VirD2 gene product from the nopaline plasmid pTiC58 has at least two activities required for virulence

Todd R. Steck⁺, Tzu-Se Lin and Clarence I. Kado*

Davis Crown Gall Group, Department of Plant Pathology, University of California, Davis, CA 95616, USA

Received August 22, 1990; Revised and Accepted November 8, 1990

ABSTRACT

Virulence genes *virD1* and *virD2* are required for T-DNA processing in *Agrobacterium tumefaciens*. The regions within *virD2* contributing to T-DNA processing and virulence were investigated. Some insertional mutations in *virD2* prevented T-DNA border endonucleolytic cleavage and produced an avirulent phenotype. However, a non-polar insertion immediately after bp 684 of the 1344 bp open reading frame of *virD2* did not inhibit endonucleolytic cleavage but still caused a loss of virulence. This suggested that in addition to T-DNA border cleaving activity, the VirD2 protein has another virulence function which resides in the C-terminal half of the protein. Comparative nucleotide sequence analyses of *virD2* showed that the first 684 bp were 81% homologous to *virD2* of an octopine Ti plasmid whereas the remaining 660 bp were only 44% homologous. A plasmid containing the *virD* region from octopine Ti plasmid could restore both virulence and processing to a nopaline *virD2* mutant. No complementation resulted when a nopaline *virD2* clone containing a region similar to eukaryotic nuclear envelope transport sequences was deleted from the 3' end. These results suggest that *virD1* and only the first half of *virD2* are required to encode for the T-DNA processing endonuclease, and that the 3'-half of *virD2* encodes a function separate from endonuclease activity that is required for virulence.

INTRODUCTION

Induction of the crown gall tumor disease requires the virulence (*vir*) region genes located on a large Ti (tumor-inducing) plasmid harbored in *Agrobacterium tumefaciens*. Upon induction of these genes a 10–25 kb sector of the plasmid (the T-DNA) is transferred to the plant cell and integrated into the plant genome (for recent review see 1). The *vir* region in the nopaline plasmid pTiC58 spans 28.6 kb (2,3) and consists of six operons (in order: *virA*, *virB*, *virG*, *virC*, *virD*, and *virE*) of which *virD* encodes trans-acting proteins that are required to process and transfer the T-DNA. Ti plasmids are classified based on genes carried in the

T-DNA region which encode enzymes that catalyze the production of opines (e.g. nopaline and octopine). There are few known differences in *vir* gene organization, gene product functions and nucleotide sequences of the open reading frames (ORFs) between the operons common to both nopaline and octopine Ti plasmids.

In strains carrying octopine Ti plasmids, linear single-stranded forms of T-DNA (4–8), both double- and linear single-stranded forms of T-DNA (9), and a small amount of circular double-stranded forms of T-DNA (10,11) have been observed in response to *vir* gene induction. The initial step in T-DNA processing of octopine plasmids is border nicking by the octopine *virD1* and *virD2* encoded endonuclease (5,12). VirD1 also contains a topoisomerase activity (13). Single-stranded forms of T-DNA have also been observed in strains carrying nopaline-derived mini-Ti plasmids (14,15).

Our examination of the native nopaline plasmid pTiC58 indicates that a predominant form of processing results in double-stranded cleavage of the T-DNA borders (16). The processing events were dependent upon the *virA*, *virG*, and *virD* operons. To determine the region of the *virD* operon required for processing, and whether the observed differences in processing between plasmid types may be due to the *virD*-encoded endonuclease, we examined T-DNA processing in various *virD* mutants, the ability of octopine *virD* clones to complement nonpolar *virD* mutants, and compared the *virD2* DNA sequence from pTiC58 and the octopine plasmid pTiA6.

METHODS AND MATERIALS

Bacterial strains, enzymes and chemicals

A. tumefaciens LBA4301 Rec, Rm^r (17), LBA4011 (17) and NT1RE (18) were grown at 29°C in medium 523 (19) containing 25 ug/ml rifampicin (Rm) and, when appropriate, 25 ug/ml neomycin (Nm) or 50 ug/ml erythromycin. A list of plasmids used in this study is given in Table 1. Enzymes were purchased from Boehringer Mannheim Biochemicals and used according to manufacturer's specifications. (α -³²P) dCTP was obtained from Amersham Corp. (Arlington Heights, IL). Antibiotics and other chemicals were from Sigma Chemical Co. (St. Louis, MO).

* To whom correspondence should be addressed

⁺ Present address: Department of Biological Sciences, Lilly Hall of Life Sciences, Purdue University, West Lafayette, IN 47907, USA

Construction of insertion mutants in *virD2*

Plasmid DNA was isolated and manipulated according to Maniatis et al. (20) unless otherwise stated. All *vir* region mutants except pJK705 have been described previously (2). pJK705 was constructed by marker exchange as follows: the neomycin phosphotransferase II (*npII*) gene, isolated from transposon Tn5 as a *HindIII* fragment, was ligated to gel purified partially *HindIII*-digested pUCD2-11K2. pUCD2-11K2 (21) is the broad-host-range plasmid pUCD2 (Tc^r) containing *KpnI* fragment 11 of pTiC58 (22). This fragment includes the *virG*, *virC*, and 5' region of the *virD* operons (2). *E. coli* strain HB101 was transformed with the resulting plasmid which was then transferred into *A. tumefaciens* strain LBA4011-RE (Rm^r, Er^r) by triparental mating using the helper plasmid pRK2013 (23). Transconjugants were selected by growth on medium 523 containing Rm and Nm. Recombination of *KpnI* fragment 11 containing the *npII* gene into pTiC58 was scored for by loss of Tc resistance. The location of the *npII* gene in the resulting pTiC58-derivative pJK705 was confirmed by Southern blot analysis (see below). pJK705 was then mobilized from the recombination proficient strain LBA4011 into the Rec- strain LBA4301 by screening for Rm^r, Er^r and UV^s colonies.

Virulence assays

Virulence assays were performed on *Datura stramonium*, a very sensitive host for LBA4301 harboring pTiC58 (24). Plants approximately 4–6" high were inoculated by puncturing the stem with a straight pin followed by addition of 5 µl of a cell culture at a concentration of approximately 2 × 10⁹ cells/ml. Plants were scored for tumor formation 25 days after inoculation.

Isolation of T-DNA intermediates

Bacterial cultures were grown overnight in medium 523 and then diluted 5-fold into MSSP-medium (25) and incubated at 29°C for 20 hr. The cultures were then diluted 100-fold into MSSP supplemented with 5% medium 523 containing antibiotics at one-fifth the concentration used when grown in medium 523. The *vir* genes of early exponential cells were induced by the addition of 100 µM acetosyringone (AS), and after 16 hr four ml of the

cell culture were collected by centrifugation. The pelleted cells were suspended in 50 µl TEN buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 10 mM NaCl), followed by addition of 25 µl of 600 µg/ml proteinase K (Sigma Chemical Co.) and 25 µl of 10% NaDodSO₄ in TEN buffer. Cells were incubated at 37°C for 60 min, then pipetted through a 1 ml tip to dissociate clumps. NaCl was added to the lysate to a final concentration of 150 mM and the solution incubated at 65°C for 30 min. The lysate was extracted twice with redistilled phenol/chloroform (1:1, v/v), twice with chloroform, and the nucleic acids were precipitated with isopropanol. The precipitated DNA was suspended overnight on ice in TEN buffer containing 0.1 µg/ml RNase A (Sigma Chemical Co.) and stored at -20°C.

Southern blot analysis

Isolated DNA was digested with *Asp718* (an isoschizomer of *KpnI*), and subjected to electrophoresis at 5 V/cm through 0.7% agarose gels in 1 × TBE buffer (20). Transfer of DNA to a Zeta-probe nylon membrane was carried out under denaturing conditions (26) as described by BioRad (Richmond, CA). DNA probes were gel purified and labeled using the random sequencing primer method of Feinberg and Vogelstein (27). The relative intensity of hybridized DNA was quantitated from preflashed Kodak X-OMAT film using a Zeineh SL-504-XL scanning densitometer.

RESULTS**Region of *virD2* required for T-DNA processing**

Double-stranded cleavage at the T-DNA left border (Fig. 1A) was assayed by Southern blot analysis of *Asp718*-digested DNA isolated from uninduced and acetosyringone(AS)-induced LBA4301 containing pJK270. pJK270 is pTiC58 containing a Tn5 insertion in a region of the T-DNA that does not affect virulence (18). These analyses of total cellular DNA from induced and uninduced cells indicated that treatment of the bacteria with the inducer AS resulted in the double-stranded cleavage of approximately 30% of the DNA (Fig. 1B). Previously we showed that this cleavage is dependent upon *virA*, *virG* and *virD* (16).

TABLE 1: Plasmids

Plasmid	Relevant characteristic	Source
pJK270	Tn5 in T-DNA of pTiC58 Tra ^c , vir ⁺	18
pJK710	pTiC58 Tra ^c virG::Tn5	2
pJK195	pTiC58 Tra ^c virD1::Tn5	2
pJK703	pTiC58 Tra ^c virD2::npII	2
pJK705	pTiC58 Tra ^c virD2::npII	this study
pJK504	pTiC58 Tra ^c virD4::Tn5	2
pUCD2480	pSa with a <i>Sall</i> fragment containing the 3' region of <i>virC</i> , <i>virD</i> , and the 5' region of <i>virE</i> , in addition TnCAT is inserted in the 3' region of <i>virD3</i>	this study
pUCD2481	pSa with a <i>Sall</i> fragment containing the 3' region of <i>virC</i> , <i>virD</i> , and the 5' region of <i>virE</i> , in addition TnCAT is inserted in the 5' region of <i>virD3</i>	this study
pUCD3032	pSa with <i>Sall</i> - <i>HindIII</i> fragment containing the 3' region of <i>virC</i> , <i>virD1</i> , <i>virD2</i> , and the 5' region of <i>virD3</i>	this study
pUCD3033	pUCD3032 with deletion of 7bp into 3' end of <i>virD2</i>	this study
pUCD3035	pUCD3032 with deletion of 77bp into 3' end of <i>virD2</i>	this study
pUCD3036	pUCD3032 with deletion of 142bp into 3' end of <i>virD2</i>	this study
pCP13-13	pCP13::pTi15995 <i>HindIII</i> fragments 21c-22d (<i>virCDE</i>)	S. Farrand
pCP13-57	pCP13::pTi15995 <i>HindIII</i> fragments 3-17 (<i>virGCD</i>)	S. Farrand

To define the involvement of *virD* in this process further, insertional mutants in the *virD* region (see Table 1, and Fig. 2A) were examined. T-DNA processing occurred in pJK703 and pJK504 but not in pJK195 and pJK705 (Fig. 2B). The difference in the amount of processing between pJK703 and pJK504 is within the experimental range observed in three trials.

The region of the 1344 bp ORF of *virD2* (29) required for T-DNA processing can be approximated from results with pJK703 and pJK705. A *npII* cassette is inserted in pJK705 at the *HindIII* site located 263 bp downstream of the ATG initiation codon. Likewise *npII* is inserted in pJK703 at the *EcoRI* site located 684 bp downstream of the ATG codon. Therefore, the 3' boundary of the region necessary for endonuclease activity lies between bp 263 and 684 of *virD2*.

Nopaline and octopine *virD2* sequence comparison

The *virD* operon of pTiC58, sequenced previously (28), was found to contain errors owing to early sequencing strategies. The *virD* sequence has been subsequently corrected (29). This sequence was recently reported by Wang et al. (30). However a comparison of their sequence with our data showed discrepancies at three positions. In the Wang et al. sequence (30) there is a missing guanine residue between their coordinate positions 2216 and 2217, an extra guanine residue at position 2223 and an erroneous base (A) at position 2257. We find that the 1344 bp *virD2* DNA sequences from pTiC58 and pTiA6 show 64% homology (data not shown). As the *virD2* ORF from pTiA6 is 69 bp shorter than that of pTiC58, the 3'-terminal 69 bases were not included in computation of similarity. When the pTiC58 *virD2* ORF is separated into two regions by virtue of the JK703 insertion, and each region is compared to the corresponding octopine *virD2* sequence, the first region shows 81% homology whereas the second region shows only 44% homology. Comparison of the DNA-derived amino acid sequences indicates that the first 228 amino acids are 82% homologous and the remaining 220 amino acids are only 19% homologous. The C-terminal encoding half of *virD2* from pTiC58 therefore shows considerable differences to its counterpart in pTiA6.

Figure 1: T-DNA processing in pTiC58. (A) A map of the LB region of pTiC58 with the size of *KpnI* fragment 13a (22) spanning the LB and the expected fragment sizes resulting from double-stranded cleavage at the border. (B) LBA4301 containing pJK270 with (+) or without (-) AS induction was lysed as described in Methods and Materials. DNA was digested with *Asp718*, Southern blotted and probed with *KpnI* fragment 13a as described in Methods and Materials. Gel fragment sizes are given in kb.

Figure 2: T-DNA processing in *vir* mutant strains. (A) The four ORFs of the *virD* operon and the positions of Tn5 (□) and *npII* gene (○) insertions are shown. (B) Cells were induced and lysed as described in Methods and Materials. DNA isolated from mutants indicated above each gel lane was digested with *Asp718*, Southern blotted and hybridized to *KpnI* fragment 13a (22) as described in Methods and Materials. Gel fragment sizes are given in kb.

Figure 3: Complementation assay. *Datura stramonium* plants were stem inoculated with LBA4301 containing the plasmids indicated below each picture. Plant stem sections were photographed after 25 days.

A

```

GTTGGCCCGCCAGCGAAGCGCGGCGAAGGATGGCAAGTGGCCCTTAGTACGGCAAGTGGAACTCTCGGECATCTCCACGAGC 1170
V G P Q A N A G E Q D G S S G F L V R Q A G T S R P S P P T

GCCACGACCGCGGCGAGCAGCGAAGCGATTGCTGTCTGCTACAGCCACCTCCAGCAACGAGAGGTGCTCTTCAAGCGTCCGCGT 1260
A T T R A S T A T D S L S A T A H L Q Q R R G V L S K R P R

3035
GAAGATGATGAGGAGAACGAGTGAACGCAACGCGAGAGATGAGCGCAGCAAGGCGGCGTGGGGAAATAGGAGATAGAGAAGG 1350
E D D D G E P S E R K R E R D E R S K D G R G N R R E N D

ACCGAGTGATGGCAATGAAGAGTTTACCAGACACTATGCGTGGCCGTTCTGTGGCTTCGAATGATGAAGGCGTGGGACGCGCGGA 1440
3032
TCCCATCCAGGCACAACTCAATCGTTGCTG 1470

```

Figure 4: Complementation of insertion mutants with 3' deleted *virD2*. A) The *virD2* DNA sequence from bp 1081 to 1470, and the corresponding amino acid sequence are shown. Plasmids pUCD3032, pUCD3033, pUCD3035, and pUCD3036 contain *virD1* and various 3' deletions into *virD2*. The endpoint of the deletions is indicated by a designated wedge. The sequence encoding amino acids similar to a nuclear transport consensus polypeptide (33) is indicated by a line below the DNA sequence. B) LBA4301 containing various plasmids as indicated in the figure was inoculated into the stem section of *Datura stramonium* plants. Plant stem sections were photographed after 20 days.

3'-region of *A. tumefaciens virD2* is required for virulence

When LBA4301 containing pTiC58 bearing defined mutations in the *vir* region was assayed for virulence in *Datura*, no tumors were produced. These results are consistent with our previous observation that the entire *virD* operon is required for virulence (26). To determine if the *nptII* insert in pJK703 and pJK705 would, as expected, allow transcriptional readthrough, strains were complemented with pUCD2480 and pUCD2481. These plasmids are derivatives of pSa (31) and contain the *virD* operon from pTiC58 (constructed by Peter Rogowsky) with a promoterless TnCAT cartridge (32) located in the 5' region (pUCD2480) or in the 3' region (pUCD2481) of *virD3*. As shown in Fig 3, only mutants JK705 and JK703 were complemented

Figure 5: T-DNA processing in octopine-complemented mutants. LBA4301 containing pCP13-13 with an addition plasmid and treated with AS as indicated below was lysed as described in Methods and Materials. Isolated DNA was digested with *Asp718*, Southern blotted and hybridized to *KpnI* fragment 13a (22) as described in Methods and Materials. Plasmids: lane 1, pJK710; lane 2, pJK710 + AS; lane 3, pJK195; lane 4, pJK195 + AS; lane 5, pJK705; lane 6, pJK705 + AS; lane 7, pJK703; lane 8, pJK703 + AS; lane 9, pJK270; lane 10 pJK270 + AS. Gel fragment sizes are given in kb.

by pUCD2480 and pUCD2481, indicating that neither pJK703 nor pJK705 block transcription and are therefore nonpolar. Furthermore, the lack of tumor formation in pJK703, indicates that the promoter-distal half of *virD2* is required for virulence but not directly for T-DNA processing.

Analysis of the derived amino acid sequence in the 3' half of the *virD2* ORF revealed at 56 bp from the 3' end (at nucleotide positions 18938 to 18963) homology to several sequences proposed as a transport signal for nuclear localization of proteins (33). To verify that this region of *virD2* was involved in virulence, deletions were made in the 3' terminal end of *virD2* (Fig. 4A) and assayed in *D. stramonium* for the ability to complement the *virD2* insertion mutants. A plasmid (pUCD3032) containing the entire *virD2* ORF complemented both pJK703 and pJK705, indicating that functional VirD2 was being produced from the complementing plasmid. Removal of the 3' terminal 142 bp (pUCD3036) or 77 bp (pUCD3035) prevented complementation of pJK703 and pJK705. Complementation of pJK703 and pJK705 with *virD2* lacking the 3' terminal 7 bp (pUCD3033) partially restored virulence, as judged by the formation of very small tumors (Fig. 4B).

Octopine *virD2* can substitute for nopaline *virD2*

Although there are significant differences between the octopine and nopaline Ti plasmids in the 3'-half of *virD2*, we confirmed the earlier result that pCP13-13 containing the *virD* of the octopine Ti plasmid pTi15995 restored virulence to pJK195 and pJK703 (2), indicating that the *virD* operon from both pTi15995 and pTiC58 encode similar functions. The cosmid pCP13-13 contains the entire *virD* operon inserted into the pLAFR1 derivative pCP13 (34). Because we have observed large quantities of double-stranded cleavage products from wild-type sized nopaline plasmids (16; Fig. 1 & 2), and others have observed primarily single-stranded molecules from octopine plasmids (4,5,7,8), we wanted to characterize the T-DNA processing products observed in the nopaline mutants complemented with pCP13-13. As shown in Fig. 5, the processing products seen in pJK270 and pJK703 containing pCP13-13 are double-stranded, the same type as is seen in the absence of pCP13-13. However no T-DNA processing products and no tumors were observed when pJK705 was complemented with pCP13-13. Similar virulence results were obtained when strains were complemented with pCP13-57 (data not shown).

DISCUSSION

To characterize the involvement of *virD2* in double-stranded T-DNA intermediate formation, the gene was analyzed using genetic and biochemical approaches. These studies revealed the interesting finding of two domains in *virD2*: the 5'-half of the gene is required for T-DNA border cleavage and the 3'-half is required for other function(s) associated with virulence. We found that the DNA sequences from the 1344 bp *virD2* gene from pTiC58 and *virD2* from pTiA6 show 81% homology for the first 684 bp of *virD2* and 44% homology for the remaining 660 bp. At most, the first 684 bp of the 1344 bp *virD2* ORF are required for T-DNA processing (Fig. 2B, lane 2). That the entire *virD2* ORF is not required for T-DNA processing has been shown for an octopine (15) and nopaline (30) plasmids in *A. tumefaciens*, and for subclones of *virD* from both nopaline and octopine plasmids assayed in *E. coli* (5,12,35). Based on these results, it appears that nopaline and octopine plasmids encode similar endonucleases specific for T-DNA border target sequences, and that *virD1* and at most the N-terminal half of *virD2* are required for endonuclease activity. Therefore, differences in the type of T-DNA border processing observed in nopaline and octopine plasmids are not likely to be due solely to differences in the *virD2* encoded portion of the endonuclease.

That a nonpolar mutation in the center of the *virD2* ORF does not inhibit T-DNA processing but renders the strain avirulent indicates that the VirD2 protein has at least two distinct functions required for virulence. This is consistent with results obtained with an octopine *virD2* mutant. The pTiA6 derived *virD2* nonpolar mutant pTi304 is avirulent (34) yet makes T-strands (15). Although not directly demonstrated previously, the N-terminal half of VirD2 apparently contains a moiety sufficient for endonuclease activity (Fig.1).

What function of VirD2 requires the C-terminal half of the protein? It is unlikely that the C-terminal region of VirD2 acts only to stabilize VirD2 binding to DNA. Cells containing pJK703 lysed in the absence of protease treatment result in retarded gel migration of processed T-DNA (16). Because this altered gel migration results from VirD2 bound to the end of the processed T-DNA, the *nptII* insertion JK703 in *virD2* would be expected to disrupt and reduce any binding cooperativity between the 3' and 5' regions of VirD2. Similarly, the C-terminal region of VirD2 does not appear to be involved in the type of processing that occurs at the T-DNA borders. In the presence of the octopine *virD2* region, pJK703 and pJK270 produced the same double-strand border breaks as is seen in the absence of pCP13-13. An activity of the C-terminal half of VirD2 is suggested from the observations that VirD2 is covalently attached to the T-DNA borders of both a processed octopine Ti plasmid (37,38) and a processed mini T-DNA nopaline Ti plasmid (39-41). VirD2 bound to the ends of the processed T-DNA might aid in piloting processed T-DNA to the cell membrane, perhaps facilitating interaction with a membrane porin encoded by the *virB* operon.

Interestingly, pJK705 had neither T-DNA processing nor virulence restored by pCP13-13. This is surprising since pJK705 contains a nonpolar insertion in *virD2* and pJK195, which is complemented by pCP13-13, contains a polar insertion in *virD1*. The simplest explanation for these results is that VirD1 and VirD2 interaction is required for T-DNA processing but nopaline VirD1 and octopine VirD2 proteins do not interact to an extent sufficient to generate wild type levels of endonuclease activity. The lack of complementation of pJK705 with pCP13-13 suggests that production of only nopaline VirD1 and not a functional VirD2

can prevent octopine VirD1 and VirD2 from functioning. That nopaline VirD1 in the absence of VirD2 is able to prevent complementation by octopine VirD1 and VirD2 is consistent with nopaline plasmids having a higher virulence efficiency (42-46; B. Hohn et al., 1989, Fallen Leaf Lake Conference Abstr. p. 16). Perhaps nopaline VirD1 has a higher affinity for the T-DNA borders than octopine VirD1.

A possible additional function of VirD2 is suggested by the results in which removal of the 3' terminal 77 bp prevented complementation of a nonpolar *virD2* insertion mutant whereas deletion of the 3' terminal 7 bp did complement (Fig. 4B). Between these two end-points [within bp 18938 and 18967 (29)] is located a consensus sequence for transport of proteins across the nuclear envelope into the nucleus. These results suggest that VirD2 may act as a pilot protein to guide the T-DNA through the nuclear envelope into the plant nucleus. However, we have not eliminated the possibility that the lack of complementability of pUCD3035 and pUCD3036 is due to the deletions affecting protein stability. This possibility is currently being investigated.

ACKNOWLEDGEMENTS

We thank Eva Zyprian for relating unpublished results, Stephen Farrand for pCP13-13 and pCP13-57, Peter Rogowsky for many helpful discussions, Brad Powell for help in computer homology searches, and Stan Gelvin, Eva Zyprian, Haresh Kamdar, Brad Goodner and Michael Cooley for reviewing the manuscript. This work was supported by PHHS, NIH research grant CA-11526-21 from the National Cancer Institute. Preliminary results of this work were presented at the Fallen Leaf Lake Conference, South Lake Tahoe, California, September 14-17, 1989, Abstract p. 44.

REFERENCES

1. Ream, W. (1989) *Annu. Rev. Phytopathol.* **27**,583-618.
2. Rogowsky P., Close T. J., Chimera J. A., Shaw J. J. & Kado C. I. (1987) *J. Bacteriol.* **169**,5101-5112.
3. Morel P., Powell, B. S., Rogowsky, P. M. & Kado, C. I. (1989) *Mol. Microbiol.* **3**,1237-1246.
4. Albright L. M., Yanofsky M. F., Leroux B., Ma D. & Nester E. W. (1987) *J. Bacteriol.* **169**,1046-1055.
5. Yanofsky M. F., Porter S. G., Young C., Albright L. M., Gordon M. P. & Nester E. W. (1986) *Cell* **47**,471-477.
6. Stachel S. E., Timmerman B. & Zambryski P. (1987) *EMBO J.* **6**,857-863.
7. Wang K., Stachel S. E., Timmerman B., Van Montagu M. & Zambryski P. (1987) *Science* **235**,587-591.
8. Veluthambi K., Ream W. & Gelvin S. B. (1988) *J. Bacteriol.* **170**,1523-1532.
9. Veluthambi K., Jayaswal R. K. & Gelvin S. B. (1987) *Proc. Natl. Acad. Sci. USA* **84**,1881-1885.
10. Koukolikova-Nicola Z., Shillito R. D., Hohn B., Wang K., Van Montagu M. & Zambryski P. (1985) *Nature* **313**,191-196.
11. Machida Y., Usami S., Yamamoto A., Niwa Y. & Takebe I. (1986) *Mol. Gen. Genet.* **204**,374-382.
12. Jayaswal R. K., Veluthambi K., Gelvin S. B. & Slightom J. L. (1987) *J. Bacteriol.* **169**,5035-5045.
13. Ghai J. & Das A. (1989) *Proc. Natl. Acad. Sci. USA* **86**,3109-3113.
14. Culianez-Macia F. A. & Hepburn A. G. (1988) *Molecular Plant-Microbe Interactions* **1**,207-214.
15. Stachel S. E., Timmerman B. & Zambryski, P. (1986) *Nature* **322**,706-712.
16. Steck T. R., Close T. J. & Kado C. I. (1989) *Proc. Natl. Acad. Sci. USA* **86**,2133-2137.
17. Klapwijk P. M., van Beelen P. & Schilperoort R. A. (1979) *Mol. Gen. Genet.* **173**,171-175.
18. Kao J. C., Perry K. L. & Kado C. I. (1982) *Mol. Gen. Genet.* **188**,425-432.
19. Langley R. A. & Kado C. I. (1972) *Mutat. Res.* **14**,277-286.
20. Maniatis T., Fritsch E.F. & Sambrook J. (1982) *Molecular cloning: a laboratory manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

21. Close T. J., Zaitlin D. & Kado C. I. (1984) *Plasmid* **12**,111–118.
22. Depicker A., DeWilde M., DeVos G., DeVos R., Van Montagu M. & Schell J. (1980) *Plasmid* **3**,193–211.
23. Ditta G., Stanfield S., Corbin D. & Helinski D. (1980) *Proc. Natl. Acad. Sci. USA* **77**,7347–7351.
24. Hirooka T. & Kado C. I. (1986) *J. Bacteriol.* **168**,237–243.
25. Stachel S. E., Messens E., Van Montagu M. & Zambryski P. (1985) *Nature* **318**,624–629.
26. Southern E. M. (1975) *J. Mol. Biol.* **98**,503–517.
27. Feinberg A. P. & Vogelstein B. (1983) *Anal. Biochem.* **132**,6–13.
28. Hagiya M., Close T. J., Tait R. C. & Kado C. I. (1985) *Proc. Natl. Acad. Sci. USA* **82**,2669–2673.
29. Rogowsky, P.M., Powell, B.S., Shirasu, K., Lin, T.-S., Morel, P., Zyprian, E.M., Steck, T.R. and Kado, C.I. (1990) *Plasmid* **23**,85–106.
30. Wang, K., Herrera-Estrella, A. & Van Montagu, M. (1990) *J. Bacteriol.* **172**,4432–4440.
31. Tait R. C., Close T. J., Lundquist R. C., Hagiya M., Rodriguez R. & Kado C. I. (1983) *Bio/Technology* **1**,269–275.
32. Kamoun S., Cooley M. B., Rogowsky P. M. & Kado C. I. (1989) *J. Bacteriol.* **171**,1755–1759.
33. Boulukas, T. (1987) *Cytology and Cell Physiology*, ed. G.H. Bourne, (Academic Press, Inc.)
34. Friedman, A.M., Long, S.R., Brown, S.B., Buikema, W.J. & Ausubel, F.M. (1982) *Gene* **18**, 289–296.
35. DeVos, G. & Zambryski, P. (1989) *Mol. Plant-Microbe Interactions* **2**,43–52.
36. Stachel S. E. & Nester E. W. (1986) *EMBO J.* **5**,1445–1454.
37. Young C. & Nester E. W. (1988) *J. Bacteriol.* **170**,3367–3374.
38. Ward E. R. & Barnes W. M. (1988) *Science* **242**,927–930.
39. Herrera-Estrella A., Zhong-mei C., Van Montagu M. & Wang K. (1988) *EMBO J.* **7**,4055–4062.
40. Howard, E., Winsor B. A., De Vos G. & Zambryski P. (1989) *Proc. Natl. Acad. Sci. USA* **86**,4017–4021.
41. Durrenberger, F., Cramer, A., Hohn, B. & Koukolikova-Nicola, Z. (1989) *Proc. Natl. Acad. Sci. USA* **86**,9154–9158.
42. Marks M. S., Kemp J. M., Woolston C. J. & Dale P. J. (1989) *Plant Science* **63**,247–256.
43. Boulton, M. I., Buchholz, W. G., Marks, M. S., Markham, P. G. & Davies, J. W. (1989) *Plant Mol. Biol.* **12**,31–40.
44. Zyprian, E. & Kado, C.I. (1990) *Plant Mol. Biol.* **15**, 245–256.