transconjugant was inoculated on at least six tomato plants (*Lycopersicon esculentum* cv. Rutgers) grown in a growth chamber. Plants were scored for symptoms of systemic PSTV infection (epinasty and rugosity) after 14 to 18 days. The pCGN211a and pCGN211b contain a Hind III fragment from pTiC58 (*1*) containing the right T-DNA border; all other constructs with borders contain the left or right border regions of the T_L-DNA from pTiA6 (*1*). Details of the construction of these plasmids are available from the authors.

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ders, one border, or no border. The structure of these plasmids and the results of the infection assays are shown in Fig. 1. The plasmid lacking borders did not cause symptoms; in contrast, all the plasmids with at least one border gave rise to PSTV infection. The location of the PSTV cDNA relative to the borders had no effect. For example, placing the PSTV trimer either between the two borders or outside them resulted in the appearance of symptoms. Although either the left or right borders alone were capable of transfer, we observed a difference in the timing of infection. The lag of several days for PSTV infection after some inoculations in which the PSTV copies were linked to the left border only may reflect a lower transfer efficiency.

These experiments directly demonstrate that borders are involved in T-DNA transfer and that either the left or right border can effect transfer to the plant cell. Other studies have shown that the border sequences are likely to be involved in the transfer or integration of T-DNA (*4, 5*). Surprisingly, PSTV cDNA that was located outside the T-DNA borders was transferred to the plant. Since the borders mark the boundaries of the integrated T-DNA (*13, 14*), the border sequences may be directly involved in integration as well as transfer of the T-DNA.

Table 1. Effect of virulence mutations on the infectivity of *Agrobacterium* containing PSTV DNA.

Locus	Mutant	Plasmid	Infectivity
*vir*A	A1007	pCGN201	–
	A1016	pCGN201	–
	A1030	pCGN201	–
*vir*B	A1019	pCGN201	–
	A2000	pCGN201	–
	A2002	pCGN201	–
*vir*C	A1021	pCGN201	–
*vir*D	A2010	pCGN201	–
*vir*E	A1026	pCGN201	+
	A2011	pCGN201	+

Plasmid pCGN201 has the PSTV trimer located between the T-DNA borders (Fig. 1). The A1000 series of Tn5 mutants were isolated and mapped by Garfinkel and Nester (*6*), and the A2000 series (Tn3) by Klee *et al.* (*8*). The infectivity assay is described in detail in Fig. 1. At least six plants were tested for each strain; results were uniformly positive or negative for each strain.

Alternatively, the association of the borders with the integration end points of T-DNA may simply be the result of a linear intermediate with borders near the termini as a result of processing within the bacterium.

We have tested a number of virulence mutants of *Agrobacterium* for their ability to initiate PSTV infections on tomato plants (Table 1). *Agrobacterium* strains with mutations in the virulence loci A, B, C, and D lacked the ability to promote infection with PSTV. Thus, the genes corresponding to these loci must be required for the transfer of T-DNA into the plant cell. However, the two mutants of the *vir*E locus that we tested both gave rise to systemic PSTV infections. Therefore, this locus is not required for transfer of the PSTV-containing T-DNA into the plant. The distinct avirulence of these strains suggests a critical role for the *vir*E gene product in the normal course of tumorigenesis by *Agrobacterium*.

The appearance of viroid symptoms reflects bona fide Ti plasmid–mediated transfer of T-DNA from *Agrobacterium* to plant cells. Symptom appearance required the presence of at least one border and of the majority of the virulence genes. The results for pCGN202 (Fig. 1) and the *vir*A, B, C, and D loci (Table 1) collectively show 54 out of 54 separate inoculations on tomato plants in which the *Agrobacterium* strain contained multimers of the PSTV cDNA but did not result in PSTV infections. Thus, nonspecific "escapes" of plasmid DNA or bacterial transcripts cannot account for the uniformly positive results seen with the other strains. The reliability of the assay is also indicated by the fact that all plants inoculated with a given strain developed symptoms within a few days of each other.

These data indicate that the *vir*E gene is not required for transfer of T-DNA from *Agrobacterium* to the plant cell. However, the recent report (*15*) of a circular T-DNA intermediate in *Agrobacterium* taken together with the data in Table 1 suggested to us that the *vir*E gene product could be involved in the formation of this intermediate. If T-DNA vectors like pCGN201 resembled the intermediate closely enough, it would not be necessary for the *vir*E gene product to form the intermediate. To test this, an *Agrobacterium vir*E mutant containing pVCK232 was inoculated onto a tomato plant. The plasmid pVCK232 (*16*) has the same replicon as pCGN201 but contains the entire tumorigenic T-DNA of pTiA6. Since the strain did not induce tumors on tomato, the *vir*E locus does not seem to be involved in the formation of transfer intermediates. Moreover, Otten *et al.* recently showed that *vir*E mutants can successfully transfer their T-DNA to plants if they are coinoculated with a wild-type strain (*17*). This unusual form of interbacterial complementation prompted these authors to suggest that the *vir*E gene product is secreted.

The assay of PSTV infection has allowed us to subdivide the process of plant transformation by *A. tumefaciens* into two stages: the first requires the *vir*A, B, C, and D loci, but not *vir*E; the second stage requires *vir*E. We suggest that the first stage represents

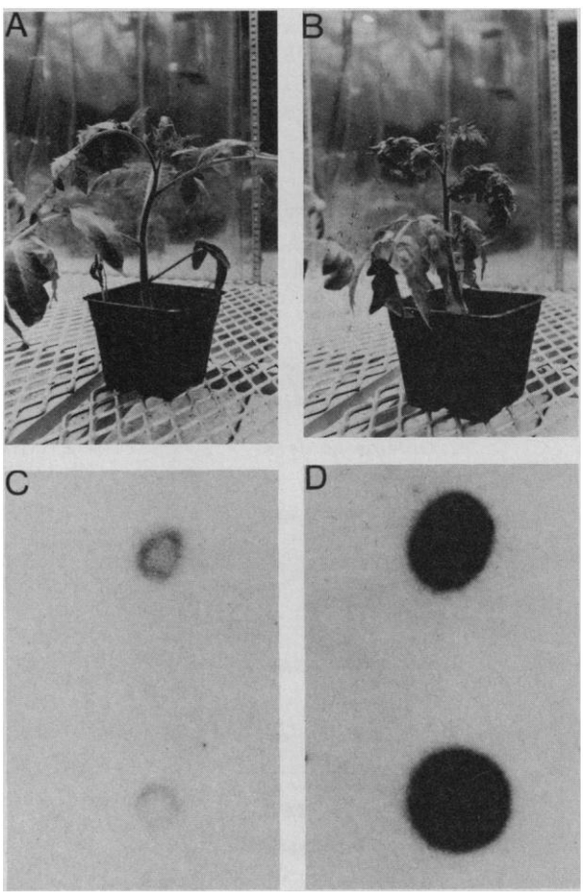

Fig. 2. PSTV infection of tomato plants resulting from the transfer of a viroid cDNA by *Agrobacterium*. (A) Leaves of normal, uninfected tomato. (B) Leaves of PSTV-infected tomato. (C) Hybridization analysis of leaf tissue from uninfected tomato plants showing background hybridization to a radioactive probe made from cloned PSTV cDNA. (D) Hybridization analysis of PSTV RNA in leaf tissue from tomato plants showing disease symptoms. The hybridization analysis was performed essentially as described by Owens and Diener (*19*) but with the addition of a phenol-chloroform extraction prior to spotting of samples onto nitrocellulose.

DNA transfer from the bacterium to the plant cell and the subsequent steps involve the *vir*E gene product(s) and the integration of T-DNA into plant host chromosomes.

Experiments in which *Agrobacterium* is cocultivated with regenerating plant protoplasts show that this bacterium is a highly efficient vector for DNA transfer, giving transformation rates of up to 50 percent (*18*). The availability of mutants of *Agrobacterium* that efficiently transfer DNA into plant cells, but do not integrate the DNA, might provide a means for the isolation or identification of autonomously replicating sequences or of centromeres from plants. *Agrobacterium* also has the potential to become a "suicide" vector for the introduction of transposable elements into plants or for the assessment of plant transformation systems on the basis of homologous recombination.

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20. These experiments were reviewed by an institutional biosafety committee prior to initiation of the investigations; all plant material was contained in a single committed growth chamber and was autoclaved after analysis.

21. We thank J. Hille, who suggested analogous experiments with the cauliflower mosaic virus; R. Owens and R. Goodman, who proposed the use of *Agrobacterium* to transfer cDNA copies of viroids; and those colleagues who reviewed this manuscript before submission. We thank R. A. Owens for PSTV cDNA clones, K. R. Chonoles for technical assistance, and L. Comai and B. Rose for some of the border-containing plasmids.

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The Cyclopean Ear: A New Sense for the Praying Mantis

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The praying mantis, thought to be deaf, possesses a sensitive and specialized acoustic sense. Neural recordings show that the auditory system responds primarily to ultrasound between 25 and 45 kilohertz with thresholds of 55 to 60 decibels. Other insects with auditory tympana possess paired, laterally placed ears; the mantis has only a single ear that is located in the ventral midline between the metathoracic legs. Some species of mantis abruptly and dramatically alter their flight path when stimulated with ultrasonic pulses, suggesting a behavioral response to insectivorous echo-locating bats.

ALL ANIMALS THAT HEAR USING true ears have two functional hearing organs. Tympanal auditory organs occur in members of four insect orders (*1*) and in all cases the ears are widely separated on the body allowing maximum opportunity for these small animals to obtain directional information about the sound source (*2*). We report tympanate hearing in another insect order, the Dictyoptera (mantises, cockroaches, and termites). Moreover, unlike other animals, the praying mantis possesses a single ear located in the ventral midline: it is an auditory cyclops.

Our evidence for audition in the praying mantis, *Mantis religiosa,* is first based on extracellular neural responses recorded from the ventral nerve cord of wild-caught adults (*3*). Stimulation with sound over a broad range of frequencies and intensities elicited strong stimulus-locked responses. At most, four action potential size classes could be distinguished. In the best frequency range for the response, the shortest latency to the first spike was 10 to 14 msec. The composite response was tonic, persisting throughout a 300-msec tone burst, and showed very high initial spike rates (up to 750 spikes per second) followed by instantaneous rates of 150 to 400 spikes per second.

The frequency tuning of the overall extracellular response is shown in Fig. 1 (solid line). These mantises hear best in the ultrasonic range. They are at least 30 times less sensitive at frequencies below 10 kHz as they are in their range of maximum sensitivity: 25 to 45 kHz. The shape of the mantis auditory tuning curve is similar to those of lacewings, many moths, and to that of an interneuron important in the cricket auditory system (*4*). In the best frequency range, the mantis hearing sensitivity [55 to 60 dB SPL (sound pressure level)] is comparable to those of lacewings and crickets (50 to 60 dB SPL), though less than that of moths (40 to 45 dB SPL).

We were not able to show any directional component in the auditory response. Sound stimuli at 35 kHz presented from either the side ipsilateral or contralateral to the recording electrode elicited an identical neural response as long as the sound pressure level of the stimuli from each side, measured at the animal, was the same.

By intracellular recording in the metathoracic connective (*5*), we discovered two types of cells that gave strong responses to sound. One of these types was penetrated twice, but only briefly. It was characterized by a best frequency range of 20 to 40 kHz, latencies of 20 to 30 msec, best sensitivities of 55 to 60 dB SPL, and a phasic firing pattern: over a broad stimulus intensity range it responded with only one to two spikes at the stimulus onset.

The second cell type encountered with intracellular recording showed temporal and frequency response patterns closely resembling the extracellular recordings. The tuning curve for one of these cells is shown in Fig. 1. It differs from the extracellular curve only at the low and high extremes of the frequencies tested. Both the latency and the number of spikes per stimulus varied with intensity in exactly the same manner as those for the extracellular response. This cell type fired tonically throughout a 300-msec tone burst; the initial rates of 550 to 600 spikes per second drop after 3 to 5 spikes to a steady rate of 100 to 200 spikes per second.

Figure 1 also shows the anatomy of one of these tonically firing auditory interneurons. It has a large (7 to 12 μm) ascending axon that lies in the dorsolateral quadrant of the connective near the sheath. Processes of this cell branch widely in the hemiganglion ipsilateral to the axon, and a major branch also crosses the midline. The soma was not filled in any of our preparations.

Transections at several levels of the ventral

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