PARENTAL JUNCTIONS IN RECOMBINANT CAULIFLOWER MOSIAC VIRUS DNAS

By

VALERIE RAY VADEN

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PARENTAL JUNCTIONS IN RECOMBINANT

CAULIFLOWER MOSAIC VIRUS DNAS

Thesis Approved:

Ulrich Melcher
Thesis Adviser

Charles O. Gardner Jr.

Richard C. Essenberg

Norman N. Durham
Dean of the Graduate College

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NOMENCLATURE

bp	base pairs
cm	centimeter
dH_2O	distilled water
dCTP	deoxycitidine triphosphate
DNA	deoxyribonucleic acid
G1	gap 1
h	hours
kbp	kilo base pairs
kd	kilodalton
M	Molar
ma	milliamps
mg	milligram
ml	milliliter
mM	millimolar
nm	nanometer
nt.	nucleotide
nts.	nucleotides
ORF	open reading frame
RNA	ribonucleic acid
tRNA	transfer RNA
U	units
ug	microgram

ul	microliter
v	volts
w.t.	wild-type
°C	degrees Centigrade
3′	three prime
5′	five prime
~	approximately

CHAPTER I

INTRODUCTION

General Background

Cauliflower mosaic virus (CaMV) is the type member of the caulimoviruses, the only known taxonomic group of plant viruses whose genetic material is double-stranded DNA. This characteristic genome has caused CaMV to be studied not only as a potential vector for introducing foreign DNA into plants for crop improvement, but also as a model system for studying the genetic interactions occurring within plant cells (as reviewed by Hull and Covey, 1983).

CaMV systemically infects members of the Crucifer family, such as broccoli, cauliflower, and turnip. Both the virus particles and purified DNA are infectious to these plants when inoculated mechanically. Typical symptoms elicited by the plants include mottling of the leaves (mosaic), vein clearing, stunted growth, and rugosity of the leaves (Covey, 1985).

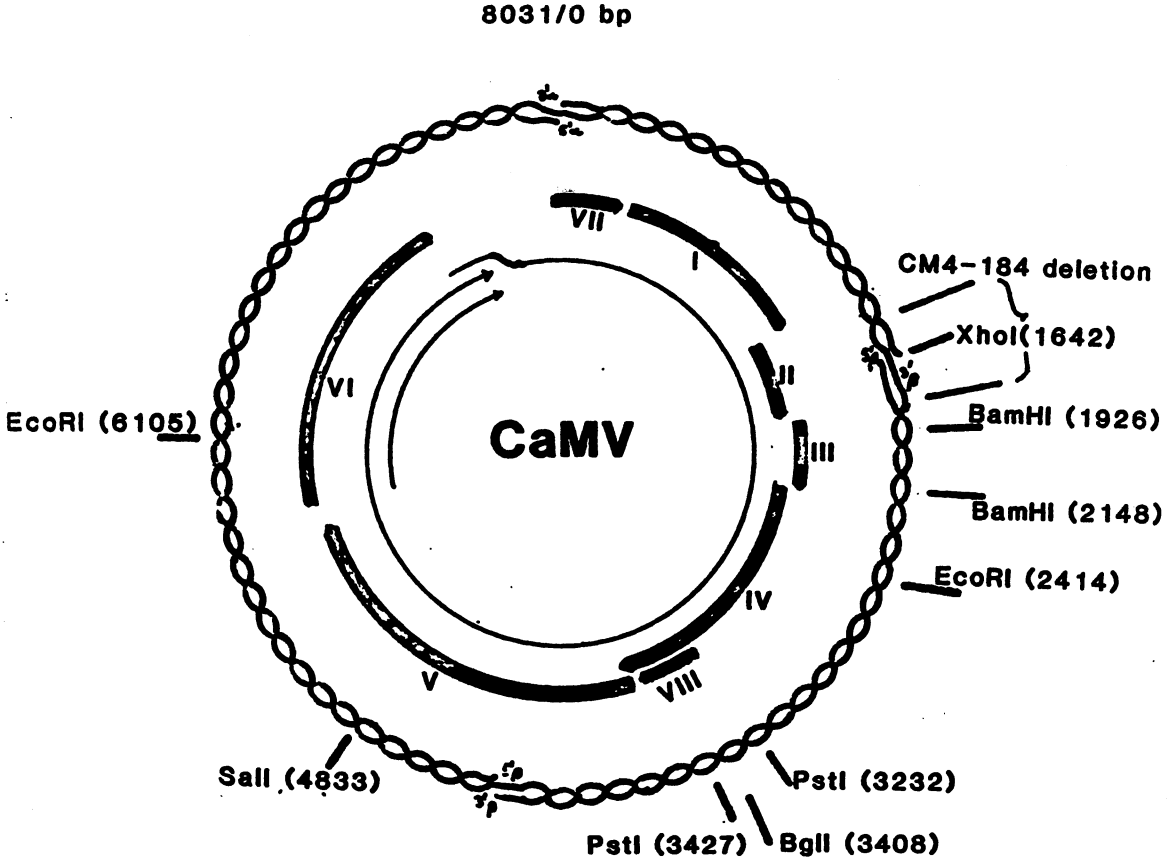
CaMV is an icosahedral virus with an outer diameter of 50 nm, and an inner diameter of 20 nm. The virion's molecular weight was calculated to be 22.8×10^6 daltons, and it is 17% DNA by weight (Covey, 1985).

Structure of CaMV DNA and RNA

The DNA of CaMV is approximately 8 kilobase pairs (kbp), with one strand defined as the "plus" strand and the other as the "minus" strand (Figure 1). The plus-strand contains the open reading frames (ORFs) and the minus-strand is that which is transcribed into RNA. CaMV DNA is unusual in that it contains small regions of triple-strandedness. This triple-strandedness creates gaps, or discontinuities, with one gap occurring in the minus-strand and depending on the isolate, one or two occurring in the plus-strand. At each discontinuity the 5′ nucleotide is in a fixed position, while the 3′ nucleotide overlaps the 5′end, and this overlapping can vary, from ten to twenty bases at gaps 1 and 2 (G1 and G2, respectively), to forty bases at gap 3 (G3) (Richards, et al., 1981).

Eight long open reading frames have been found by nucleotide sequencing. These ORFs are all located on only one of the two strands of DNA (the plus strand), and could encode for polypetides with molecular weights greater than 10 kilodaltons (kd) (Franck, et al., 1980). The functions of ORFs I and III have not yet been elucidated, but deletions in these regions have suggested that they are essential for propagation of the virus in plants (Howell, et al., 1981 and Gardner, 1983). Xiong, et al. (1984) showed that ORF III is expressed *in vivo* and codes for a 15 kd protein (P15). Giband, et al. (1986) found that the ORF III product associ-

Figure 1. Map of the cauliflower mosaic virus genome. (From D. L. Steffen's Ph.D. dissertation.)


8031/0 bp
CaMV
VII
I
II
III
IV
V
VI
VIII
CM4-184 deletion
XhoI(1642)
BamHI (1926)
BamHI (2148)
EcoRI (2414)
PstI (3232)
BglI (3408)
PstI (3427)
SalI (4833)
EcoRI (6105)

ates with viral particles, probably at the capsid surface, and that it also exhibits DNA-binding activity. ORF II has been shown to be involved as a "helper component" in transmission of the infection to plants by aphids via the synthesis of an 18,000 molecular weight polypeptide (Armour et al., 1983; Daubert et al., 1983; Woolston et al., 1983). The 58,000 molecular weight protein coat precursor is encoded by ORF IV and processed into the 42,000 molecular weight capsid protein (Franck, et al., 1980; Hahn and Shepherd, 1982; and Burger and DuPlessis, 1983). That the coat protein is found only in the inclusion bodies of infected plant cells led to speculations that encapsidation is closely linked to replication (Giband, et al., 1984). ORF VI codes for the inclusion body matrix protein (Odell and Howell, 1980 and Covey and Hull, 1981). This protein has also been shown to play a role in the type of local symptom produced by the plant in response to infection (Schoelz, et al., 1986).

Evidence is mounting that ORF V (79,000 molecular weight) is the gene coding for the viral reverse transcriptase. Computer analysis has shown it shares amino acid sequence homology with retroviral reverse transcriptase, and it is the most highly conserved coding region among the CaMV isolates (Volovitch, et al., 1984; Toh, et al., 1983). Takatsuji, et al. (1986), obtained reverse transcriptase activity from yeast expressing a cloned ORF V gene, while Laquel, et al. (1986), demonstrated that the enzyme activity

associated with viral replication complexes could be inhibited with antibodies raised against a synthetic peptide corresponding to a portion of the gene V protein. Its role in replication will be discussed later.

Two major polyadenylated RNAs, the 19S and 35S transcripts, have been isolated from infected plants. Both are transcribed from the minus-strand of DNA. The 19S transcript has its 5' and 3' ends at nucleotides 5765 and 7615, respectively (numbering system is that of Franck et al., 1980). The 19S transcript is the messenger RNA (mRNA) for ORF VI's inclusion body protein. The 35S species is a longer-than-genome-length transcript of the entire DNA minus-strand, having its 5' end at nucleotide 7470 and its 3' end at nucleotide 7615 (Covey, et al, 1981, and Guilley, et al., 1982). This transcript is 180 nucleotides longer than the minus-strand and is considered to be the primary template for DNA synthesis by reverse transcription (Olszewski, et al., 1982; Pfeiffer and Hohn, 1983).

CaMV Replication Model

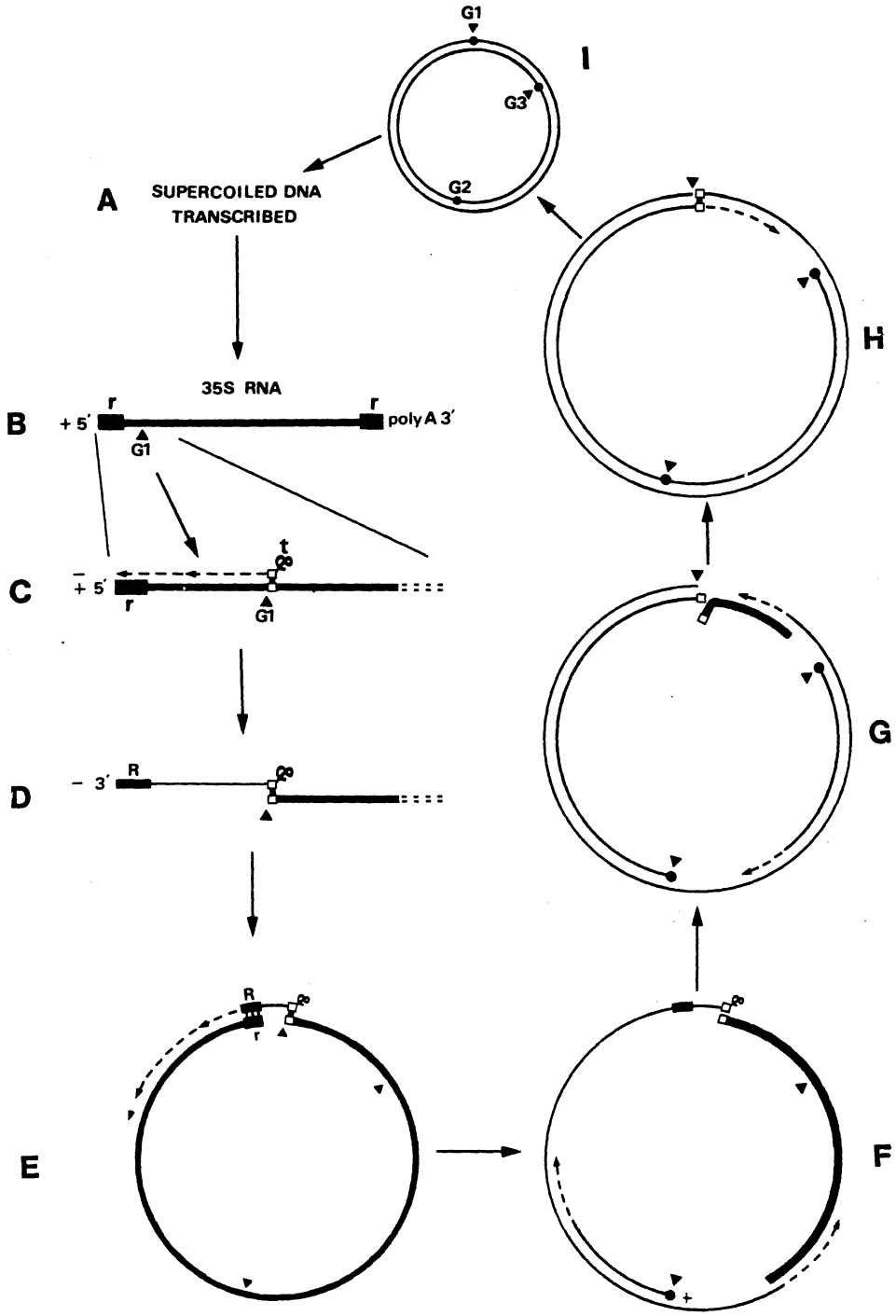
The mode of replication for CaMV currently being investigated involves a viral reverse transcriptase. A viral reverse transcriptase has not been previously reported to occur in plants and would therefore be a unique system for plants. The reverse transcription step is similar to that of retroviral and hepadnaviral reverse transcriptions. The similarities include a tRNA primer for the synthesis of

minus-strand DNA on an RNA template, the formation of strong-stop DNA with accompanying template switch, the presence of purine-rich regions associated with plus-strand priming, and a second template switch at the minus-strand priming site.

Little is known about how the virus enters the initially infected cells. That protoplast infection *in vitro* required polycations which promote adsorption of viral particles onto the cell surface prompted speculations that viral factors may promote adsorption of viral particles onto subcellular structures *in vivo* (Furusawa, et al., 1980; Howell and Hull, 1978). Little is known of the initial uncoating of the virions. Since proteases are required for releasing DNA *in vitro* perhaps they are also required *in vivo* (as reviewed by Hull and Covey, 1985).

Features of CaMV replication have been summarized by several groups (Hull and Covey, 1983; Pfeiffer and Hohn, 1983). The model of replication by reverse transcription is depicted in Figure 2. At an early stage in the infection, the DNA must enter the nucleus. After association with host proteins, the gaps will be repaired and the molecule supercoiled. Transcription of this supercoiled DNA occurs via the host cell RNA polymerase II. After transcription, the polyadenylated RNA moves to the cytoplasm for reverse transcription. This genome-length RNA contains terminal direct repeats of 180 nucleotides ("r" in Figure B). Initiation of reverse transcription occurs with the 3' end of a host-cell $tRNA^{met}$ primer binding to 14 nucleotides

Figure 2. A model for the replication cycle of CaMV DNA. (Adapted from Hull and Covey, 1983.)


G1
I
G3
G2
A
SUPERCOILED DNA
TRANSCRIBED
35S RNA
B
+ 5′
r
r
poly A 3′
G1
t
C
+ 5′
r
G1
D
– 3′
R
H
G
R
r
E
F
+

immediately to the right of G1 as depicted in C of Figure B. Reverse transcriptase copies the RNA to its 5' end at nucleotide 7435, resulting in a small minus-strand DNA molecule of approximately 600 nucleotides that contains the terminally repeated sequence, which is considered equivalent to the "strong stop" DNA of retroviruses (D in Figure B). The repeated sequence in the minus-strand DNA ("R") base-pairs with the repeated sequence at the 3' end of the same 35S RNA or of a different one (E in Figure B). Here, the reverse transcriptase switches templates, creating the first template "jump". Minus-strand synthesis continues, and the copied template RNA is degraded, probably by RNase H activity. The minus-strand synthesis continues past nucleotides 4218 and 1632 (Gaps 2 and 3, respectively), which are purine-rich regions that serve as plus-strand primer-binding sites. It has been suggested that primers at these sites may be residual RNA that was not degraded due to the resistance of the rG:dC base pairs to RNase H attack, or may be some unidentified RNA with the consensus sequence (Pfeiffer and Hohn, 1983). From these positions, synthesis of DNA plus-strands is initiated (F and G of Figure B). The tRNA primer sequence is copied into the the plus-strand, and the tRNA primer complementary sequence is copied into the the minus strand. These tRNA sequences base pair, allowing the reverse transcriptase its second template switch. Minus- and plus-strand synthesis continue (H in Figure 2), producing a

double-stranded gapped CaMV DNA molecule (I in Figure 2).

Some important variations between the reverse transcription system of retroviruses and hepatitis B virus (HBV) and that of CaMV also exist (as reviewed by Hohn et al., 1985). CaMV's reverse transcription occurs in the cytoplasmic inclusion bodies while the same for retroviruses occurs in the pre-virus particles, although pre-virions may be present in the inclusion bodies; CaMV uses between one and three plus-strand primer sites while HBV and retroviruses use only one; retroviruses form DNA with long terminal repeats that can integrate into the host cell chromosomes while CaMV forms relaxed and supercoiled circular DNA and has not yet been shown to integrate. Each of these three systems also differ in their viral genetic materal: HBV particles contain incomplete double-stranded DNA (containing regions of single-strandedness); retroviruses contain RNA; and CaMV particles contain a complete double-stranded genome with single-stranded overhangs (the gaps) (Hohn, et al., 1985).

Recombination in CaMV

Recombination is defined as "any set of pathways in which elements of nucleic acid interact with a resultant change of linkage of genes or parts of genes" (Clark, 1971). Several recombination events have been observed to occur between CaMV DNAs. Lebeurier, et al. (1980) had demonstrated that the infectivity of cloned CaMV DNA was dependant upon its being excised from the vector DNA at the restriction

enzyme site used in cloning. Homologous, intergenomic recombination events, by yielding infectious progeny, "rescued" coinoculated non-infectious CaMV DNAs that contained non-overlapping mutations (Howell, et al., 1981; Lebeurier, et al., 1982; Daubert, et al., 1983; and Choe, et al., 1985). Analysis of the resulting progeny virions revealed that they contained wild-type (w.t.) DNA, and therefore most likely resulted from recombination as opposed to complementation.

Walden and Howell (1982) proposed two general mechanisms for homologous recombination observed in CaMV. In the first, infectious progeny are produced from coinoculated linear DNA molecules with complementary overhanging ends (ie., "sticky ends"). This type of recombination could occur by the formation of mixed dimers, or even higher multimers, that can result in wild-type genomes via single cross-over events. The other recombination mechanism involves viral DNAs that do not have complementary sticky ends. Infectious DNAs are produced via a double cross-over or internal gene conversion between the mutant DNAs. Recombinants of this type arise readily (Walden and Howell, 1982 and Choe et al., 1985).

Intragenomic recombination has also been shown to occur in CaMV. Lebeurier et al. (1982), used circular plasmid DNAs containing cloned, partially deleted tandem dimers CaMV DNA to infect plants; while Walden and Howell (1983) obtained infectious virions with intact recombinant plasmids containing complete CaMV genomes flanked by homologous viral DNA

arms. The frequency of infection increased with increasing length of the flanking homologous DNA arms (Walden and Howell, 1983).

Evidence is accumulating that some CaMV recombinants result from the mechanisms of reverse transcription. Replicative recombination could result if, while undergoing an intramolecular template switch during the viral replication cycle, the reverse transcriptase actually "jumped" onto a second RNA template, thus creating an intermolecular template switch. A plasmid containing, in tandem, one complete genome of the CaMV CM4-184 isolate and a 3.3 kbp fragment of a different CaMV isolate ("S"), was used with the transforming DNA of *Agrobacterium tumefaciens* to obtain infectious CaMV virions (Grimsley et al., 1986). Analysis of the progeny revealed the majority probably arose by means of the mechanisms of reverse transcription, i.e., template switching. DNA sequencing of the naturally occurring deletion mutant, CM4-184, believed to be a clonal derivative of CM1841, revealed that it contained regions similar to the CM1841 isolate and also to the CaMV Cabbage S (Cabb S) isolate (Dixon et al., 1986). The regions of recombination between the two parental isolates could be explained by the switching of templates during reverse transcription. Recombinant CaMV DNAs resulting from the co-inoculation of two mutant DNAs contained regions from both parental DNAs (Choe et al., 1985). Nucleotide sequencing of the regions surrounding Gap I of the CaMV genome revealed

unique nucleotides from one parent upstream of Gap I and unique nucleotides from the other parent downstream of Gap I (Melcher, unpublished). These findings are consistent with recombination by reverse transcription as, in accordance with the CaMV model for replication, Gap I arises as a result of template switching. Geldreich et al., (1986) reported recombinant genomes that possibly resulted from *in vivo* dimerization of two genomes, one defective, that were restricted at the same site. The subsequent production of a hybrid 35S RNA could have given rise to the recombinant progeny.

CHAPTER II

MATERIALS AND METHODS

Cleavage of DNAs by Restriction Enzymes

Restriction enzymes were used to digest CaMV DNAs for three purposes: one, to free the CaMV DNA from its plasmid vector to use in inoculation of turnip plants; two, to provide the desired CaMV DNA fragments to sequence; and three, as a means of producing DNA fragments having characteristic banding patterns on agarose or polyacrylamide gels in analysis of the recombinant CaMV DNAs.

The restriction enzymes and buffers used are shown in Table 1. Most were used in analysis of the recombinant DNAs, with the exceptions of SalI, which was used to cleave DNAs from their plasmid vector, and BamHI and XbaI, which were used in producing fragments to sequence. The plasmids used in this study are shown in Table 2.

Phenol Extraction and Ethanol Precipitation of DNA

A standard procedure often used in the preparation of DNA was the removal of protein by extracting an aqueous solution of DNA with water saturated phenol. The phenol

Table I. Restriction enzymes and buffers used in study. All reaction mixtures contained 100 ug/ml bovine serum albumin and were incubated for 2 hours or more at 37°C, except for TaqI reaction mixtures, which were incubated under paraffin oil at 60°C.

TABLE I

RESTRICTION ENZYMES AND BUFFERS

Enzyme	Buffer
AccI	60 mM NaCl, 6 mM Tris (pH 7.5), 6 mM $MgCl_2$, 6 mM 2-mercapto-ethanol
AluI	50 mM NaCl, 6 mM Tris (pH 7.6), 6 mM $MgCl_2$, 6 mM 2-mercapto-ethanol
BamHI	50 mM NaCl, 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol
BglI	60 mM NaCl, 10 mM Tris (pH 7.4), 10 mM $MgCl_2$, 1 mM 2-mercapto-ethanol
ClaI	50 mM NaCl, 6 mM Tris (pH 7.9), 6 mM $MgCl_2$, 6 mM 2-mercapto-ethanol
EcoRI	50 mM NaCl, 100 mM Tris (pH 7.2), 5 mM $MgCl_2$, 2 mM 2-mercapto-ethanol
HaeIII	6 mM NaCl, 6 mM Tris (pH 7.4), 6.6 mM $MgCl_2$, 6 mM 2-mercapto-ethanol
HindIII	50 mM NaCl, 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol
HinfI	100 mM NaCl, 6 mM Tris (pH 7.5), 6 mM $MgCl_2$, 6 mM 2-mercapto-ethanol
MspI	6 mM KCl, 10 mM Tris (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol
PstI	50 mM NaCl, 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol
SalI	150 mM NaCl, 6 mM Tris (pH 7.9), 6 mM $MgCl_2$, 6 mM 2-mercapto-ethanol
TaqI	6 mM NaCl, 6 mM Tris (pH 7.4), 6 mM $MgCl_2$, 6 mM 2-mercapto-ethanol
XbaI	100 mM NaCl, 6 mM Tris (pH 7.4), 6 mM $MgCl_2$

Table II. Plasmids used in study. All CaMV DNAs are cloned into the SalI site of pBR322 except W DNA, which is cloned into the XhoI site of pACYC177 (see text).

TABLE II

PLASMIDS USED IN STUDY

Plasmid	Isolate	Mutation	Reference
pDLS19	NY8153	deletion 3233-3427	Melcher et al., (1986)
pUM124	Cabb S	deletion 886-1032	Choe et al, (1985)
pIC23	W	deletion 6317-6664	Choe et al, (1985)
pUM24	Cabb S	deletion 6299-6338, SmaI linker	Choe et al, (1985)
pLW414	CM4-184		Howell, et al., (1980)
pLW214	CM4-184	EcoRI linker at 1285	Howell, et al., (1981)
pLW76	CM4-184	deletion 777-1208	Howell, et al., (1981)
pIC141	Recombinant		Choe et al, (1985)
pIC143	Recombinant		Choe et al, (1985)
pIC148	Recombinant		Choe et al, (1985)

denatures proteins and removes them from the nucleic acids.

An amount of 1 M Tris-HCl, pH 8.0, that was equal to one-tenth the sample volume was added to the sample. Also added were one-fiftieth volume of 5 M NaCl and one volume of water-saturated phenol. These combined volumes were mixed by vortexing and then centrifuged for 5 minutes in either a Beckman model B microfuge, an Eppendorf model 5415 microfuge at 10-12,000 rpm, or at 10,000 rpm for 15 minutes in a Beckman J-21 centrifuge. The upper aqueous layer was transferred to a new Eppendorf microfuge tube, avoiding transferring the interphase and lower phenol layer. To remove any traces of phenol, three to five volumes of ether were added, followed by vortexing. The upper ether layer and any interphase were removed. To precipitate the DNA, two and one-half volumes of cold 95% ethanol were added, then the contents of the tube were mixed by inversion. The DNA precipitate was allowed to form at either -20°C overnight or at -70°C for approximately 1 hour. This precipitate was recovered by centrifugation as described above. After decanting the liquid, a wash of 0.5 ml of cold 70% ethanol aided in removing any solutes that may have been trapped in the precipitate. The pellet and wash was centrifuged and the ethanol decanted. The DNA pellet was dried by vacuum aspiration in a dessicator and then dissolved in the desired amount of DNA dissolving buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM NaCl) or, depending on the fate of the DNA, water.

Care and Maintainance of Turnip Plants

The host plants used in this study were turnips (*Brassica rapa*, cv. Just Right). Seeds were planted and the seedlings transplanted in Jiffy Mix Brand potting soil and transplanted at age 2 weeks. The plants were fertilized twice weekly. 9 to 12 days after transplanting, the plants were inoculated with the plasmid and viral DNAs as described below.

Growth conditions included daily waterings, 12 h of light with temperature of 21°C, and 12 h dark with temperature of 19°C.

Inoculation With Mutant CaMV DNAs

Recombinant CaMV DNAs were obtained from turnip plants infected as the result of various coinoculations of turnip plants with two mutant viral DNAs. These mutant CaMV DNAs were first released from their plasmid vector by digestion with SalI or XhoI as described previously. Completion of digestion was confirmed by electrophoresing 0.2-0.3 ug of DNA on 0.8 or 1% agarose slab gels. The vector DNA, pBR322, was not separated from the inoculation mixture prior to inoculation.

Until inoculation, the enzyme-DNA mixture was stored at -20°C. The inoculum consisted of the two mixtures of the mutant CaMV DNAs, 9.2% (v/v) 20x SSC (3.0 M NaCl, 0.3 M sodium citrate), and 200 ug celite. After removing the two

primary leaves of the turnip plants, 20 ul of inoculum were applied to three leaves and gently rubbed into the leaves with gloved fingers. The DNA concentrations in the mixtures were such that each plant was inoculated with 1 ug of DNA. Examinations for symptoms of mottling, chlorotic lesions, and stunting were performed for ten to thirty days after inoculation.

Inoculation With Virally Infected Leaf Tissue

To either maintain or increase the supply of recombinant viral DNAs, turnip plants were inoculated with homogenates of infected turnip leaves. The infected leaves were first ground in a mortar with 3 ml of 1% K_2HPO_4 and acid-washed sea sand. The mixture was passed through cheese cloth to filter out the larger particles. This method provided approximately 2 ml of inocula. 10 mg of celite were added to the mixture and then the plants were inoculated as previously described.

CaMV DNA Isolation From Infected Leaves

Virus was isolated from systemically infected turnip leaves using a modified procedure of Gardner and Shepherd (1980). 2 g of leaf tissue were quick frozen with liquid nitrogen and ground to a fine powder. 10 ml of TEU solution (0.2 M Tris-HCl, pH 7.5., 0.02 M EDTA, and 1.5 M urea) were stirred into the powder. After pouring the mixture into a 30 ml Corex tube kept on ice, 2 ml of 10% Triton X-100 were used

as a rinse for the mortar, then poured into the Corex tube. The crude homogenate was pelleted by centrifugation at 10,000 rpm in a Beckman Type JA-20 rotor for 15 minutes. Decanting the supernatant through Miracloth caught any floating debris. At this point, the samples were either stored at -20°C or processed further. 2 ml of TEU-sucrose solution (15% w/v sucrose, 2% v/v Triton X-100, to volume with TEU solution) were pipetted into a 17mm plastic Oak Ridge centrifuge tube to serve as a sucrose cushion through which to pellet the virus. The leaf supernatant was gently pipetted onto this sucrose pad and centrifuged at 34,000 rpm for 2.5 hr at 4°C in either a Beckman Type Ti 65 or Ti 75 rotor.

After centrifugation, the liquid was carefully aspirated out of the Oak Ridge tube with a pasteur pipet attached to a vacuum aspirator, and 2.0 ml of viral resuspension buffer (0.1 M Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$) were added to the pellet. The pellet and buffer were transferred to 1.5 ml Eppendorf microfuge tubes, to which 2 ul of 1 mg/ml DNAse I (from bovine pancreas) was added. After incubation at 37°C for 10 minutes, 4 ul of 0.5 M EDTA, 50 ul of 2.5 mg/ml proteinase K, and 12.5 ul of 20% SDS were added. This was also followed by an incubation for 10 minutes or longer, but at 65°C. The proteins were removed and DNA concentrated by phenol extraction and ethanol precipitation as previously described. The dried DNA pellet was then dissolved in 30 ul of DNA dissolving buffer.

Sticky-End Ligation

The isolated, recombinant CaMV DNAs were cloned into the bacterial plasmid pBR322 (Bolivar, et al., 1977). This vector is a molecule of double-stranded DNA with 4,363 base pairs containing genes conferring resistance to ampicillin (amp^r) and tetracycline (tet^r). Both CaMV and pBR322 contain a single SalI restriction enzyme site, which was utilized for ligation purposes. This site in pBR322 lies within the tetracycline resistance gene and insertion of DNA into this site renders the plasmid unable to confer tet^r on tet^- bacterial host cells.

Each 20 ul of ligation mixture consisted of 0.37 ug of alkaline-phosphatase treated pBR322 DNA, 7.5 ul of SalI-digested recombinant CaMV DNA isolated from turnip leaves, 2 ul of 10x ligation buffer (0.66 M Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP), and 200 U/ul T_4 DNA ligase. These mixtures were incubated at 15°C overnight, with the ligation of CaMV inserts confirmed by electrophoresing 13.5 ul of the ligation mixture on 0.8-1.0% agarose gels.

Transformation and Screening

The ligation mixtures were used to transform competent cells (HB101). The mixtures were first diluted ten-fold with sterile dH_2O. The competent bacteria were thawed 10 minutes in ice water and then a volume twice the diluted ligation mixture was added and mixed by vortexing. After incubating

on ice for 10 minutes, the tubes were frozen for 100 seconds in an ethanol-dry ice bath. The tubes were thawed at room temperature and incubated on ice for 5 more minutes. Upon completion of incubations at 42°C for 1 minute and on ice for 2 minutes, 200 ul of nutrient broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, pH 7.5, to 1 L with dH_2O) were added. The tubes were incubated at 37°C; their contents spread on nutrient agar plates containing 20 ug/ml ampicillin, and incubated at 37°C overnight.

The colonies that arose were replica-plated onto nutrient agar (nutrient broth plus 15 g agar/L) plates containing 10 ug/ml tetracycline and onto ampicillin-containing plates. Any amp^r and tet^s colonies were further analyzed by isolation of the plasmid via the small-scale alkaline-SDS procedure of Ish-Horowicz and Burke (1981) (described below).

Plasmid DNA Isolation---Small Scale

To confirm the successful ligation of the recombinant viral DNA into the pBR322 vector, the tet^s colonies were increased in number by inoculating 5 mls of nutrient broth plus 50 ul of 2 mg/ml ampicillin with these colonies. After shaking overnight at 37°C, 1 ml was used in isolating the plasmid via the procedure of Ish-Horowicz and Burke (1981). The 1 ml was microfuged for 1 minute in a 1.5 ml Eppendorf tube. The pelleted cells were resuspended in 100 ul of Solution I (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0) by vortexing and then incubated at room temperature for

5 minutes. 200 ul of Solution II (0.2 M NaOH, 1% SDS) were added and the tubes mixed by inversion. After incubating these on ice for 5 minutes, 150 ul of cold 5 M KOAc (Solution III) were added. Gentle inversion and another incubation on ice followed. Next, the tubes were microfuged for 2 minutes and the supernatant transferred to a new microfuge tube. 250 ul of isopropanol were then added, followed by mixing by inversion and an incubation at room temperature for at least 15 minutes.

Plasmid DNA Isolation---Large Scale

To produce stock quantities of plasmid DNA, the procedure of Maniatis, et al. (1982) was used. A loopful of bacteria from frozen-storage was streaked onto ampicillin plates so as to obtain isolated colonies and incubated overnight at 37°C. An isolated colony from the plate was used to inoculate 5 ml of nutrient broth containing 50 ul of 2 mg/ml ampicillin. The 5 ml nutrient broth tubes were shaken overnight at 37°C and then used to inoculate 250 ml of nutrient broth containing 2.5 ml of 2 mg/ml ampicillin. The 250 ml nutrient broth flasks were shaken at 250 to 300 rpm overnight at 37°C.

The resulting bacteria were pelleted in 250 ml centrifuge bottles by centrifugation in a Beckman Type JA-14 rotor at 8,000 rpm for 10 minutes. The supernatant was decanted and the bacterial pellet resuspended in 5 ml of Solution I. The resuspended bacteria were transferred to a 50 ml centri-

fuge tube. After sitting at room temperature for 10 minutes, the tubes were put in ice and 10 ml of Solution II were added. Upon mixing by inversion, the tubes were further incubated on ice for 10 minutes. 7.5 ml of solution III were added and the tubes were again mixed by inversion. This was followed by another incubation on ice for 10 minutes. After centrifugation in a Beckman Type JA-20 rotor at 12,000 rpm for 15 minutes, 18 ml of the supernatant were pipeted into 12 ml of isopropanol in 30 ml centrifuge tubes. The tube's contents were mixed by inversion and allowed to sit at room temperature for 15 minutes or longer. After this precipitation incubation, the tubes were centrifuged in the Type JA-20 rotor at 12,000 rpm for 10 minutes and the supernatant decanted. The pellet was washed with 10 ml of 70% ethanol by centrifuging again, followed by decanting the supernatant and drying in a vacuum dessicator. Upon dissolving in 1 ml TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) the pellet was transferred to an Eppendorf microfuge tube and microfuged for 5 minutes. The supernatant was decanted into a new microfuge tube and stored at 4°C until further purification by cesium chloride and ethidium bromide density gradient centrifugation.

Cesium Chloride and Ethidium Bromide Purification of Plasmid DNA

Further purification of the plasmids from RNA and/or proteins was accomplished by centrifugation in a density gradient of cesium cloride (CsCl) with the DNA intercalating

agent ethidium bromide (EthBr). Covalently closed, circular DNA bands at a higher density in cesium chloride gradients than does linear DNA as it binds less EthBr. EthBr also makes the DNA bands visible in ultraviolet light.

Into each Beckman "Quick Seal" 13x51 mm polyallomer centrifuge tube 4.95 g dry CsCl, 4.5 ml plasmid sample plus distilled water (dH_2O), and 450 ul 10 mg/ml EthBr was added. The tubes were balanced, heat sealed, and centrifuged in a Beckman VTi65.2 ultracentrifuge rotor at 20°C for either 48 hours at 34K rpm or 24 hours at 50-55K rpm. The plasmids were removed in semi-darkness by illuminating the tubes with UV light, puncturing them with a size 18G hypodermic needle, and withdrawing the plasmid band into a 1 cc syringe. This band was located near the middle of the tube, with the band of linear or relaxed DNA 1-2 cm above it, and the RNA band stuck vertically along one wall of the tube.

The plasmid DNA-EthBr complex was separated from the CsCl by ethanol precipitation. Phenol extraction removed the EthBr from the DNA. After ethanol precipitating the plasmids and redissolving same in 200 ul of DNA dissolving buffer, the concentrations of the plasmid preparations were determined by measuring the absorbance at 260 and 280 nm using a Hitachi Model 100-80A spectrophotometer.

Storage of Bacteria

The plasmid-containing *E. coli* were stored frozen until needed. Bacteria were grown overnight by shaking an isolated

colony in 5 ml of nutrient broth. To freeze the bacteria, 0.85 ml of the bacteria/nutrient broth mixture was added to 0.15 ml sterile glycerol. The bacteria and glycerol were placed in 1.5 ml cryovial tubes, mixed, and placed in a -70°C freezer.

3' End Labeling of DNA

The 3' end of the DNA fragments to be sequenced were labeled with alpha-^{32}P-dCTP. These fragments resulted from a BamHI digest, in which the enzyme cleaved the DNA at the sequence G'GATCC.

Added to each dried DNA pellet were: 12 ul triply-distilled H_2O; 1 ul each of 20 mM dATP, 20 mM dGTP, and 20 mM dTTP; 2 ul 10x reverse transcriptase buffer (0.5 M KCl, 0.4 M Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 50 mM DTT); 2.5 ul 10 mCi/ml alpha-^{32}P-dCTP; and 0.5 ul 20 U/ul reverse transcriptase. This mixture was vortexed lightly and incubated for 30 minutes at 37°C. The enzyme was removed by phenol extraction, with the addition of phenol only; i.e., no salt or Tris buffer was added. Upon ethanol precipitation, the doubly-end-labeled DNA fragments were digested by XbaI, which, with the exception of one fragment, produced singly-end labeled fragments containing the regions to be sequenced. Removal of proteins was by phenol extraction with Tris buffer and phenol only, followed by ethanol precipitation.

The dried pellet was dissolved in 20 ul triply-distilled H_2O and 5 ul 5x TBE stop solution [50% 10x TBE (0.89 M Tris,

0.89 M boric acid, 20 mM EDTA), 50% glycerol, and a few crystals of xylene cyanol and bromphenol blue]. The sample was electrophoresed through a 10% polyacrylamide [acrylamide:bis-acrylamide (30:1)] gel overnight at 3 ma, then at 15 ma the next morning, until the xylene cyanol marker was approximately three-quarters down the length of the gel. The power supplies used were either a BioRad Model 1420 A, a Buchler Model No. 3-1155, or two Gelmans, models unknown. Exposure of Kodak X-Omat AR x-ray film to the parafilm-covered gel and subsequent development of the film allowed the desired DNA fragments in the gel to be excised from the rest of the gel. To aid in the elution of the DNA from the gel, the clumps of gel were smashed through 1 cc sterile syringes (without needles). The fragments were eluted from the gel matrix by shaking the gel/DNA mixture in 1.5 ml DNA fragment elution butter (0.2 M NaCl, 0.2 M Tris-HCl, pH 8.0, 2 mM EDTA) overnight at 37°C. Separation of the gel from the DNA solution was accomplished by filtering the mixture through a 3 cc syringe with its end screened by a circular filter of Whatman 3MM paper. Further purification of the fragments occurred on DEAE cellulose columns.

Purification of End-Labeled DNA Fragments by DEAE Cellulose Columns

Preparation of the DEAE cellulose columns consisted of poking a small amount of glass wool into the end of a short pasteur pipette and adding approximately 1 cm DE-52 cellulose

powder (suspended in DNA fragment elution buffer). The columns were rinsed 2-3 times with more fragment elution buffer. The DNA fragments were passed through the column twice. Then the columns were rinsed twice by filling them with DNA fragment elution buffer before eluting with 400 ul hot (90°C) DNA column elution buffer (1.5 M NaCl, 0.2 M Tris-HCl, pH 8.0, 2 mM Na-EDTA). The fragments were eluted into new 1.5 ml Eppendorf microfuge tubes that contained 1 ul of 10 mg/ml yeast RNA to aid in precipitating the DNA. 1.0 ml of cold 95% ethanol was added and the fragments were precipitated at -20°C overnight. After washing with 70% ethanol, 200 ul triply-distilled H_2O, 100 ul 0.3 M sodium acetate,and 750 ul 95% ethanol were added, and precipitation followed as previously.

Maxam and Gilbert Sequencing Reactions

In Maxam and Gilbert sequencing reactions (Maxam and Gilbert, 1980), the DNA is partially cleaved at each of the four bases in four reactions. The procedures used are shown in Table 3.

Prior to the Maxam and Gilbert sequencing reactions, the DNA in ethanol was pelleted, washed with 70% ethanol, dried, and dissolved in 21 ul triply-distilled H_2O. Into four Eppendorf microfuge tubes the following were added: 13 ul G+A buffer (0.71 mg/ml calf thymus DNA) and 7 ul DNA, 200 ul G buffer (0.01 mg sonicated calf thymus DNA, 50 mM sodium

Table III. Maxam and Gilbert DNA sequencing reactions.
*Buffers are described in text.

TABLE III

PROCEDURE FOLLOWED FOR CLEAVAGE OF DNA
BY MAXAM AND GILBERT SEQUENCING REACTIONS

G+A	G	C+T	C
13 ul G+A buffer 7 ul DNA	200 ul G buffer 3.5 ul DNA	7 ul C+T buffer 7 ul DNA	10.5 ul C buffer 3.5 ul DNA
0°C	0°C	0°C	0°C
2 ul pyridine formate	1 ul DMS*	21 ul hydrazine	21 ul hydrazine
20°C 60 minutes	20°C 4 minutes	20°C 10 minutes	20°C 10 minutes
Freeze in liquid N_2	50 ul DMS stop	200 ul hydrazine stop	200 ul hydrazine stop
Lyophilize 1 hour		750 ul 95% ethanol	
		-70°C, 5 minutes	
10 ul triply-distilled H_2O		Centrifuge: 10K rpm, 10 minutes	
Freeze in liquid N_2		250 ul 0.3 M sodium acetate, 750 ul 95% ethanol	
Lyophilize 1 hour		-70°C, 5 minutes	
		Centrifuge: 10K rpm, 10 minutes	
		750 ul 95% ethanol	
		-70°C, 5 minutes	
		Centrifuge: 10K rpm, 10 minutes	
		100 ul 1.0 M piperidine	
		90°C, 30 minutes	
		Freeze in liquid N_2	

TABLE III (Continued)

G+A	G	C+T	C
	Lyophilize		
	100 ul triply-distilled H_2O		
	Freeze in liquid N_2		
	Lyophilize		
	50 ul triply-distilled H_2O		
	Freeze in liquid N_2		
	Lyophilize		

cacodylate, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA) and 3.5 ul DNA, 7 ul C+T buffer (1.25 mg/ml sonicated calf thymus DNA) and 7 ul DNA, and 10.5 ul C buffer (4.73 M NaCl and 0.121 mg/ml sonicated calf thymus DNA) and 3.5 ul DNA. These reaction tubes were kept on ice. The reactions to disrupt the ring structure of the DNA base were performed by the following: into the G+A tube, 2 ul of pyridine formate were added and incubated at 20°C for 60 minutes; into the G tube, 1 ul of dimethyl sulfate (DMS) was added and incubated at 20°C for 4 minutes; and into both the C+T and C tubes, 21 ul of hydrazine were added, and both tubes were incubated at 20°C for 10 minutes. These reactions were stopped by adding 50 ul DMS stop (1.5 M Na-acetate, pH7.0, 1.0 M 2-mercaptoethanol, 100 ug/ml yeast RNA) to the G tube and 200 ul hydrazine stop (0.3 M Na-acetate, 0.1 mM EDTA, 25 ug/ml yeast RNA) to both the C+T and C tubes. The G+A reaction was stopped by quick-freezing the tubes in liquid nitrogen. At this point, the G, C+T, and C tubes were treated alike by adding 750 ul 95% ethanol, precipitating at approximately -70°C in a dry-ice and ethanol bath for 5 minutes, and centrifuging in either a Beckman Type JA 18.1 or a Sorvall model SM-24 at 10K rpm for 10 minutes. After decanting the supernatant, 250 ul 0.3 M sodium acetate and 750 ul 95% ethanol were added. These were then precipitated and centrifuged as previously. Again, the supernatent was decanted, followed by another addition of ethanol, and another precipitation and centrifugation as

previously. These tubes were then dried in a vacuum dessicator. Meanwhile, the G+A tube was lyophilized for 1 hour until the pyridine formate was removed. Then 10 ul triply-distilled H_2O were added, and the tube was again frozen in liquid nitrogen and lyophilized for another hour. After these steps, the tubes were treated alike. To each, 100 ul 1.0 M piperidine were added, followed by incubation at 90°C for 30 minutes. The piperidine reaction catalyzed the beta-elimination of phosphates from the sugars, thus breaking the DNA strand. Following the incubation, the tubes were quick-frozen in liquid nitrogen and lyophilized until the piperidine was removed. Next, 100 ul triply-distilled H_2O were added, followed by freezing and lyophilizing as above. This was repeated once more, except only 50 ul triply-distilled H_2O were added. Upon completion of these reactions, the samples were stored at -20°C until use.

Several differences exist between the above-described procedure and that of Maxam and Gilbert (1980). While Maxam and Gilbert (1980) used 10 ul of DNA for the G+A and C+T tubes and 5 ul of DNA for the G and C tubes, 7 ul and 3.5 ul respectively, were used here. Also, Maxam and Gilbert (1980) did not utilize a C+T buffer and their C buffer was made of only 5 M NaCl. As described above, 21 ul hydrazine were used, whereas Maxam and Gilbert (1980) added 30 ul. While pyridine formate was utilized in the G+A reactions, Maxam and Gilbert (1980) reported using piperidine formate.

Immediately prior to separation by size via electro-

phoresis on a slab gel, the fragments were dissolved in 5 ul sequencing buffer charge [80 ul formamide, 20 ul 5x sequencing stop solution (5x TBE and a few crystals of xylene cyanol and bromphenol blue)] incubated at 90°C for 1 minute, transferred to ice, and loaded onto the sequencing gel. The gels used were either 8% or 20% acrylamide-urea (Maxam and Gilbert, 1980) and electrophoresis occurred at 1500-2000v.

CHAPTER III

RESULTS

Analysis of Existing Recombinants

Sequencing of Junctions in Recombinants IC143 and IC148

Recombinants pIC141, pIC143, and pIC148 resulted from the coinoculation of turnip plants with the mutant plasmids pIC11 and pUM41, which were both unable to individually infect turnip plants. pUM41 is a Cabb S-derived plasmid with a 4bp deletion in ORF III, nucleotides (nts.) 2041 to 2044, and an 8bp SmaI linker inserted at this position (Choe et al., 1985). pIC11 is derived from the plasmid pLW303X, with a deletion in ORF VI at nts. 6261 to 6420 (Choe et al., 1985). The nucleotide numbering system used is that of Franck et al., (1980).

pLW303X had been reported to be the ligation product of Xho I-digested Cabb B-JI CaMV DNA and Xho I-digested pACYC177 (Walden and Howell, 1982; and Chang and Cohen, 1978). Restriction mapping of pLW303X produced inconsistencies with the published map of Cabb B-JI (Hull, 1980; and Choe et al., 1985). Due to the uncertainty as to the identity of the viral DNA cloned in pLW303X, the DNA was designated as isolate "W" (Choe et al., 1985).

Recombinants pIC141, pIC143, and pIC148, were analyzed by restriction mapping and found to contain restriction enzyme sites unique to each parent (Choe et al., 1985). Among the three recombinants, 8 junctions between parental alleles were detected (Figure 3). To determine the junctions between the parental alleles at the nucleotide level, and perhaps any previously undetected junctions, sequencing of specific restriction fragments was performed. The 3' ends of fragments resulting from the BamHI digestion of the 3 plasmids and pLW303S were labeled with ^{32}P-dCTP. A second digestion by XbaI produced three fragments for each plasmid near junction 2 (see Figure 3). These three fragments extended from nts. 1702 to 1925, 1926 to 2147, and 2148 to 2548, and were 225, 222, and 397 bp in length, respectively (Table 4).

Sequencing of the three fragments revealed junctions between W and Cabb S alleles for two of the three recombinants (pIC143 and pIC148). All the regions sequenced in the recombinants and in W that were different from the known sequence of Cabb S were identical to the known sequence of Cabb B-JI (J. Stanley, personal communication). pIC141 had no Cabb S-derived nucleotides in the regions 1720 to 1883, 1949 to 1999, and 2163 to 2321; all were like W and Cabb B-JI sequences (Table 5).

The nucleotides sequenced in pIC143 were from 1720 to 1883, 1927 to 2148, and 2164 to 2340 (Table 4). A nucleotide

Figure 3. Restriction sites in cloned forms of redombined CaMV DNAs. Top line: linear representation of allelic restriction site location. Bars above the line are sites cleaved by the indicated enzymes in Cabb S, but not in W DNA, while bars below the line are sites cleaved in W, but not in Cabb S DNA. Symbols used are: HF, HinfI; T, TaqI; K, KpnI; M, MspI; HH, HhaI; PS, PstI; X, XbaI; AL, AluI; HD, HindIII; E, EcoRI; PV, PvuII; HP, HpaI, HG, HgaI; HA, HaeIII; B, BglI; AC, AccI. The remaining lines depict the distribution of alleles in three CaMV DNA recombinants. Bars above the line indicate resistance or susceptibility to restriction characteristic of Cabb S DNA, while bars below the line identify W characteristic restriction. Apparent Cabb S-W sequence junctions are designated J1, J2, J3, and J4. (Taken from Choe et. al., 1985.)

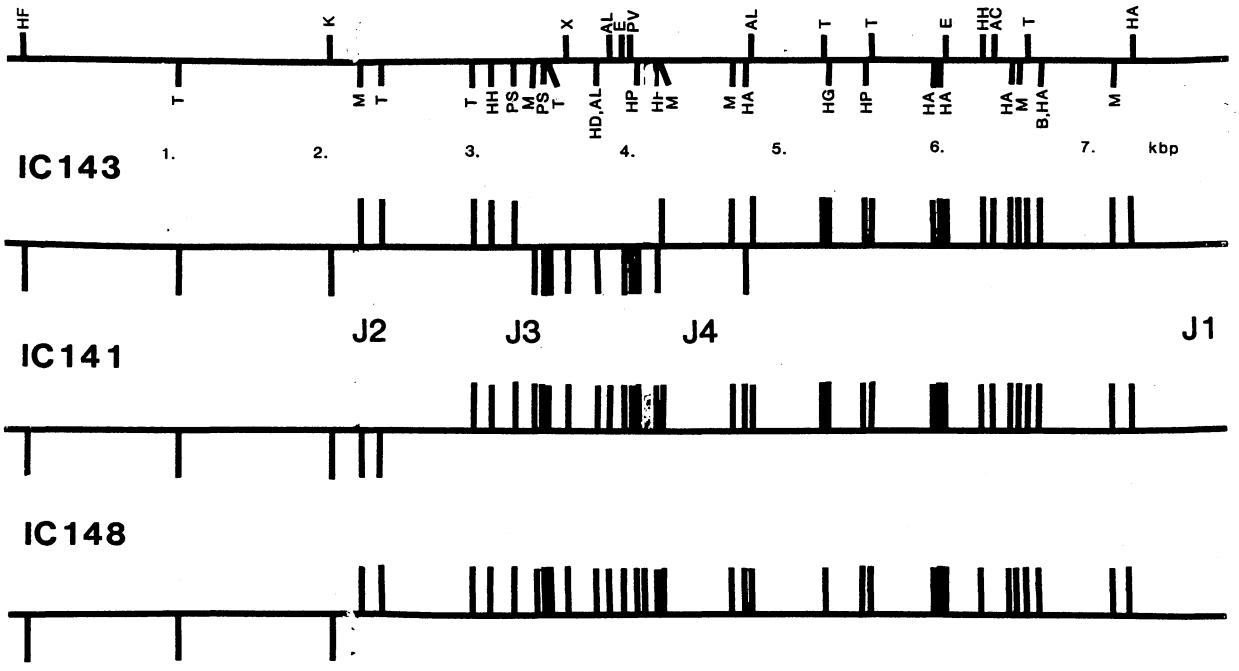

HF
K
X
AL
E
PV
AL
T
T
E
HH
AC
T
HA
T
T
M
T
T
HH
PS
M
PS
T
HD,AL
HP
H
M
M
HA
HG
HP
HA
HA
HA
M
B,HA
M
1.
2.
3.
4.
5.
6.
7.
kbp
IC143
J2
J3
J4
J1
IC141
IC148

Table IV. Sequenced regions of viral DNAs. Numbering used is that of Franck et al., (1980).

TABLE IV

SEQUENCED REGIONS OF VIRAL DNAS

DNA	Sequences Read Between Nucleotides 1701 and 1926	Sequences Read Between Nucleotides 1927 and 2148	Sequences Read Between Nucleotides 2149 and 2545
pIC141	1720-1883	1949-1999	2163-2321
pIC143	1720-1883	1927-2148	2164-2340
pIC148	1720-1883	1987-2106	2180-2321
pLW303S	1720-1883	1927-2148*	2266-2380

*(Melcher, unpublished)

Table V. Unique parental nucleotides detected in recombinant DNAs. Nucleotides not sequenced are so indicated by "-".

TABLE V

UNIQUE PARENTAL NUCLEOTIDES DETECTED IN RECOMBINANT DNAS

Nucleotide	Cabb B-JI	Cabb S	Recombinants pIC141	pIC143	pIC148
1729	C	A	C	C	C
1762	A	G	A	A	A
1777	T	A	T	T	T
1847	A	G	A	A	A
1920	C	T	-	-	-
1959	G	A	G	G	-
1961	T	C	T	T	-
1970	G	A	G	G	-
1990	G	C	G	G	G
2009	C	T	C	C	C
2012	C	T	C	C	C
2042	C	T	C	C	C
2044	A	C	A	A	A
2045	T	C	T	T	T
2048	G	A	G	G	G
2081	G	A	G	G	G
2087	C	T	C	C	C
2099	T	C	T	T	T
2233	C	T	C	T	T
2239	C	T	C	T	T
2281	T	C	T	C	C
2284	C	T	C	T	T
2296	G	A	G	A	A
2299	A	G	A	G	G

difference between the parental DNAs occurs at nt. 2111, in which W and Cabb B-JI have the purine adenine, while Cabb S has the pyrimidine cytosine. Up to, and including the difference at 2111, the region of pIC143 sequences is identi- to W and Cabb B-JI. There are no further nucleotide differ- ences between the parental DNAs until nt. 2224. At this position, W and Cabb B-JI have a guanine, while Cabb S has an adenine. At 2224, pIC143's sequence was found to have an adenine like Cabb S. Likewise, from 2224 to the end of the region sequenced (nt. 2340), IC143 exhibits nucleotides like those of Cabb S.

The nucleotides sequenced in pIC148 were from 1720 to 1883, 1987 to 2086, and 2180 to 2321 Table 4). Up to and including nt. 2224, pIC148's sequences were found to be identical to W and Cabb B-JI. The next nucleotide difference occurs at nt. 2233, at which W and Cabb BJI have a cytosine, while Cabb S has a thymine. At nt. 2233, pIC148 also has a thymine, and continues to show nucleotides like Cabb S at positions of parental differences to the end of the sequenced region.

Of the regions sequenced, nts. 1720 to 1830, 1949 to 1999, 2078 to 2132, and 2163 to 2321, no junction between parental DNAs was detected for pIC141 (Table 5); all the sequences were identical to the Cabb B-JI sequences. However, a junction has been detected occurring between the parental nucleotide differences that occur at nts. 2578 and

2623 (Melcher, unpublished). The possible mechanisms that gave rise to this junction and the above-mentioned junctions will be discussed later.

Creation and Analysis of New Recombinants

Creation of New Recombinants

New recombinant CaMV DNAs were produced by coinoculating turnip plants with pairs of mutant CaMV DNAs. The pairs were chosen so that their mutations were not less than 2500 bp apart (see Table 6). With the exception of the combination of pDLS19 and pLW76, each coinoculation included one partner that had previously been characterized as having the ability to recombine readily with most other mutants (Choe et al., 1985). These high-frequency recombining (Hfr) partners were pUM124, pUM41, and pUM24. The combinations of mutant plasmids used are shown in Table 6.

As a positive control, pUM24 and pUM41 were coinoculated onto a total of 8 plants. This combination had previously produced 5/5 infected plants (Choe, et al., 1985), however, these control inoculations produced no infected plants. Yet the fact that infected plants were produced from mutant combinations revealed that recombinations could occur. It should be noted that all of the combinations involving pUM41 as a mutant partner (pUM41 +pHL3H, pIC21, and pUM24) produced no infected plants. For each of these combinations, the

Table VI. Mutant combinations for recombinational rescue. Each Hfr partner is of the isolate Cabb S, with the exception of pDLS19, which is not a known Hfr and is from the NY8153 isolate. Negative and positive control inoculations are described in the text.

TABLE VI

MUTANT COMBINATIONS FOR RECOMBINATIONAL RESCUE

Hfr Partner	ORF Modified	Other Partner	Isolate	ORF Modified	No. Infected Plants/Total Plants Inoculated
pUM124	I	pDLS19	NY8153	IV	1/6
pUM124	I	pHL3H	CM4-184	V	0/12
pUM124	I	pIC21	W	VI	1/46
pUM124	I	pIC23	W	VI	1/16
pUM41	III	pHL3H	CM4-184	V	0/10
pUM41	III	pIC21	W	VI	0/11
pUM24	VI	pLW214	CM4-184	I	2/6
pUM24	VI	pLW76	CM4-184	I	1/11
pUM24	VI	pCMS34	NY8153	III	0/11
pDLS19	IV	pLW76	CM4-184	I	3/12

pUM41 plasmid was not CsCl-purified, but only obtained via a large-scale alkaline-SDS preparation. It had previously been found that plasmids prepared in this manner could recombine to produce infectious recombinants (Melcher, unpublished), so no steps were taken to further purify the plasmid.

For each combination of mutants inoculated, negative control inoculations were also conducted. These consisted of turnip plants inoculated with only one of the two mutant partners which, alone, should not be able to infect the plants. Indeed, no plants inoculated with a single mutant plasmid became diseased.

Of the coinoculations shown in Table 6, the following combinations did not give rise to any infected plants: pUM124 + pHL3H, pUM41 + pHL3H, pUM41 + pIC21, and pUM24 + pCMS34. While six mutant combinations produced infectious recombinants, the recombinant DNAs resulting from the coinoculations of pUM124 + pIC21 and pDLS19 + pLW76 were not successfully cloned into the *E. coli* plasmid pBR322.

The coinoculations of pUM124 +pDLS19, pUM124 + pIC23, pUM24 +pLW76, and pUM24 + pLW214 produced infectious recombinants, were successfully ligated into pBR322, used to transform HB101, and screened for plasmids with viral DNA inserts. The plasmids from a chosen colony were isolated and CsCl purified. The newly-cloned recombinants, pVR1243, pVR244-A, pVR244-B, and pVR246 were found to be infectious, while another recombinant, pVR1249, was not infectious. pVR1249's

inability to infect host plants is most likely due to the presence of a large deletion of approximately 800-900 bp in the viral DNA. This deletion will be discussed later.

Before restriction mapping data will be discussed, a summary of the recombinants and their junctions will be presented. Possible mechanisms giving rise to the juntions will be discussed later.

VR246 resulted from the coinoculation of pUM24 + pLW76. UM24, a Cabb S-derived mutant, contains a SmaI linker insert at 6299 and a 40 bp deletion from 6299 to 6338 (Choe, et al., 1985). The other parental DNA used, pLW76, is derived from isolate CM4-184 and has a 433 bp deletion from nts. 777 to 1208 (Howell, et al., 1981).

Four junctions have been detected in VR246 (Table 7). Those detected are between nts. 1784 and 2236, 4149 and 4660, 5943 and 6045, and 7724 and 197. From nts. 197 to 1784 and 4660 to 5943, either only Cabb S unique sites or the absence of sites unique to CM4-184 were detected. Likewise, from nts. 2236 to 4149 and 6045 to 7224, either sites unique to CM4-184 or the absence of Cabb S unique sites were detected.

VR244-A and VR244-B resulted from the coinoculation of pUM24 and pLW214 onto the same plant. The mutation in pUM24 is described above, and pLW214, a CM4-184-derived plasmid, has an 8 bp EcoRI linker inserted at nt. 1285, rendering

Table VII. Diagnostic restriction sites detected in pVR246.

TABLE VII

DIAGNOSTIC RESTIRCTION SITES
DETECTED IN pVR246

Enzyme	Site	Source	pVR246	Result
AluI	197	CM4-184	-	Cabb S
HinfI	830	Cabb S	+	Cabb S
AccI	904	Cabb S	+	Cabb S
HaeIII	1170	Cabb S	+	Cabb S
HinfI	1218	Cabb S	+	Cabb S
HindIII	1513	Cabb S	+	Cabb S
HinfI	1571	Cabb S	+	Cabb S
TaqI	1643	Cabb S	+	Cabb S
HinfI	1781	Cabb S	+	Cabb S
ClaI	1783	Cabb S	+	Cabb S
TaqI	1784	Cabb S	+	Cabb S
MspI	2236	CM4-184	+	CM4-184
TaqI	2367	CM4-184	+	CM4-184
ClaI	2680	CM4-184	+	CM4-184
TaqI	2967	CM4-184	+	CM4-184
PstI	3231	CM4-184	+	CM4-184
MspI	3360	CM4-184	+	CM4-184
PstI	3426	CM4-184	+	CM4-184
HindIII	3772	CM4-184	+	CM4-184
EcoRI	3928	Cabb S	-	CM4-184
HinfI	4133	CM4-184	+	CM4-184
MspI	4149	CM4-184	+	CM4-184
MspI	4660	CM4-184	-	Cabb S
HaeIII	4752	CM4-184	-	Cabb S
HinfI	4921	CM4-184	-	Cabb S
TaqI	5541	Cabb S	+	Cabb S
HaeIII	5943	CM4-184	-	Cabb S
EcoRI	6045	Cabb S	-	CM4-184
HaeIII	6068	Cabb S	-	CM4-184
MspI	6509	CM4-184	+	CM4-184
HaeIII	6654	CM4-184	+	CM4-184
BglI	6655	CM4-184	+	CM4-184
MspI	7118	Cabb S	-	CM4-184
HaeIII	7224	Cabb S	-	CM4-184

it non-infectious (Howell, et al., 1981 and Melcher, unpublished). While two junctions have been detected in VR244-A, four have been detected for VR244-B (see Tables 8 and 9, respectively). The two in VR244-A, between nts. 5541 and 5943, and 7224 and 197, are also found in VR244-B. The other two junctions occurring in VR244-B are found between nts. 1784 and 2236, and 2680 and 3231. Both recombinants exhibit sites unique to CM4-184 between nts. 5943 and 7224. VR244-A appears to be derived from Cabb S from at least nt. 197 to 5541. VR244-B exhibits Cabb S unique sites (or lack CM4-184 unique site) from nts. 197 to 2091 and from 3231 to 5541. CM4-184 unique sites were detected in VR244-B from nts. 2236 to 2680.

VR244-B also differed from VR244-A in that it contained a small deletion (80-200 bp) between nts. 3960 and ~4310.

VR1249 arose from the coinoculation of pUM124 and pDLS19. pUM124, a Cabb S-derived plasmid, has a 147 bp deletion between nts. 886 and 1032 (Choe et al., 1985). DLS19 is derived from the NY8153 isolate and has a 195 bp deletion between the PstI sites at 3233 and 3427 (Melcher et al., 1986). No junction was detected for VR1249 by restriction analysis (Table 10). Only sites unique to Cabb S (or the absence of sites unique to NY8153) were detected. VR1249 has been found to be a recombinant of its parental DNAs as sequenced regions from nts. 7945 to 87 have revealed that

Table VIII. Diagnostic restriction sites detected in pVR244-A.

TABLE VIII

DIAGNOSTIC RESTRICTION SITES
DETECTED IN pVR244-A

Enzyme	Site	Source	pVR244-A	Result
AluI	197	CM4-184	-	Cabb S
HinfI	830	Cabb S	+	Cabb S
HaeIII	1170	Cabb S	+	Cabb S
HinfI	1218	Cabb S	+	Cabb S
HindIII	1513	Cabb S	+	Cabb S
HinfI	1571	Cabb S	+	Cabb S
TaqI	1643	Cabb S	+	Cabb S
HinfI	1781	Cabb S	+	Cabb S
ClaI	1783	Cabb S	+	Cabb S
TaqI	1784	Cabb S	+	Cabb S
MspI	2236	CM4-184	-	Cabb S
TaqI	2367	CM4-184	-	Cabb S
ClaI	2680	CM4-184	-	Cabb S
PstI	3231	CM4-184	-	Cabb S
MspI	3360	CM4-184	-	Cabb S
PstI	3426	CM4-184	-	Cabb S
HindIII	3772	CM4-184	-	Cabb S
EcoRI	3928	Cabb S	+	Cabb S
HinfI	4133	CM4-184	-	Cabb S
MspI	4149	CM4-184	-	Cabb S
MspI	4660	CM4-184	-	Cabb S
HaeIII	4752	CM4-184	-	Cabb S
AluI	4766	Cabb S	+	Cabb S
HinfI	4921	CM4-184	-	Cabb S
TaqI	5541	Cabb S	+	Cabb S
HaeIII	5943	CM4-184	+	CM4-184
EcoRI	6045	Cabb S	-	CM4-184
HaeIII	6068	Cabb S	-	CM4-184
MspI	6509	CM4-184	+	CM4-184
HaeIII	6654	CM4-184	+	CM4-184
BglI	6655	CM4-184	+	CM4-184
MspI	7118	Cabb S	-	CM4-184
HaeIII	7224	Cabb S	-	CM4-184

Table IX. Diagnostic restriction sites detected in pVR244-B.

TABLE IX

DIAGNOSTIC RESTRICTION SITES
DETECTED IN pVR244-B

Enzyme	Site	Source	pVR244-B	Result
AluI	197	CM4-184	-	Cabb S
HinfI	830	Cabb S	+	Cabb S
HaeIII	1170	Cabb S	+	Cabb S
HinfI	1218	Cabb S	+	Cabb S
HindIII	1513	Cabb S	+	Cabb S
HinfI	1571	Cabb S	+	Cabb S
TaqI	1643	Cabb S	+	Cabb S
HinfI	1781	Cabb S	+	Cabb S
ClaI	1783	Cabb S	+	Cabb S
TaqI	1784	Cabb S	+	Cabb S
MspI	2236	CM4-184	+	CM4-184
TaqI	2367	CM4-184	+	CM4-184
ClaI	2680	CM4-184	+	CM4-184
PstI	3231	CM4-184	-	Cabb S
MspI	3360	CM4-184	-	Cabb S
PstI	3426	CM4-184	-	Cabb S
HindIII	3772	CM4-184	-	Cabb S
EcoRI	3928	Cabb S	+	Cabb S
HinfI	4133	CM4-184	-	Cabb S
MspI	4149	CM4-184	-	Cabb S
MspI	4660	CM4-184	-	Cabb S
HaeIII	4752	CM4-184	-	Cabb S
AluI	4766	Cabb S	+	Cabb S
HinfI	4921	CM4-184	-	Cabb S
TaqI	5541	Cabb S	+	Cabb S
HaeIII	5943	CM4-184	+	CM4-184
EcoRI	6045	Cabb S	-	CM4-184
HaeIII	6068	Cabb S	-	CM4-184
MspI	6509	CM4-184	+	CM4-184
HaeIII	6654	CM4-184	+	CM4-184
BglI	6655	CM4-184	+	CM4-184
MspI	7118	Cabb S	-	CM4-184
HaeIII	7224	Cabb S	-	CM4-184

Table X. Diagnostic restriction sites detected in pVR1249.

TABLE X

DIAGNOSTIC RESTRICTION SITES
DETECTED IN pVR1249

Enzyme	Site	Source	pVR1249	Result
ClaI	2636	NY8153	-	Cabb S
HindIII	3249	Cabb S	+	Cabb S
BglI	3414	Cabb S	+	Cabb S
PstI	3427	NY8153	-	Cabb S
HindIII	3772	NY8153	-	Cabb S
EcoRI	3928	Cabb S	+	Cabb S
MspI	4149	NY8153	-	Cabb S
MspI	4660	NY8153	-	Cabb S
EcoRI	6045	Cabb S	+	Cabb S
AccI	6330	Cabb S	+	Cabb S
MspI	7118	Cabb S	+	Cabb S
HinfI	7794	Cabb S	+	Cabb S

VR1249's DNA is like Cabb S from nts. 7945 to the last nt. before Gap I, 8024, and VR1249's DNA shares homology with NY8153 from nts. 1 to 87 (Melcher, unpublished). The implications of this junction will be discussed later. The most probable reason for there being no NY8153 sites detected is the presence of a large deletion of 800 to 900 bp between nts. 124 and 1514. This deletion rendered pVR1249 non-infectious.

VR1243 arose from the coinoculations of pUM124 (described above) and pIC23. pIC23 is derived from pLW303X, which contains the DNA referred to as "W" (described above). IC23 has a 348 bp deletion from nts. 6317 to 6664. Two junctions were detected in VR1243, between nts. 1218 and 2236 and between nts. 7118 and 7224 (Table 11). The region that exhibited Cabb S unique sites and the absence of W sites extends from nts. 2236 to 7118. Likewise, the region indistinguishable from W DNA extends from nts. 7224 to 1218.

The above conclusions as to the positions of junctions between parental DNAs were base on analyses by restriction mapping using the following restriction enzymes: AccI, AluI, BglI, ClaI, EcoRI, HaeIII, HindIII, HinfI, MspI, PstI, and TaqI.

Analysis by Restriction Mapping

PstI. The recombinants and either their parental DNAs or DNAs very similar to their parents were digested with PstI

Table XI. Diagnostic restriction sites detected in pVR1243.

TABLE XI

DIAGNOSTIC RESTRICTION SITES
DETECTED IN pVR1243

Enzyme	Site	Source	pVR1243	Result
AccI	904	W	+	W
HinfI	1218	Cabb S	−	W
MspI	2236	W	−	Cabb S
PstI	3231	W	−	Cabb S
MspI	3360	W	−	Cabb S
PstI	3427	W	−	Cabb S
HindIII	3773	W	−	Cabb S
EcoRI	3928	Cabb S	+	Cabb S
HaeIII	4752	W	−	Cabb S
HaeIII	5939	W	−	Cabb S
HinfI	6001	Cabb S	+	Cabb S
EcoRI	6045	Cabb S	+	Cabb S
HaeIII	6068	Cabb S	+	Cabb S
HaeIII	6411	Cabb S	+	Cabb S
AccI	6630	Cabb S	+	Cabb S
HaeIII	6655	W	−	Cabb S
MspI	7118	Cabb S	+	Cabb S
HaeIII	7224	Cabb S	−	W
ClaI	7980	Cabb S	−	W

and electrophoresed through a 1% agarose gel. The resulting gels are shown in Figures 4 and 5. The parental DNAs' wild type PstI restriction maps are shown in Figure 6. The PstI results were used to determine orientation of the recombinant DNA in the pBR322 vector.

The CaMV DNAs in pDLS19, pUM124, pLW414, and pUM24 and pLW76 (Figure 4, lanes A, C, and I, and Figure 5, lanes C and A, respectively) are inserted in pBR322 in the same orientation as is the CaMV DNA in pCS101. The Cabb S parents (pUM124 and pUM24) have only one PstI site in the viral DNA (at nt. 5386) (Figure 6) and one in the pBR322 DNA (at nt. 3609 of the pBR322 map). This results in two fragments of 1955 bp and 10,285 bp for pUM124 or 1955 bp and 10,400 bp for pUM24. If their recombinants were to contain only the one Cabb S site, but be inserted in opposite orientation, the resulting fragments would be 3506 bp and 8879 bp. The three recombinants displaying these fragments, (and therefore their CaMV DNAs are in opposite orientation), are pVR1249 (Figure 4, lane B, and pVR244-A, and pVR244-B (Figure 5, lane G and H respectively). These lanes also reveal that these three recombinants did not contain any extra PstI sites from the other parent involved in the coinoculations: the site at nt 3427 in DLS19 and the two sites at 2811 (3232) and 3006 (3427) in pLW414. (The restriction sites for the CM4-184 parents are listed first and the approximate equivalent nt.

Figure 4. Agarose gel electrophoresis of PstI-digested plasmids (First gel). A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) pUM24, G) pVR244-A, H) pVR244-B, I) pLW414

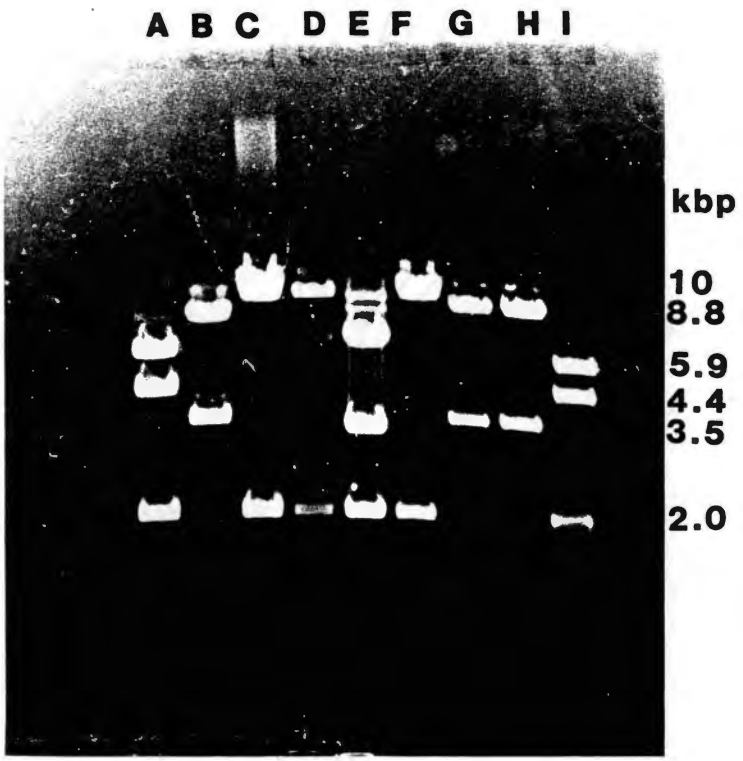

A B C D E F G H I
kbp
10
8.8
5.9
4.4
3.5
2.0

Figure 5. Agarose gel electrophoresis of PstI-digested plasmids (Second gel). A) pLW76, B) pVR246, C) pUM24

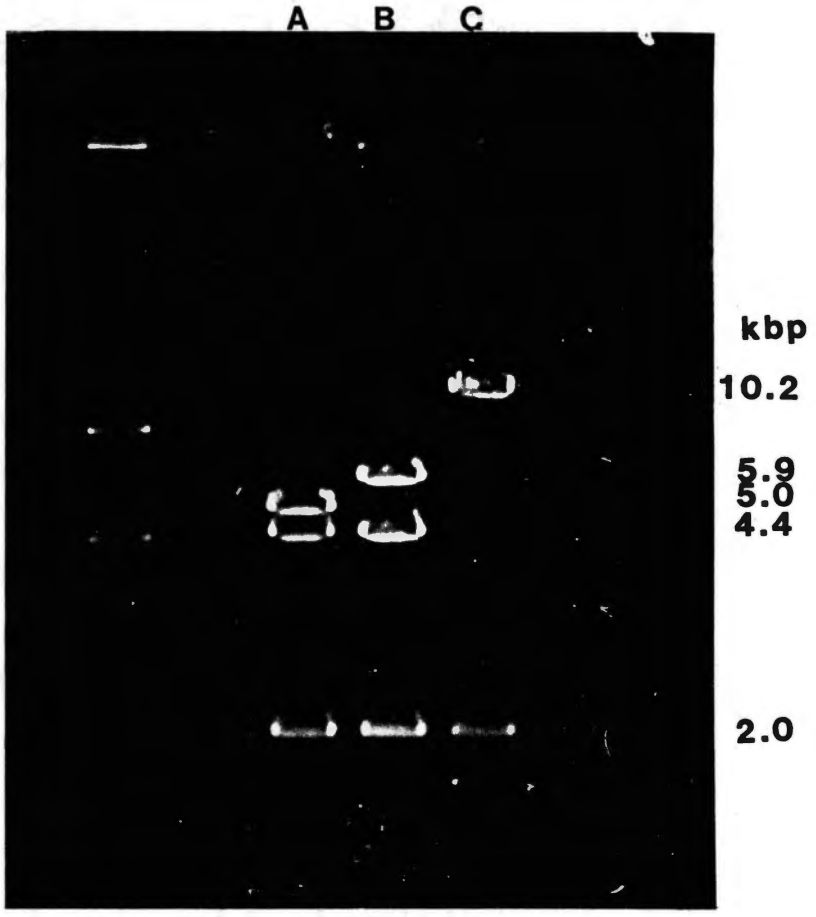
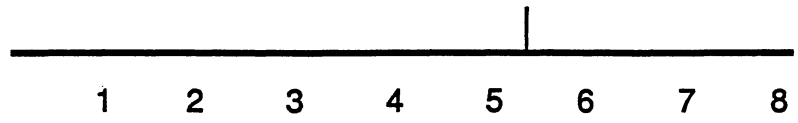
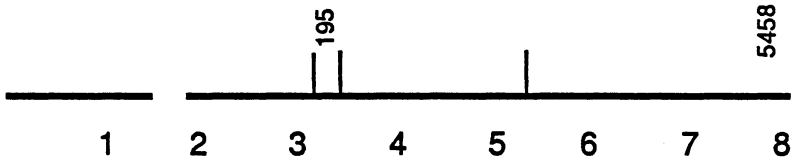

A
B
C
kbp
10.2
5.9
5.0
4.4
2.0

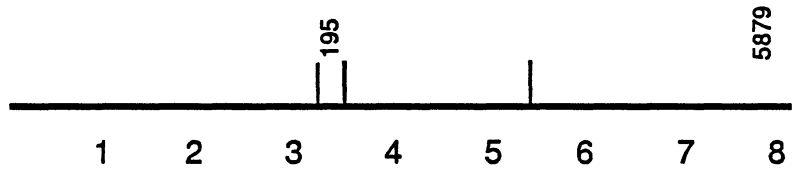
Figure 6. PstI restriction maps for wild-type parental CaMV DNAs. Numbers indicate kbp.



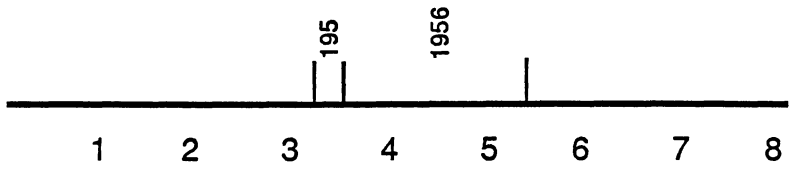
Cabb S



CM4-184



NY8153



W

number as if in the other parents is given in parenthesis.) The 800 to 900 bp deletion in VR1249 produced a fragment of approximately 8000 bp instead of the expected 8879 bp.

The two plasmids with their recombinant DNAs inserted in the same orientation as the parents are pVR1243 (Figure 4, lane D and pVR246 (Figure 5, lane B). This is shown by the presence of the 1955 bp fragment in both lanes. pIC23 (lane F of Figure 4) most likely contains the PstI sites found in Cabb B-JI, i.e., the sites at nts. 3232, 3427, and 5386, which produce fragments of 195 bp and 1956 bp (Figure 6). The other fragment of 5870 bp is interrupted by the pACYC177 vector, and cannot be deduced because a restriction map is unavailable. If pVR1243 contained the two sites at 3232 and 3427, the expected bands would be 4366 bp, 195 bp, and 5870 bp, plus the 1955 bp fragment. Instead, the bands of 10,432 bp and 1955 bp are present, indicating only the site at 5386 is present in the viral DNA.

pLW76 (Figure 5, lane A) contains fragments of 5026 bp, 195 bp, 4363 bp, and 1955 bp due to the sites at nts. 4962 (5386), 2811 (3232), 3006 (3426), and the pBR322's site at 3609 (Figure 6). As shown in lane B, VR246 also exhibits a fragment of 1955 bp which indicates a site is present at nt. 5386. If pVR246 were to contain the PstI site at 3232, which produces a fragment of 5026 in pLW76, and if pVR246 were to not contain the deletion of 421 bp characteristic of CM4-184 and not contain the pLW76 deletion of 433 bp, a 5869 bp

fragment would result. As can be seen in lane B of Figure 5, pVR246 contains a fragment that has migrated less distance than the 5026 bp fragment of pLW76.

AccI. Fragments electrophoresed through either 0.8% or 1% agarose gels after digestion with AccI are shown in Figures 7 and 8. The parental DNAs' wild-type restriction map is shown in Figure 9. AccI cleaves at nucleotide sequences very similar to those recognized by the restriction enzyme SalI, so AccI not only cleaves at the site of ligation (nt. 4836), but also at several other AccI sites throughout the recombinant plasmids. Since cleavage occurs at nt. 4836, viral DNA fragments appear separate from the vector DNA.

Only one site difference occurs between pUM24 and pLW76 (Figure 7, lanes J and L, respectively). The AccI site at nt. 904 is deleted in pLW76, yielding a 3895 bp fragment. pUM24 has the site at 904, yielding 2858 and 1886 bp fragments. pVR246 (lane L) also had the 2858 and 1886 bp fragments, indicating it has the AccI site at 904.

Although pUM24 and pLW414 share the same AccI sites, the presence of the 421 bp natural deletion in pLW414 (Figure 8, lane D) gives a fragment of 2434 bp, instead of the 2858 bp fragment in pUM24 (Figure 8, lane A). This 2858 bp fragment (Figure 8, lane A) appears as a band of double intensity, as it co-migrates with a 2768 bp fragment of pBR322. The 2768 bp fragment appears as a band of single intensity in pLW414.

Figure 7. Agarose gel electrophoresis of AccI-digested plasmids (First gel). A) pDLS19, B) pVR1249, C) pVR1243, D) pUM124, E) pIC23 F) pUM24, G) pVR244-A, H) pVR 244-B, I) pLW214, J) pUM24, K) pVR246, L) pLW76

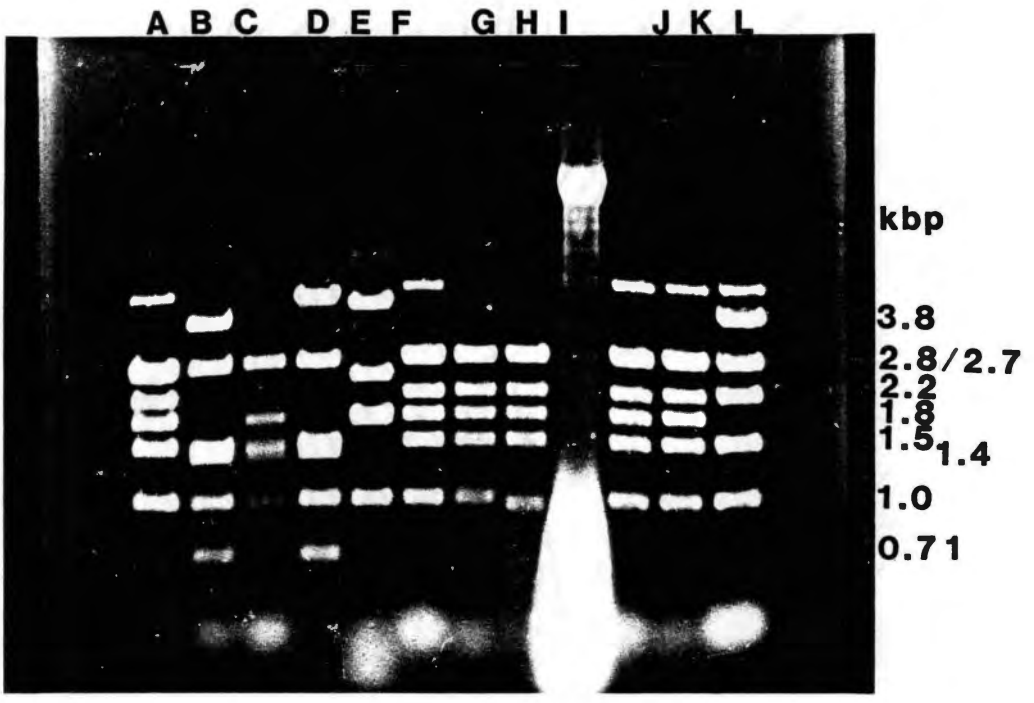

A B C D E F G H I J K L
kbp
3.8
2.8/2.7
2.2
1.8
1.5
1.4
1.0
0.71

Figure 8. Agarose gel electrophoresis of AccI-digested plasmids (Second gel). A) pUM24, B) pVR244-A, C) pVR244-B, D) pLW414

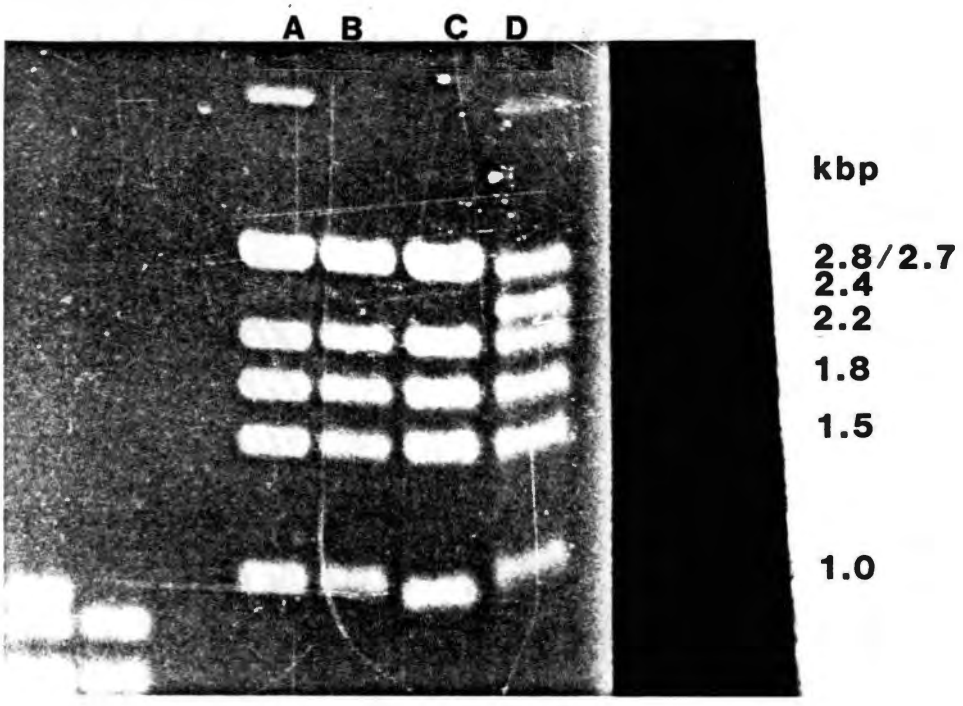

A
B
C
D
kbp
2.8/2.7
2.4
2.2
1.8
1.5
1.0

Figure 9. AccI restriction maps for wild-type parental CaMV DNAS. Numbers indicate kbp.

Cabb S

CM4-184

NY8153

W

As can be seen from lanes B and C of Figure 8, both pVR244-A and pVR244-B (respectively) had a band of double intensity, indicating the presence of the 2858 bp fragment. No 2434 bp fragment is present. Therefore, pVR244-A and B do not contain the 421 bp natural deletion that is characteristic of CM4-184. This is also true for pVR246 (Figure 7, lane K) as it displayed the doubly-intense bands indicating the presence of the 2858 bp fragment, and did not display the 2434 bp fragment.

As can be seen in Figure 8, lane C, the fragment that migrated the farthest through the gel migrated slightly further down the gel than the corresponding bands in the other lanes. These bands in lanes A, B, and D represent fragments of 1074 bp, resulting from cleavage at the AccI sites at nts. 3762 and 4836 which are common to both Cabb S and CM4-184. Therefore, in pVR244-B, the fragment between the sites at 3762 and 4836 contains a deletion. As there is no vector DNA included in this fragment, and as the vector fragments have migrated equivalent distances, the deletion in pVR244-B is only in the viral DNA. This deletion was calculated to be between 80 and 200 bp in length (data not shown).

The site at nt. 904 is present in both NY8153 and Cabb S but is not present in pUM124 as it is deleted due to the pUM124 mutation (Figure 9 and Figure 7, lanes A and D,). pDLS19's site at 904 produces a 2660 bp fragment which

includes the 195 bp deletion in pDLS19 and also produces an 1894 bp fragment. The deleted 904 site in pUM124 causes a 4597 bp fragment to result. A site at 6330 is unique to pUM124 and yields fragments of 1492 bp and 712 bp. As can be seen in lane B, pVR1249 also exhibits the 1492 and 712 bp fragments, indicating it contains the site at nt. 6330. The site at 904 is not present in pVR1249 and its absence can most likely be attributed to the 800 to 900 bp deletion based upon evidence to be presented. If the site at 904 was missing and no deletion was present, a 4744 bp fragment would result. Instead, as shown in Figure 7, lane B, the fragment migrates approximately the same distance as does the 3895 bp fragment of pLW76 (lane L).

The site differences between pUM124 and pIC23 (Figure 7, lanes D and E, respectively) are 6330 in pUM124 and 904 in pIC23 (see also Figure 9). The site at 904 in pIC23 yields a fragment of 1895 bp while the site at 6330 in pUM124 yields two fragments of 1494 bp and 712 bp. As seen in lane C, pVR1243 has the 1895 bp fragment, indicating the presence of the W site at 904. The 712 bp and 1494 bp fragments are also present and therefore the Cabb S site at 6330 is present in pVR1249.

<u>Bgl I.</u> Figures 10 and 11 show the distribution of the fragments of the Bgl I-digested plasmids through a 0.8% agarose gel. The parental DNAs' wild-type restriction maps

Figure 10. Agarose gel electrophoresis of BglI-digested plasmids (Second gel). A) pUM24, B) pUM24, C) pLW76

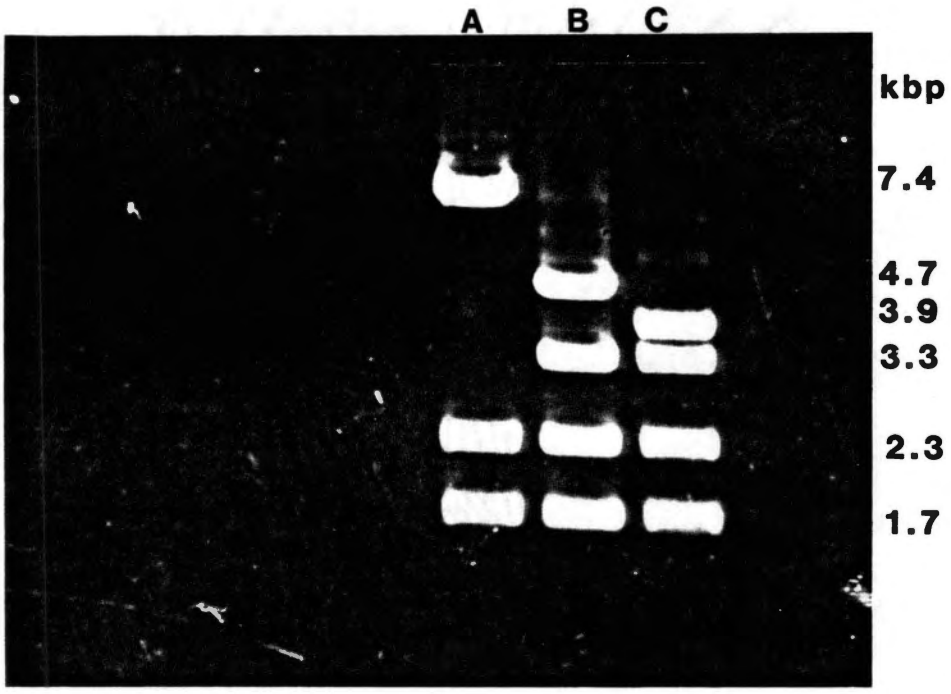
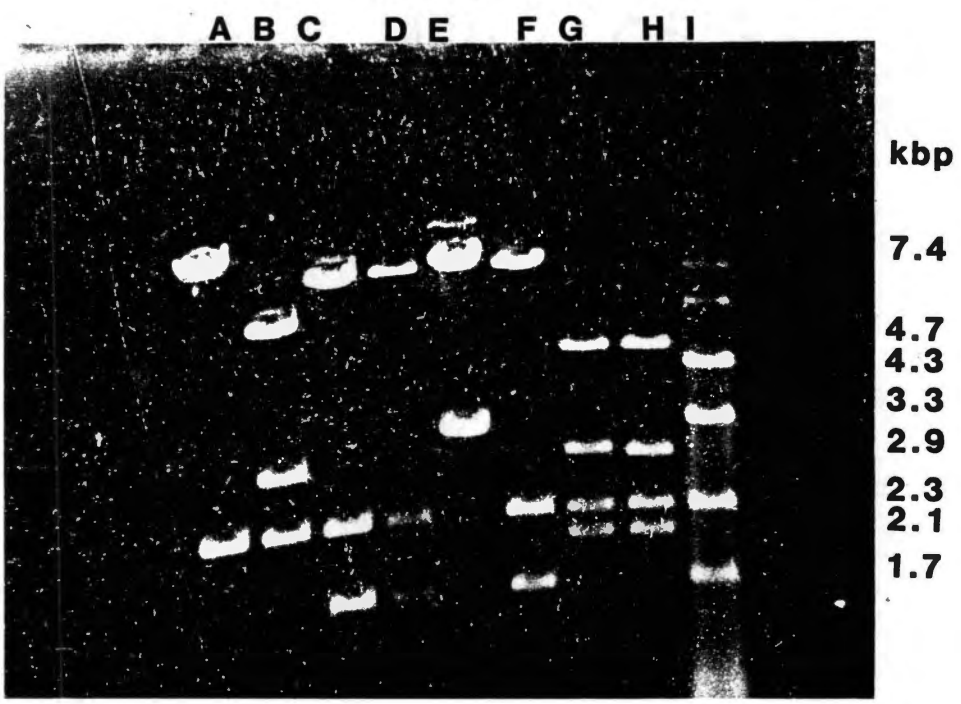

A
B
C
kbp
7.4
4.7
3.9
3.3
2.3
1.7

Figure 11. Agarose gel electrophoresis of BglI-digested plasmids (First gel). A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) pUM24, G) pVR244-A, H) pVR244-B, I) pLW214

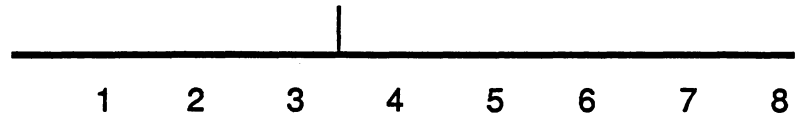

A B C D E F G H I
kbp
7.4
4.7
4.3
3.3
2.9
2.3
2.1
1.7

are shown in Figure 12. The site at nt. 6235 (6655) in pLW76 is the only unique site between pLW76 and pUM24 (Figure 10, lanes C and A, respectively and Figure 12). The site at 6655 in pLW76 gives fragments of 3351 bp and 3928 bp, while its absence produces a 7450 bp fragments in pUM24. The 3928 bp fragment is a 4782 bp fragment with the CM4-184 natural deletion and the pLW76 mutation. Lane B shows that pVR246 did not have the 7450 bp fragment of Cabb S, suggesting the site at 6655 is present in pVR246. Indeed, the 3351 bp fragment was present, as was a fragment larger than 3928 bp. This larger fragment is most likely the 4782 bp fragment that would result from the absence of the 421 bp CM4-184 deletion and the absence of the pLW76 mutation.

The diagnostic fragments between pUM24 and pLW214 (Figure 11, lanes F and I, respectively) are essentially the same as they were for pUM24 and pLW76 above, with the exception of pLW214 having a 4369 bp fragment instead of pLW76's 3928 bp fragment. Both pVR244-A and pVR244-B (lanes G and H, respectively) had the 4790 bp fragment that is characteristic of the CM4-184 site at 6655. Due to these two plasmids' opposite orientation in the vector DNA, the other fragment resulting from cleavage at the site at 6655 is not 3351 as it was above, but is 2095 bp.

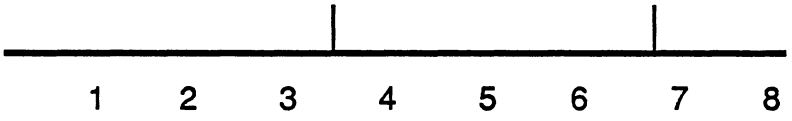
While Cabb S (pUM24) and NY8153 have a single BglI site in the viral DNA at nt. 3414, pDLS19 does not as this site is

Figure 12. BglI restriction maps for wild-type parental DNAs. Numbers indicate kbp.

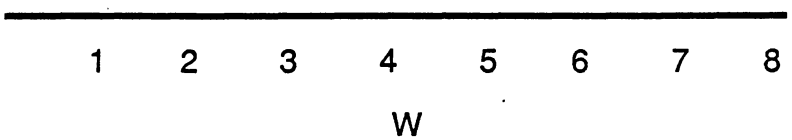


Cabb S

CM4-184



NY8153



W

deleted due to the DLS19 mutation. Therefore, no BglI sites are in DLS19 and a fragment of 9644 bp results (Figure 11, lane A). pUM124's site at 3414 yields fragments of 1699 bp and 7335 bp. If pVR1249 were to have the site at 3414, the resulting fragments would be 2954 bp and 6878 bp due to pVR1249's opposite orientation. While the 2954 bp fragment is present, the other fragment is actually less than 6878 bp due to pVR1249's deletion of approximately 800 to 900 bp.

pUM124 and pIC23 (Figure 11, lanes C and E, respectively) both have only one BglI site, at nt. 3407. W actually has another, at 6656, but this site is deleted in IC23's mutation. Therefore, pVR1243 should only have one site at 3407, and should therefore have fragments of 1699 bp and 7482 bp. These fragments can be seen in Figure 11, lane D.

ClaI. The agarose gel with the ClaI-digested fragments electrophoresed through it is shown in Figure 13. The restriction maps for the wild-type parental DNAs are shown in Figure 14. The diagnostic sites between CM4-184 and Cabb S include the site at nt. 2260 (2680) in pLW76 and the sites at nts. 820, 1783, and 2855 in pUM24. The CM4-184 site at 820 (Figure 14) is deleted due to the pLW76 mutation, so the site at nt. 2680 yields fragments of 1871 bp and 1276 bp (Figure 13, lane K), whereas the presence of the site at nt. 820 in CM4-184 yields two fragments of 864 bp and 1440 bp (Figure 14). pUM24's site at 820 and the site at 7980 (common to

Figure 13. Agarose gel electrophoresis of ClaI-digested plasmids. A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) "pLW414", G) pVR244-A, H) pVR244-B, I) pUM24, J) pVR246 K) pLW76

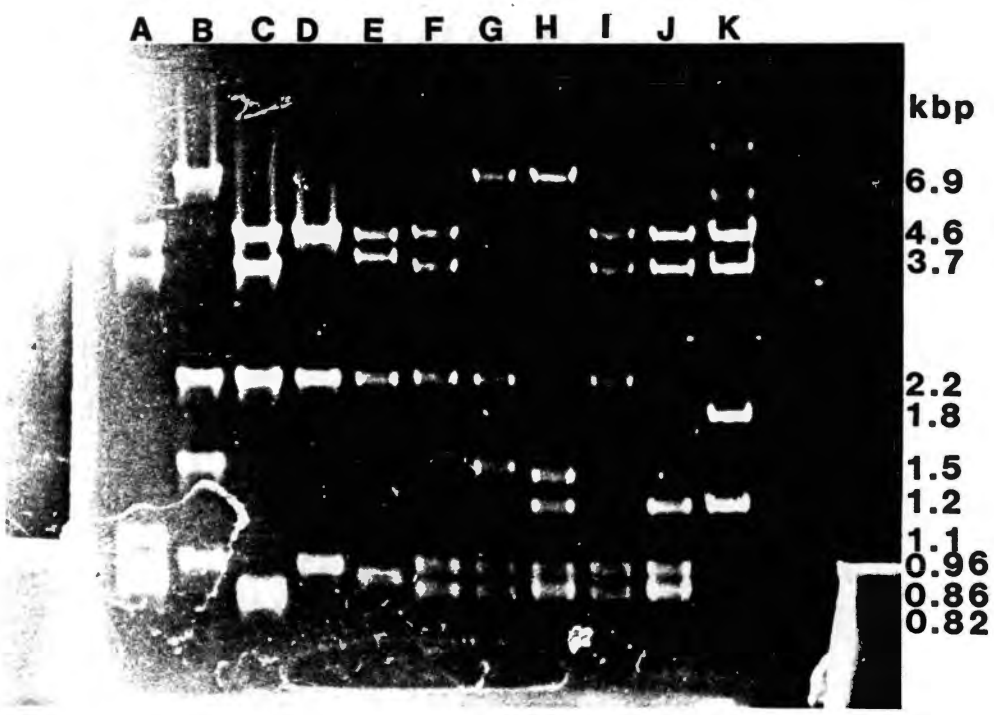
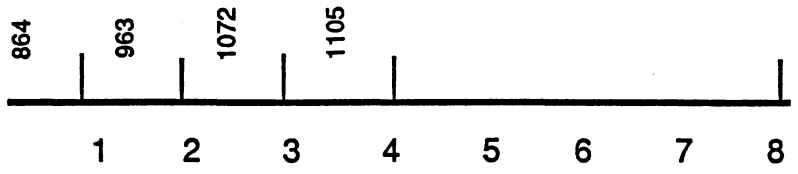
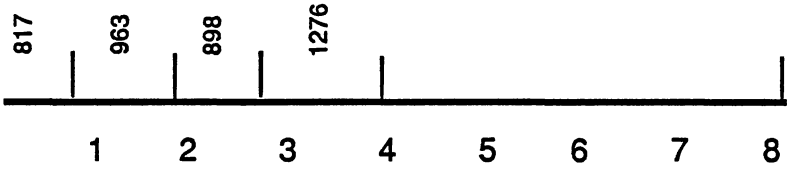

A B C D E F G H I J K
kbp
6.9
4.6
3.7
2.2
1.8
1.5
1.2
1.1
0.96
0.86
0.82

Figure 14. ClaI restriction maps for wild-type parental CaMV DNAs. Numbers indicate kbp.

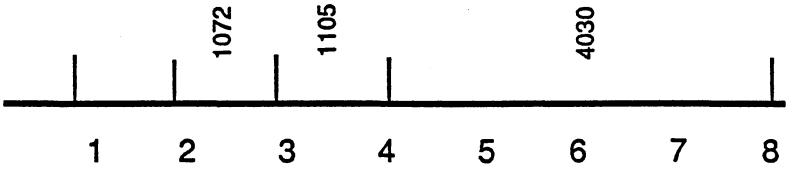


Cabb S

CM4-184



NY8153



W

both parents yields a fragment of 3739 bp while the site at 820 and another Cabb S-unique site at 1783 yields a fragment of 963 bp (Figure 13, lane I). Cleavage at nt. 2855 does not occur, as this is a DNA-adenine-methylating (dam) site (Marinus and Morris, 1973); instead of the expected fragments of 1072 bp and 1105 bp, a 2177 bp fragment results. The site at nt. 1783 also aids in producing the 2177 bp fragment. pVR246 had the CM4-184 site at nt. 2680 since the 1276 bp fragment was present (shown in lane J). The 963 bp fragment was also present, indicating the Cabb S sites at 820 and 1783 were present, while the 864 bp fragment indicates the 820 site was present.

While pLW414 was originally supposed to be the DNA in lane F, examination of this lane suggests that its plasmid is similar to, if not actually, pUM24. Therefore, to interpret the restriction pattern for pVR244-A and pVR244-B, pLW76's fragments (lane K) were used. pLW76 is expected to differ from pLW414 only in the absence of the 1871 bp fragment from pLW76 since the site at 820 is present in pLW414. pVR244-A (lane G) had the 2177 bp fragment of pUM24, indicating the CM4-184 site at 2680 is not present, as corroboroated by the absence of the 1276 bp fragment. The 1276 bp fragment did show for pVR244-B (lane H) indicating the site at 2680 was present in pVR244-B. The 963 bp fragment in both pVR244-A and -B indicates both contain the site at 1783. The site at nt. 1783 could only have come from Cabb S, as this site is not

present in CM4-184 due to the 421 bp deletion that is characteristic of CM4-184.

In pVR244-A and -B, the viral DNA site at 3960 and the pBR322 site at 23 give a fragment of 1504 bp, which is present in both pVR244-A and -B. But as can be seen in lane H, the 1504 bp fragment is actually smaller for pVR244-B than the same fragment is for pVR244-A (lane G). The shorter fragment is the result of the 80 to 200 bp deletion in pVR244-B.

One site between NY8153 and Cabb S can be used diagnostically: the site at nt. 2636 in NY8153, which yields a 1276 bp fragment (which is 1081 bp in pDLS19) and an 898 bp fragment (Figure 13, lane A and Figure 14). Neither of these fragments appear for pVR1249 (Figure 13, lane B), therefore the NY8153 site at 2636 is not present in VR1249. Although an approximately 963 bp fragment occurs in pVR1249, it is unlikely that it has resulted from cleavage at the site at nt. 820, as this site has most likely been deleted in pVR1249 and since no fragment of 864 bp or 817 bp (depending on parental source) appears. The 6877 bp fragment of pVR1249 (lane B) appears to be the equivalent size as the 6877 bp fragments of pVR244-A and -B (lanes G and H, respectively); i.e., pVR1249's 6877 bp fragment does not appear to contain the 800-900 bp deletion. Therefore, the site at 7980 is most likely present and the site at 820 deleted.

The only diagnostic site between pUM124 and pIC23 is the inaccessibility of 7980 in W due to dam methylation (Figure

14). The dam site yields a 4636 bp fragment (which is 4546 in pIC23) between the sites at 3960 and 820 (Figure 13, lane E). The fragment resulting from cleavage at the sites at 820 and 1783 cannot be resolved in pIC23, as the fragment is interrupted by the pACYC177 vector. This fragment would be 963 bp in Cabb S, but is 817 bp in pUM124. Since VR1243 is inserted into the pBR322 vector, it would have either fragments of 3771 bp and 864 bp if the Cabb S site at 7980 were present, or it would have a fragment of 4636 bp if the site at 7980 were absent. As shown in lane D, pVR1243 has neither the 3771 bp fragment nor the 864 bp fragment. Instead, pVR1243 has a band of double intensity at 4600, due to one fragment of 4610 from pBR322, and one fragment of 4636 bp.

EcoRI. The EcoRI-digested plasmids were electrophoresed through a 1% agarose gel, shown in Figure 15. The EcoRI restriction maps for the parental wild type DNAs are shown in Figure 16. While all the sites present in pLW76 (lane K) are also present in pUM24 (lane I), UM24 contains two extra sites that are not found in LW76. The two extra sites are at 3928 (which creates fragments of 4618 bp and 1415 bp) and at 6045 (which creates fragments of 396 bp and 62 bp). The absence of these two sites in pLW76 produce fragments of 6068 bp and 459 bp. pVR246 (lane J) shares these same two fragments, indicating it lacks the Cabb S sites at 3928 and 6045. pVR246 also does not exhibit the 1152 bp fragment that pLW76

Figure 15. Agarose gel electrophoresis of EcoRI-digested plasmids. A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) "pLW414", G) pVR244-A, H) pVR244-B, I) pUM24, J) pVR246, K) pLW76

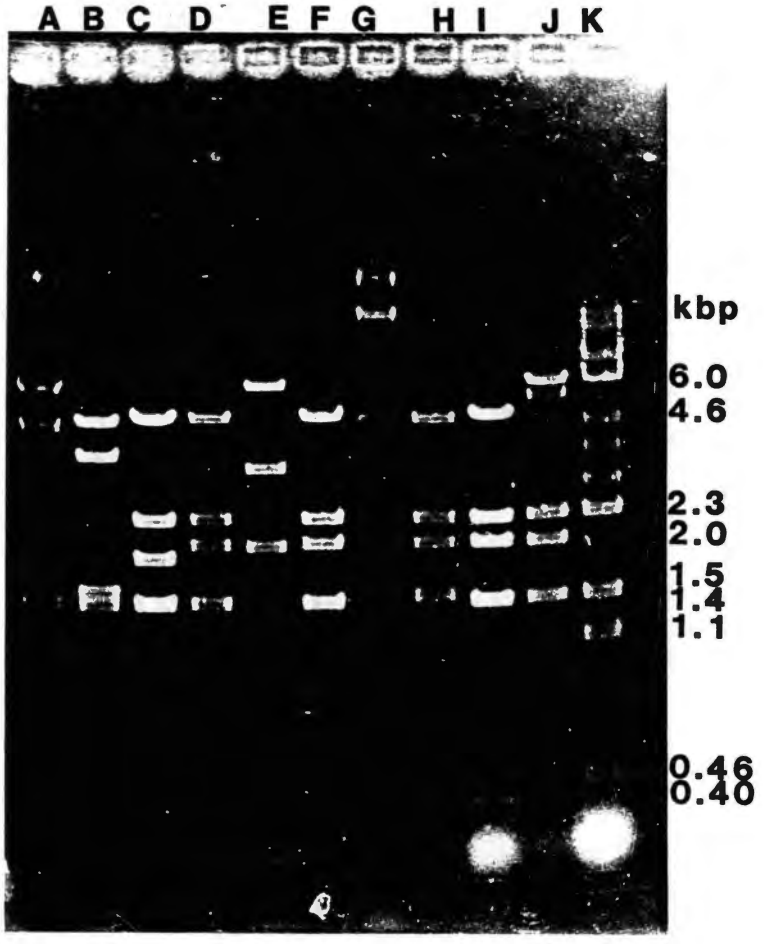
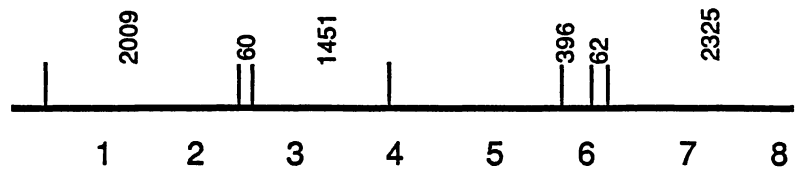
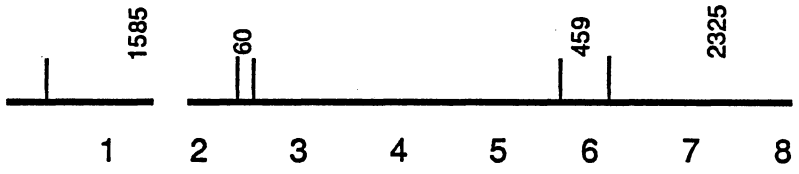

A B C D E F G H I J K
kbp
6.0
4.6
2.3
2.0
1.5
1.4
1.1
0.46
0.40

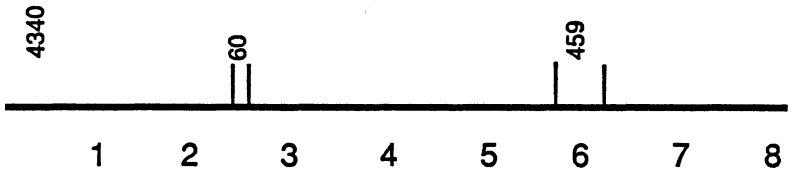
Figure 16. EcoRI restriction maps for wild-type parental CaMV DNAs. Numbers indicate kbp.



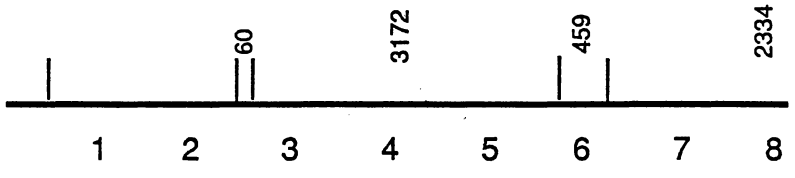
Cabb S



CM4-184



NY8153



W

does, as this fragment contains the 421 bp deletion of CM4-184 and the 433 bp deletion of pLW76. Instead, pVR246 has the 2009 bp fragment like pUM24, indicating the deletions in pLW76 are not present in VR246.

As above, pLW414 appears to instead be pUM24. Therefore, pLW76 will again be used to resolve the restriction pattern for pVR244-A and pVR244-B. The only expected difference between pLW76's fragments and pLW414's was that the 1152 bp fragment of pLW76 should instead be 1585 bp in pLW414. Both pVR244-A and pVR244-B had the similar EcoRI restriction patterns (lanes G and H, respectively). The 459 bp fragment was present in both recombinants, indicating the Cabb S site at 6045 is not present. With these plasmids' opposite orientation, a fragment of 3112 bp would result if no site at 3928 was present. This fragment was not found in either pVR244-A or -B. Instead, fragments of 1561 bp and 1451 bp are seen, indicating both sites contain the Cabb S site at 3928. As the parent used in the inoculation was pLW214 and not pLW414, the pattern for pLW214 would show two fragments of 877 bp and 708 bp instead of the 1585 bp fragment of pLW414. The two fragments result from the insertion of an extra EcoRI site at nt. 1285. Neither pVR244-A or -B exhibit these fragments, indicating they do not contain the pLW214 mutation.

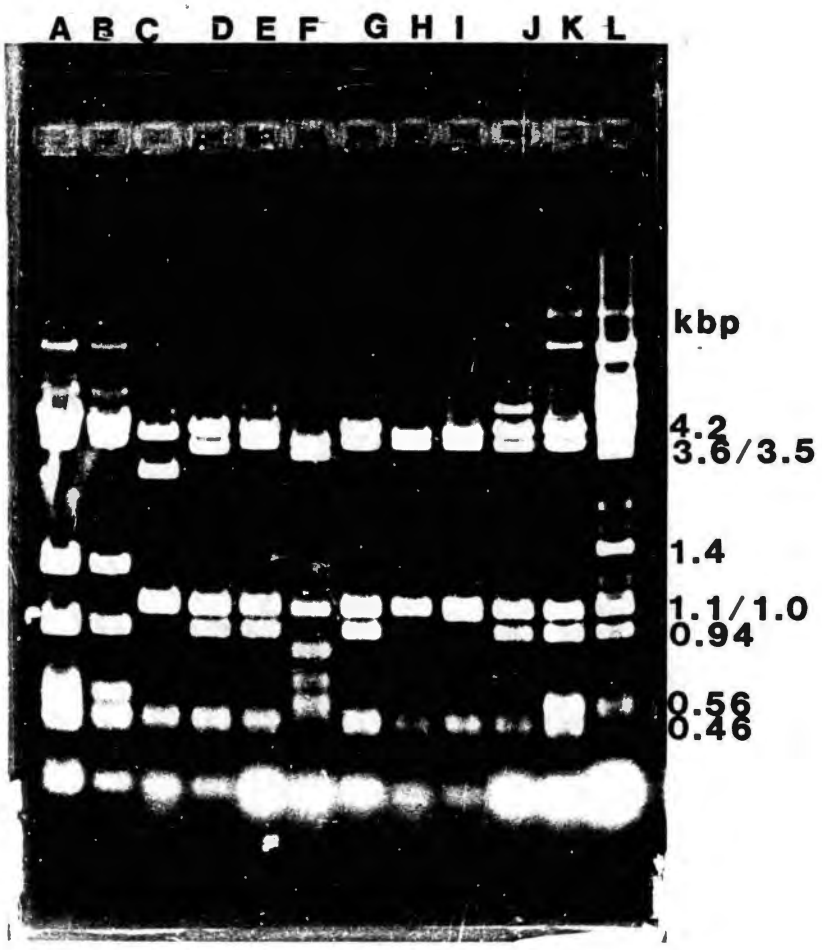
Three sites are distinct between pDLS19 and pUM124 (Figure 15, lanes A and C, respectively). While the sites

found in pDLS19 are common to pUM124, pUM124 has three extra EcoRI sites at 408, 3928, and 6045. These sites yield fragments of 2325 bp and 1862 bp, 1451 bp and 4618 bp, and 396 and 62 bp, respectively. In pDLS19, the absence of a site at 408 yields a 4340 bp fragment, the absence of a site at 3928 yields a 5873 bp fragment, and the missing site at 6045 results in a 459 bp fragment. pVR1249 (lane B) exhibits the 396 bp fragment, indicating a site occurs at nt. 6045. The 1561 and 1451 bp fragments, as found in pVR244-A and -B, indicate the presence of the Cabb S site at 3928 and are also indicative of pVR1249's opposite orientation. While the site at 408 is not detected in pVR1249, the resulting fragment is not 4402 bp as would be expected, but due to VR1249's deletion, is approximately 3500 to 3600 bp.

pIC23 (Figure 15, lane E) does not share the Cabb S sites at 3928 or 6045, yet it does have the site at 408. pVR1243 (lane D) displayed the 1451 and 4618 bp fragments like pUM124 (lane C), indicating the Cabb S site at 3928 is present. Likewise, the 396 bp fragment is present in pVR1243, indicating the site at 6045 is present in pVR1243.

HindIII. The HindIII-digested plasmids were electrophoresed through a 1% agarose gel as shown in Figure 17. The HindIII restriction maps of the parental wild-type DNAs are

Figure 17. Agarose gel electrophoresis of HindIII-digested plasmids. A) pDLS19, B) pDLS19, C) pVR1249, D) pUM124, E) pVR1243, F) pIC23, G) "pLW414", H) pVR244-A, I) pVR244-B, J) pUM24, K) pVR246, L) pLW76

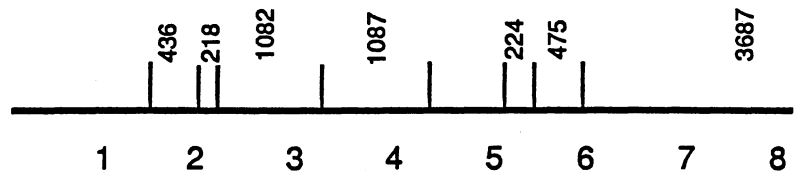

A B C D E F G H I J K L
kbp
4.2
3.6/3.5
1.4
1.1/1.0
0.94
0.56
0.46

shown in Figure 18.

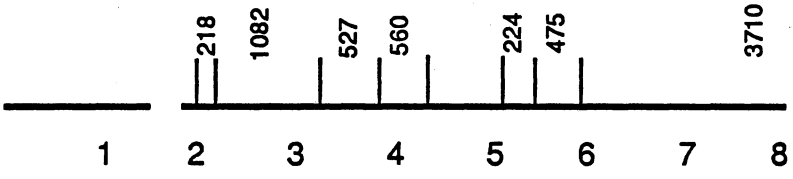
Two site differences exist between pLW76 and pUM24. In pLW76 (Figure 17, lane L), a HindIII site at 3352 (3773) gives rise to two fragments of 527 bp and 560. The site at 3772 is not present in pUM24 (lane J) and a 1087 bp fragment is the result. Shown in lane K are bands of double intensity at 527 and 560, indicating the CM4-184 site at 3772 is present in pVR246. A Cabb S site at nt. 1513, producing a 436 bp fragment and a 3687 bp fragment (3655 bp in pUM24) (Figure 18), is not present in the CM4-184 plasmids, as this site was deleted in the CM4-184 natural deletion. Instead, in the CM4-184 plasmids, the two sites at nts. 1528 (1948) and 5427 (5847), which are common to both CM4-184 and Cabb S, produce a fragment of 3710 bp (3277 bp in pLW76) (Figure 14). Lane K of Figure 17 shows that pVR246 had the 436 bp fragment and a fragment of 3.6 kbp, indicating pVR246 had the HindIII site at nt. 1513.

pLW414 (Figure 17, lane G) again appears to be pUM24 instead. The HindIII banding patterns for pLW414 and pLW76 are essentially the same except for minor differences due to the mutations which are contained within non-diagnostic fragments. Therefore, pLW76 (lane L) can be used to analyze the gel patterns for pVR244-A and pVR244-B. The 436 bp fragment and the 3687 bp fragment were present in both pVR244-A and pVR244-B, indicating the Cabb S site at 1513 is present.

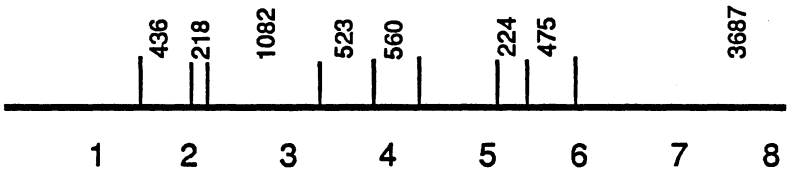
Figure 18. HindIII restriction maps for wild-type parental CaMV DNAs. Numbers indicate kbp.



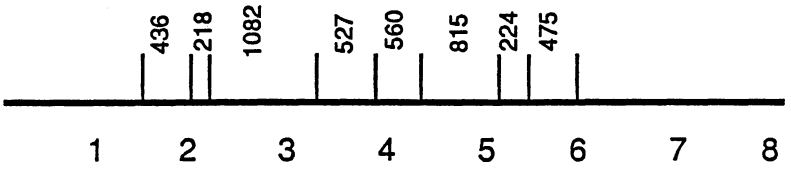
Cabb S



CM4-184



NY8153



W

The 3772 site is present in pDLS19 (Figure 17, lanes A and B) and, while the corresponding 564 bp fragment occurs, the DLS19 mutation deleted the site at 3249. The resulting fragment is 1410 bp in length due to cleavage at nt. 2168, a site that is found in both Cabb S and NY8153. The Cabb S site at 3249 is present in pUM24 and yields fragments of 1082 bp and 1087 bp, which appeared as a band of double intensity in lane D. This band also appears in pVR1249 (lane C), indicating the plasmid contains the Cabb S site at nt. 3249. Since the 564 bp fragment is absent in pVR1249, the NY8153 site at 3772 is not present. Also shown in lane C of Figure 17 is a fragment that migrated further than the 3690 bp fragments that are common in the other recombinants. This fragment in pVR1249 contains the 800 to 900 bp deletion.

The only site difference between pIC23 and pUM124 (Figure 17, lanes F and D, respectively) is the presence of the 3772 site in pIC23, as shown by the characteristic double bands at 527 and 564. pUM124 does not have the HindIII site at nt. 3772 and the resulting fragment is 1087 bp, which is shown as a doubly-intense band in lane D. pVR1243 (lane E) does not exhibit the doubly-intense band at 0.53 and 0.56 kbp, but instead exhibits the doubly-intense band at approximately 1.1 kbp.

AluI. The AluI-digested plasmids were electrophoresed through both a 1% agarose gel to resolve the larger fragments

and through a 6% acrylamide gel to resolve the smaller fragments. These gels are shown in Figures 19 and 20. The AluI restriction maps for the parental wild-type DNAs are shown in Figure 21.

Between pLW76 and pUM24, the only diagnostic fragments that were discerned on the gels are the 493 and 509 bp fragments that migrated as a triply-intense band in pLW76 (lane K in both Figures 19 and 20). The two fragments indicate a CM4-184 site at nt. 197. In pUM24, the absence of the site at 197 creates a fragment of 1002 bp. pVR246 (lane J of both Figures 19 and 20) only had a single-intensity band at 500, due to the 521 bp fragment in the vector DNA. The presence of a 1002 bp fragment in lanes J, G, and H, indicates the CM4-184 AluI site at nt. 197 is absent in pVR246, pVR244-A, and in pVR244-B, respectively.

Due to their opposite orientation, pVR244-A and -B would either have a 691 bp fragment if the Cabb S site at 4766 were present, or would have a 907 bp fragment if it were absent. Both pLW414 and pUM24 (Figure 19, lane F and I, respectively) display double-intensity bands at 900 (actual lengths are 903 bp and 910 bp). Both of these fragments are due to viral and vector sites that are common to both parents. Using these as a guide for relative intensities, it can be seen that pVR244-A and -B (Figure J, lanes G and H, respectively) only exhibit the single-intensity bands caused by the vector fragment of 910 bp, and therefore do not have the 907 bp fragment that

Figure 19. Agarose gel electrophoresis of AluI-digested plasmids. A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) pLW414, G) pVR244-A, H) pVR244-B, I) pUM24, J) pVR246, K) pLW76

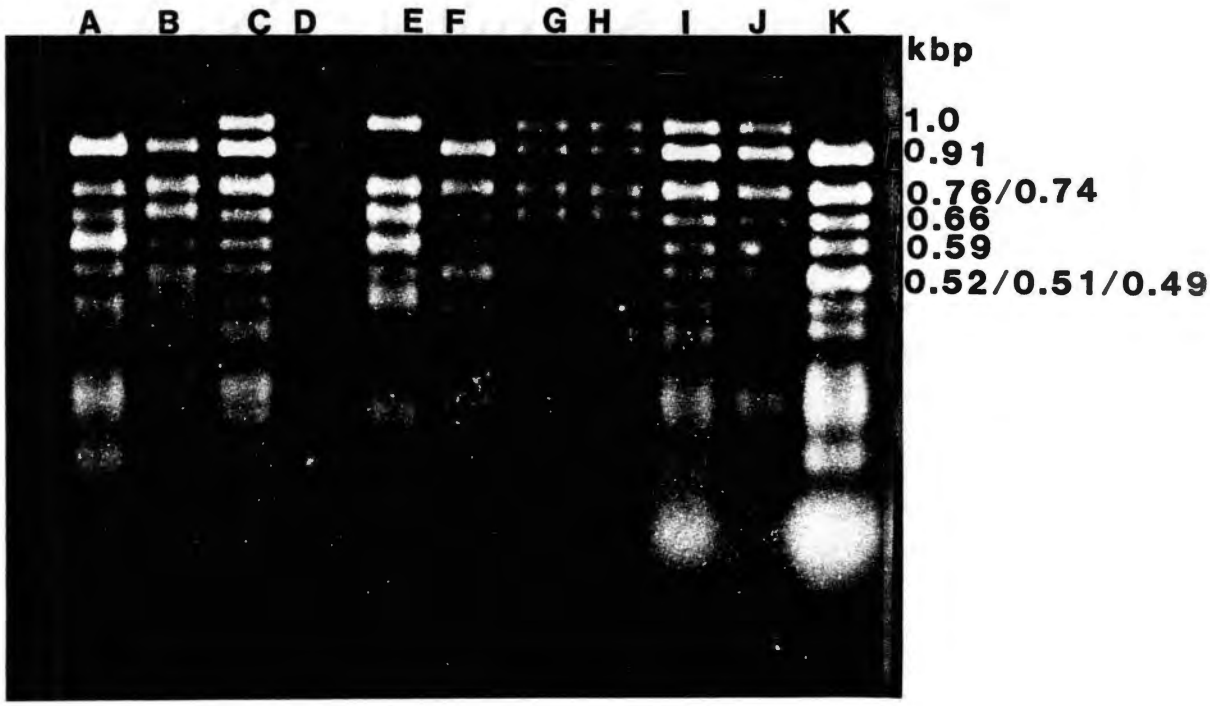

A B C D E F G H I J K
kbp
1.0
0.91
0.76/0.74
0.66
0.59
0.52/0.51/0.49

Figure 20. Acrylamide gel electrophoresis of AluI-digested plasmids. A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) "pLW414", G) pVR244-A, H) pVR244-B, I) pUM24, J) pVR246, K) pLW76

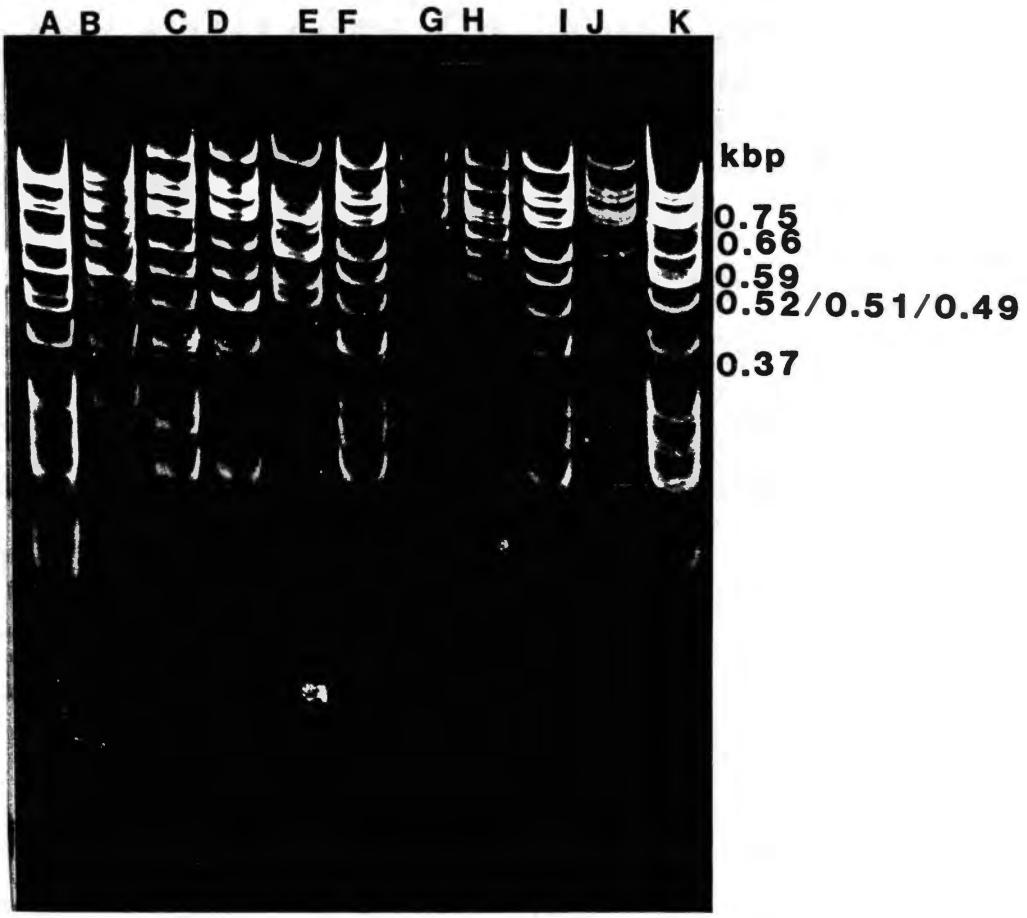
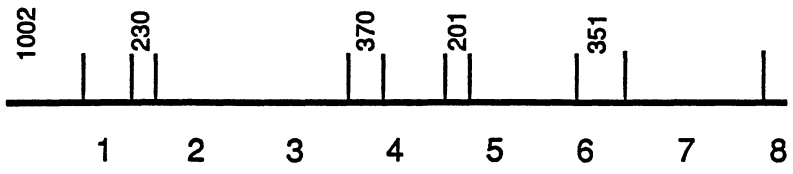

A B C D E F G H I J K
kbp
0.75
0.66
0.59
0.52/0.51/0.49
0.37

Figure 21. AluI restriction maps for wild-type parental CaMV DNAs. Numbers indicate kbp.



Cabb S

CM4-184

would indicate the absence of a Cabb S site at 4766. The 903 bp fragment found in their parents is not expected in pVR244-A and -B due to their opposite orientation.

As the AluI restriction pattern for pDLS19 and pIC23 is unknown, there must be distinct differences in parental banding patterns before any information can be learned about the recombinants' fragments and sites. For AluI, these differences are not apparent, and therefore no information was learned about pVR1249 or pVR1243.

HaeIII. The HaeIII-digested plasmids were electrophoresed through both 2% agarose and 6% acrylamide gels, shown in Figures 22 and 23, respectively. The HaeIII restriction maps of the parental wild-type DNAs are shown in Figure 24.

Several diagnostic HaeIII fragments occur between Cabb S- and CM4-184-derived plasmids. A site present in CM4-184 and absent in Cabb S is the site at 4331 (4752) (Figure 24), which yields 2 fragments of 1297 bp and 259 bp. The 259 bp fragment includes some vector DNA. In Cabb S, only one fragment of 1554 is found, as seen in pUM24, lane I of Figure 22. pVR246 (Figure 22, lane J), also displayed the 1554 bp fragment, indicating the CM4-184 HaeIII site at nt. 4752 is not present in pVR246. With their opposite orientation, pVR244-A and pVR244-B (Figure 22, lanes G and H, respectively) would

Figure 22. Agarose gel electrophoresis of HaeIII-digested plasmids. A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) pLW414, G) pVR244-A, H) pVR244-B, I) pUM24, J) pVR246, K) pLW76

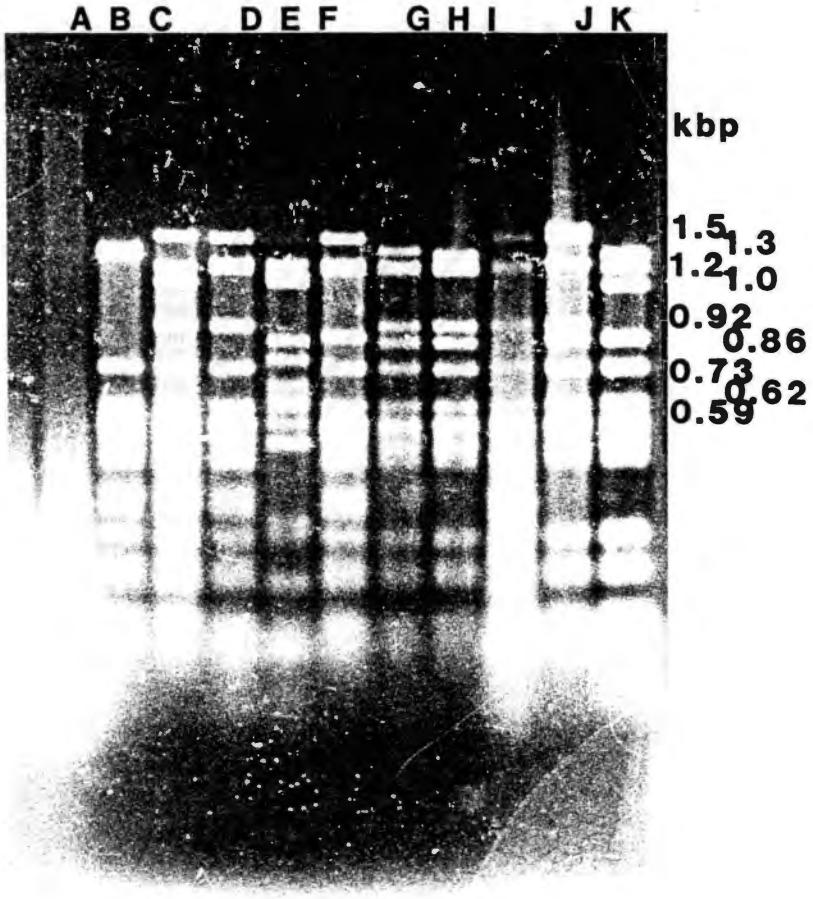

A B C D E F G H I J K
kbp
1.5
1.3
1.2
1.0
0.92
0.86
0.73
0.62
0.59

Figure 23. Acrylamide gel electrophoresis of HaeIII-digested plasmids. A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) "pLW414", G) pVR244-A, H) pVR244-B, I) pUM24, J) pVR246, K) pLW76

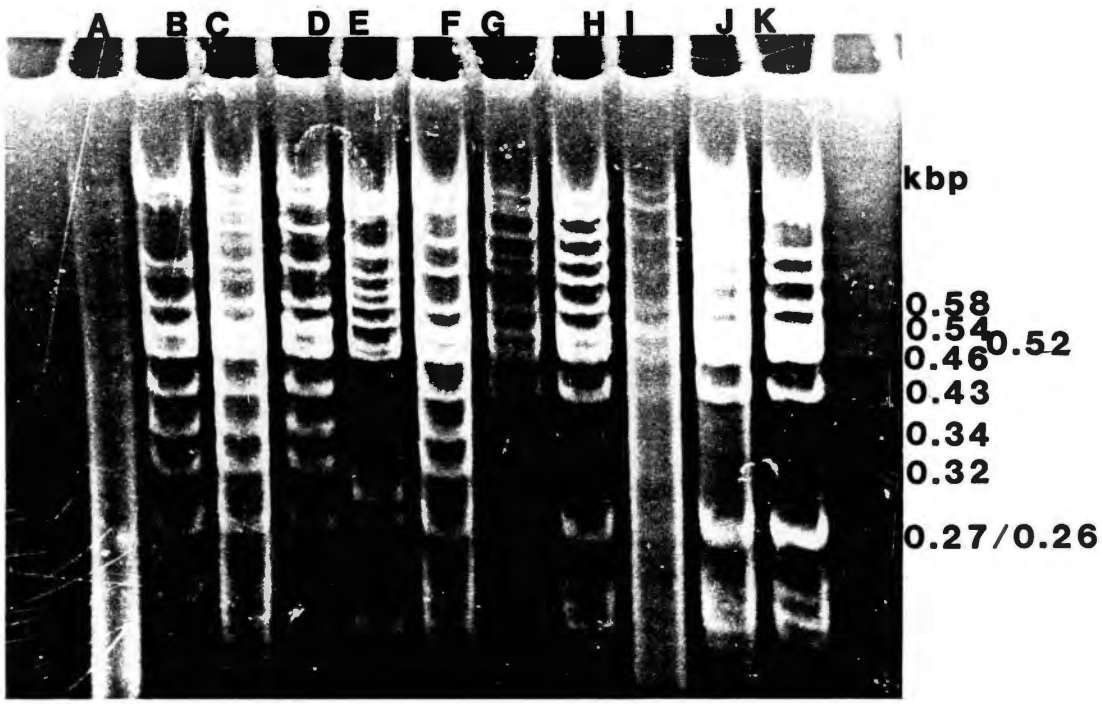
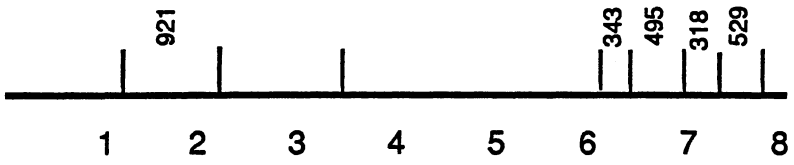
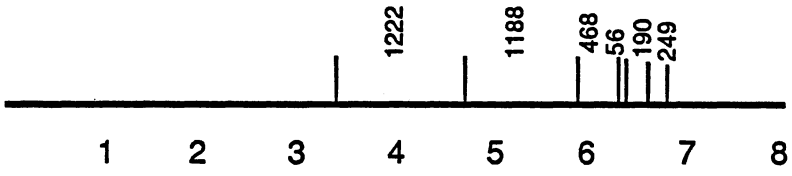

A
B
C
D
E
F
G
H
I
J
K
kbp
0.58
0.54
0.52
0.46
0.43
0.34
0.32
0.27/0.26

Figure 24. HaeIII restriction maps for wild-type parental CaMV DNAs. Numbers indicate kbp.



Cabb S

CM4-184



W

have a 140 bp fragment if the CM4-184 site at 4752 were present, or they would have a 1430 bp fragment if this site were missing. As shown in lane G of Figure 22, pVR244-A has the 1430 bp fragment and therefore did not have the CM4-184 site at 4752. pVR244-B (lane H) contained a fragment that had migrated slightly further down the gel than the 1430 bp fragment of pVR244-A. pVR244-B's fragment is most likely the fragment corresponding to the 1430 bp fragment of pVR244-A, yet is less that 1430 bp due to the presence of the 80 to 200 bp deletion in pVR244-B. The 1297 bp fragment is not present in either pVR244-A or -B, also indicating the site at nt. 4752 is absent.

CM4-184 plasmids have fragments of 1163 bp and 468 bp due to a site at nt. 5520 (5943). This 1163 bp fragment can be seen in pLW76 (lane K of Figure 22). The 468 bp fragment appeared as a doubly-intense band, having migrated with a 458 bp fragment of pBR322 DNA (Lane K of Figure 23). Cabb S does not have the site at 5943, but does have a site at nt. 6068 that is not present in CM4-184 (see Figure 24). The site at nt. 6068 yields fragments of 1287 bp and 343 bp (375 bp in pUM24) (Lane I of Figures 22 and 23, respectively). pVR246 (Figure 22, lane J) has neither the 1163 bp fragment like pLW76 nor the 1287 bp fragment like pUM24. Instead, pVR246 had a band at 1.6 kbp. A 1630 bp fragment would result if the recombinant were missing both sites 5943 and 6068 and if cleavage occurred at the site at nt. 6411, which is common to both parents. Due to their opposite orientations, pVR244-A

and -B would have an 863 bp fragment and a 468 bp fragment if the CM4-184 site at nt. 5943 were present, or they would have a 1411 bp fragment and 343 bp fragment if the Cabb S site at 6068 were present. In neither pVR244-A or pVR244-B is the Cabb S fragment of 343 bp present (Figure 23, lanes G and H, respectively), indicating the Cabb S site at 6068 is absent. If the 343 bp fragment is absent, then the 1411 bp fragment should also be absent. Indeed, the bands at 1.4 kbp are only of single intensity, due to the 1430 bp fragment described above. The fragments resulting from cleavage at the CM4-184 site at 5943, 863 bp and 468 bp, cannot be differentiated between the 855 bp fragment (described below) and the 458 bp fragment of vector DNA.

A Cabb S site at nt. 7224 yields two fragments of 318 bp and 529 bp, while the absence of this site in CM4-184 produces an 855 bp fragment. All three recombinants, pVR244-A, pVR244-B, and pVR246 (lanes G, H, and J,respectively,of both Figures 22 and 23), did not have the 318 bp fragment and did display the 855 bp fragment, indicating a Cabb S site at nt. 7224 is absent.

While both Cabb S and CM4-184 have HaeIII sites at nts. 1170 and 2091, the resulting fragments are distict due to the 421 bp deletion characteristic of CM4-184. In Cabb S, the fragment is 921 bp while it is 500 bp in CM4-184 (Figure 24). However, pLW76's extra deletion of 433 bp has deleted the site at nt. 1170 so cleavage must occur at nt. 7456 (7871), a site common to both parents. Cleavage at 7871 produces a

1046 bp fragment in pLW76 (Figure 22, lane K). In recombinants pVR244-A, -B, and pVR246, the 921 bp fragment was present, indicating all three recombinants had the Cabb S site at nt. 1170.

Lane A of both Figures 22 and 23 reveal that the HaeIII digest of pDLS19 was contaminated with nucleases, and produced a smear. Since the sequence (and therefore restriction pattern) of NY8153 is not completely known, and since the smeared pattern yields no bands distinct from pUM124 (lane C of Figures 22 and 23), no information could be learned about the HaeIII pattern of pVR1249 (lane B of Figures 22 and 23).

In W DNA, sites at nts. 5939 and 6407, 6407 and 6463, 6463 and 6653, and 6653 and 6902 produce fragments of 468 bp, 56 bp, 190 bp and 249 bp, respectively (see Figure 24). The pIC23 mutation has deleted the sites at nts. 6407, 6463, and 6653, producing instead, a fragment of 615 bp (Figure 22, lane E). The W site at nt. 5939 is not present in Cabb S, and the site at nt. 6902 is common to both parents. Cabb S contains one site (at nt. 6068) which is not present in W DNA. If the Cabb S site at 6068 were present in pVR1243, two fragments of 1284 bp and 343 bp would result. If the W site at 5939 were present in pVR1243, a fragment of 1157 bp would be present. pVR1243 displayed the 1284 bp and 343 bp fragments (lane D of Figures 22 and 23, respectively), indicating the recombinant had the Cabb S site at nts. 6068 and 6411 and did not have the W site at nt. 5939.

pVR1243 did not contain the pUM124 deletion, as this mutation creates an 1176 bp fragment in pUM124. Instead, a 1323 bp fragment (without the deletion) which is characteristic of pIC23, was displayed by pVR1243 (Figure 22, lane D). A W site at nt. 4752 gives 2 fragments of 1222 bp and 1188 bp that appeared as a doubly-intense band in lane E of Figure 22. The site at 4752 is not present in pUM124, resulting in a 1554 bp fragment. This fragment contains some vector sequences. The 1554 bp fragment was displayed by pVR1243, indicating the recombinant does not contain the W site at nt. 4752.

HinfI. The HinfI-digested plasmids are shown electrophoresed through a 6% acrylamide gel in Figure 25. The HinfI restriction maps for the wild-type parental DNAs are shown in Figure 26.

pUM24 contains HinfI sites at nts. 830, 1218, 1571, and 1781 that produce fragments of 233 bp, 388 bp, 353 bp, 210 bp, and 426 bp (see Cabb S map in Figure 26). All of these sites are not present in CM4-184 plasmids, and the result is a fragment of 1189 bp (Figure 26). The 433 bp deletion in pLW76 further reduces this fragment to 756 bp (lane K of Figure 25). As can be seen in lane J of Figure 25, pVR246 displayed the 426 bp, 353 bp, and the 388 bp fragments, indicating pVR246 contains the Cabb S sites at nts. 830, 1218, 1571, and 1781. pVR244-A and pVR244-B (lanes G and H, respectively, of Figure 25) also displayed the same diagnos-

Figure 25. Acrylamide gel electrophoresis of HinfI-digested plasmids. A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) "pLW414", G) pVR244-A, H) pVR244-B, I) pUM24, J) pVR246, K) pLW76

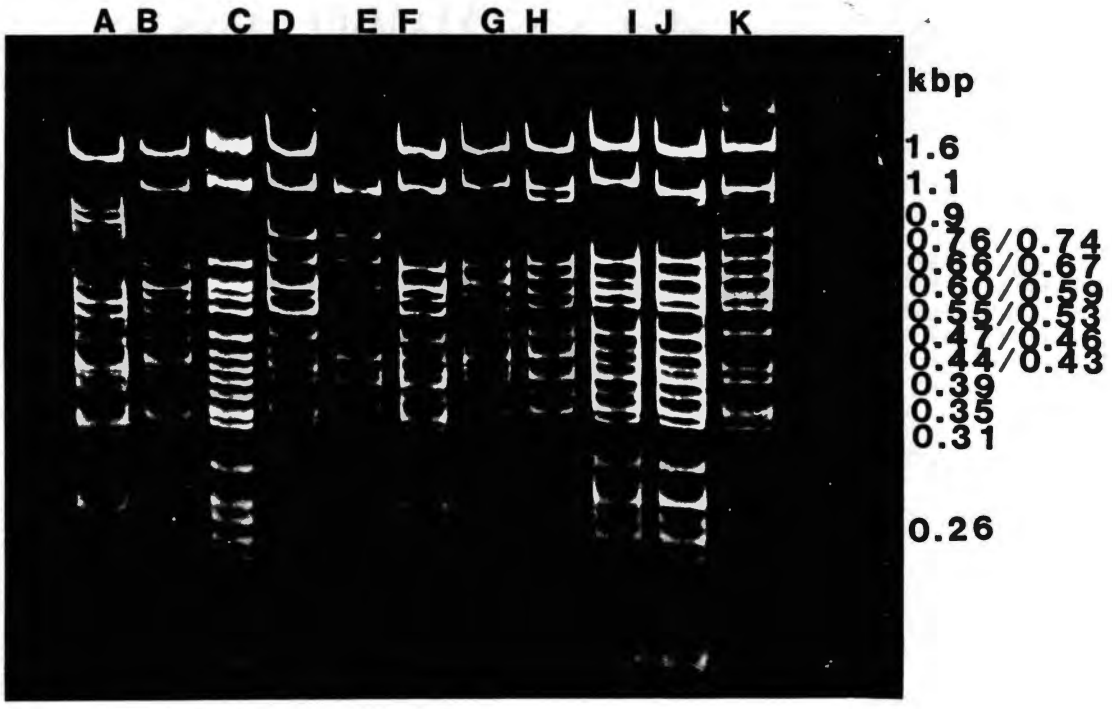
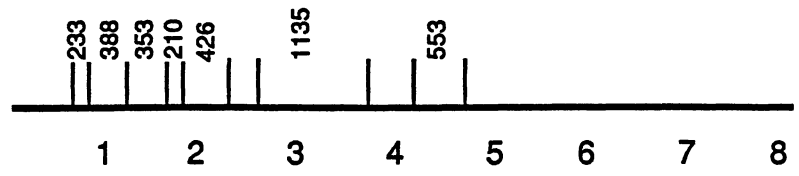
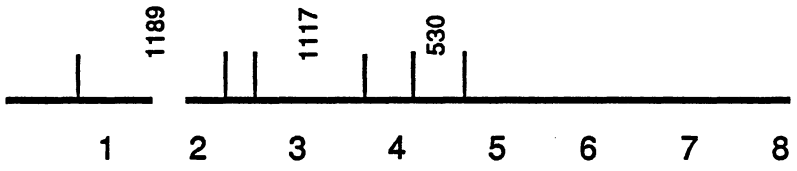

A B C D E F G H I J K
kbp
1.6
1.1
0.9
0.76/0.74
0.66/0.67
0.60/0.59
0.55/0.53
0.47/0.46
0.44/0.43
0.39
0.35
0.31
0.26

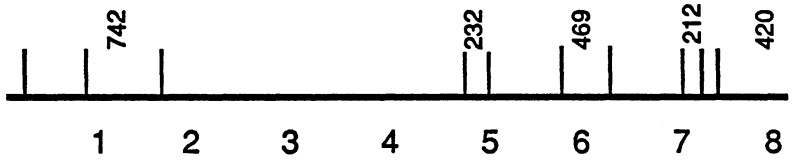
Figure 26. HinfI restriction maps for wild-type parental CaMV DNAs. Numbers indicate kbp.



Cabb S



CM4-184



W

tic fragments as did pVR246, and therefore also had the Cabb S HinfI sites at nts. 830, 1218, 1571, and 1781.

The presence of a site at nt. 3211 (3635) in CM4-184 produces an 1117 bp fragment (Figure 26 and lane K of Figure 25) while the absence of the site at 3635 produces an 1135 bp fragment in Cabb S and pUM24 (Figure 26 and lane I of Figure 25, respectively). The 1117 bp fragment was also displayed by pVR246 (lane J), while pVR244-A and -B (lanes G and H, respectively) had fragments that migrate with the Cabb S fragment of 1135. Therefore, pVR246 had the CM4-184 site at nt. 3635 while pVR244-A and pVR244-B did not.

Another site present in CM4-184 that is not present in Cabb S is the site at nt. 3709 (4133). This site produces a 530 bp fragment in CM4-184 and its absence produces a 553 bp fragment in Cabb S (Figure 25, lanes K and I, respectively, and Figure 26). The CM4-184 fragment of 530 bp appeared as a band of double intensity in pLW76 (lane K) and also in lane J (pVR246). Therefore, pVR246 had the CM4-184 site at nt. 4133. pVR244-A displayed the Cabb S fragment of 553 bp, indicating the CM4-184 site at 4133 was not present in pVR244-A. Common to both parents is a site at nt. 4110, which yields a 392 bp fragment. As can be seen in lane H of Figure 25, pVR244-B lacked the 392 bp fragment, indicating that the site at 4110 was missing. This site is most likely deleted due to the 80 to 200 bp deletion in pVR244-B. If the site at nt. 4110 were indeed missing, the 553 or 530 bp frag-

ment should also be absent. While there appeared to be a fragment migrating with the 553 bp fragments, there is further evidence to support the conclusion the site at 4110 is deleted in pVR244-B. If this site were to be deleted, the 392 bp fragment and either the 553 bp or 530 bp fragment would be combined as one fragment, and the result would be a fragment of approximately 900 bp. Indeed, a fragment of approximately 900 bp was displayed by pVR244-B in lane H of Figure 25.

A HinfI site present in CM4-184 but not in Cabb S is at nt. 4497 (4921). In pLW76, the resulting fragment is 100 bp which could not be discerned. However, due to their opposite orientations, the presence or absence of the site at 4921 in pVR244-A and -B could be deduced. If the site were absent in the recombinants, a 441 bp fragment would result. As shown in lanes G and H of Figure 25, both pVR244-A and -B displayed a 441 bp fragment, indicating they did not contain the CM4-184 site at nt. 4921.

Since the sequence of pDLS19 is unknown, only obvious fragment differences from the gel could be used in diagnosing the restriction pattern for pVR1249. As can be seen in lane A of Figure 25, pDLS19 lacks the 661 bp fragment that was present in both pUM124 (lane C) and pVR1249 (lane B), indicating a HinfI site was present at nt. 7794, which was apparently not present in pDLS19. If the site were missing in pDLS19, a 906 bp fragment would result due to cleavage at the sites at nts. 7133 and 15. Lane A did display a fragment

of approximately 900 bp, in support of the above conclusion.

Cabb S DNA contains a HinfI site at nt. 1218 that does not occur in W DNA (Figure 26). This site in Cabb S yields two fragments of 353 bp and 388 bp (241 bp in pUM124), while its absence in W produces a 742 bp fragment. Lanes D of Figure 25 displayed the 742 bp fragment, indicating pVR1243 did not have the Cabb S site at nt. 1218.

MspI. The MspI-digested plasmids were electrophoresed through 2% agarose and 6% acrylamide gels (Figures 27, 28, and 29). The MspI restriction maps for the wild-type DNAs are shown in Figure 30.

A Cabb S site at nt. 7118 is not present in CM4-184 plasmids (Figure 30). Two fragments of 846 bp and 1030 bp result from the site in Cabb S plasmids (814 bp in pUM24, lane A of Figure 27 or lane F of Figure 28). The absence of the site at 7118 in CM4-184 plus a CM4-184-unique site at 6085 (6509) yields a 1648 bp fragment and a 236 bp fragment in CM4-184 plasmids (pLW76) (lane C of Figure 27 and lane K of Figure 29, respectively). All three recombinants, pVR246, pVR244-A, and pVR244-B, displayed the 1648 bp fragment (Figure 27, lane B, and Figure 28, lanes G and H, and the 236 bp fragments (Figure 29, lanes G, H, and J), indicating they did not have the Cabb S site at nt. 7118 and they did have the CM4-184 site at 6509.

A site present in CM4-184 and absent from Cabb S is at

Figure 27. Agarose gel electrophoresis of MspI-digested plasmids (Second gel). A) pUM24 B) pVR246, C) pLW76

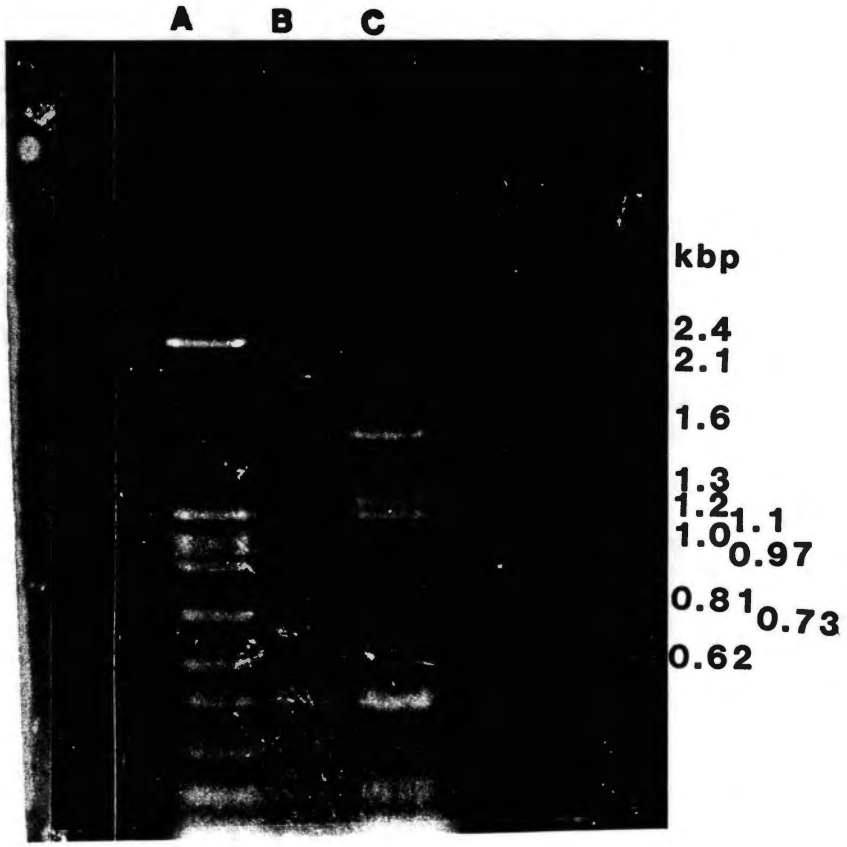

A
B
C
kbp
2.4
2.1
1.6
1.3
1.2
1.0
1.1
0.97
0.81
0.73
0.62

Figure 28. Agarose gel electrophoresis of MspI-digested plasmids (First gel). A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) pUM24, G) pVR244-A, H) pVR244-B

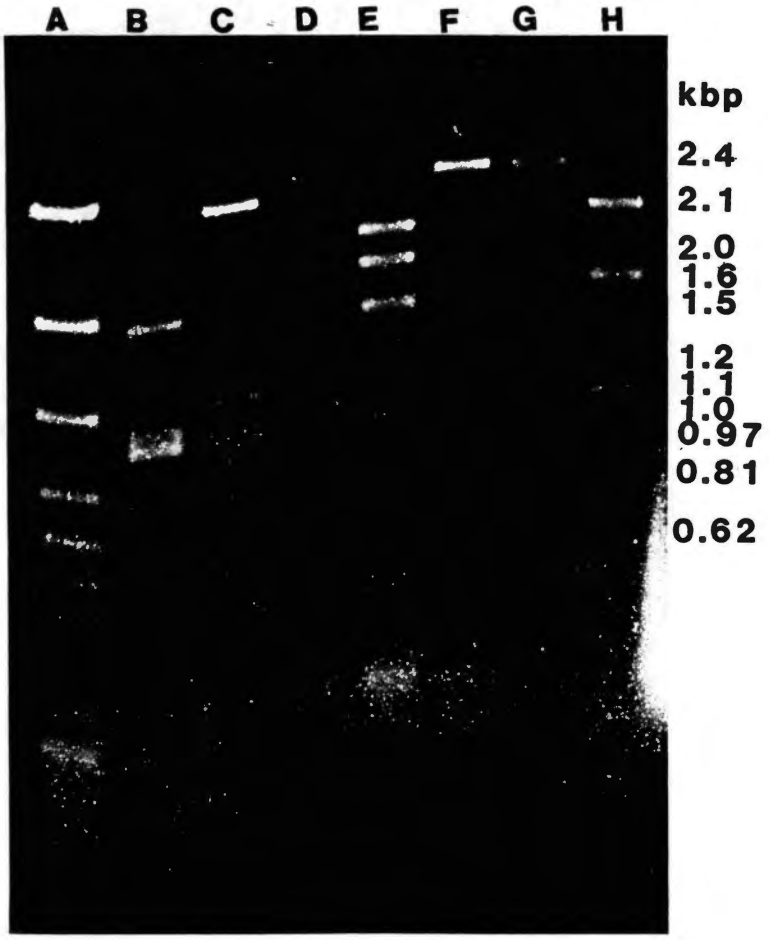

A
B
C
D
E
F
G
H
kbp
2.4
2.1
2.0
1.6
1.5
1.2
1.1
1.0
0.97
0.81
0.62

Figure 29. Acrylamide gel electrophoresis of MspI-digested plasmids. A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) pLW414, G) pVR244-A, H) pVR244-B, I) pUM24, J) pVR246, K) pLW76

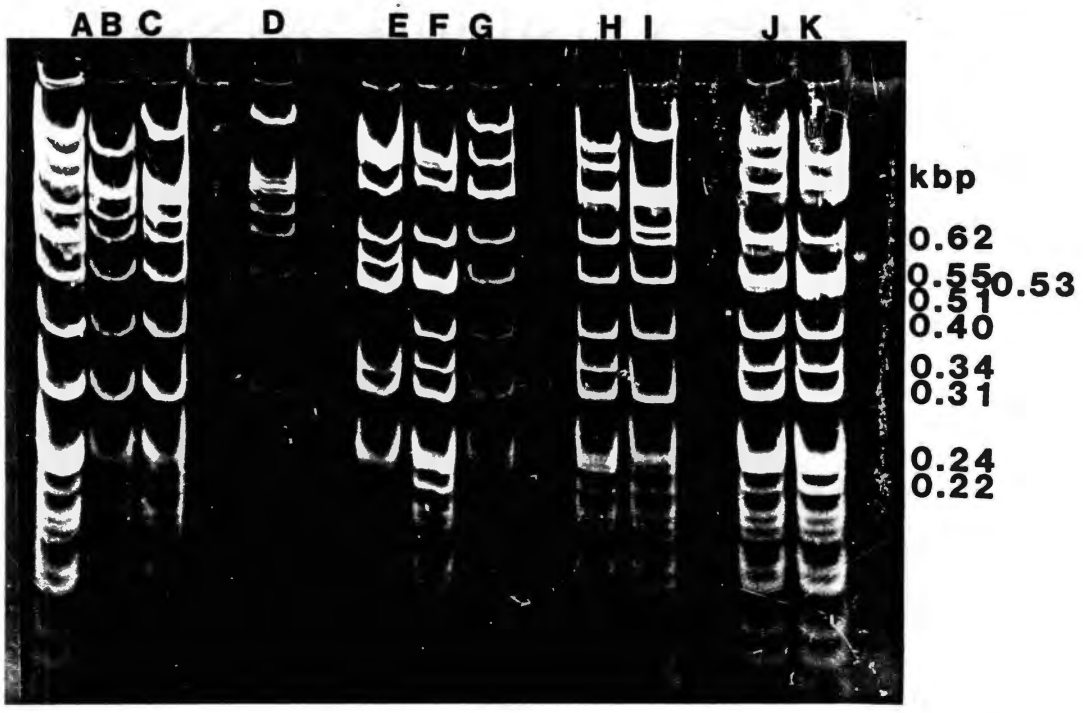
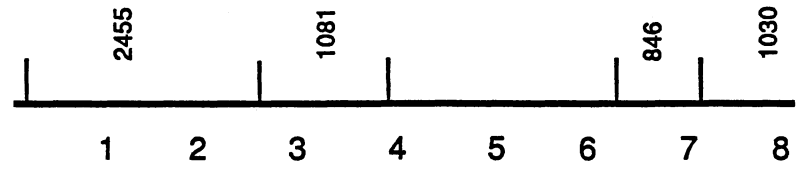
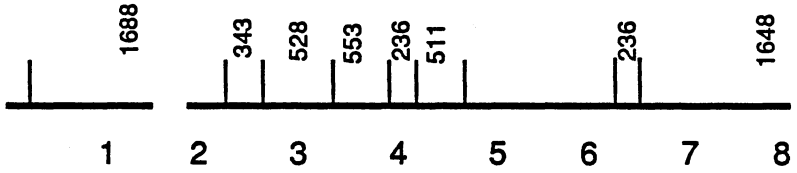

A B C
D
E F G
H I
J K
kbp
0.62
0.55
0.53
0.51
0.40
0.34
0.31
0.24
0.22

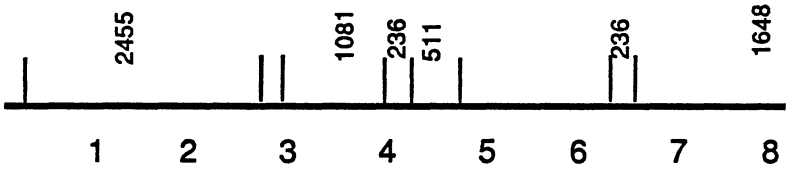
Figure 30. MspI restriction maps for wild-type parental CaMV DNAs. Numbers indicate kbp.



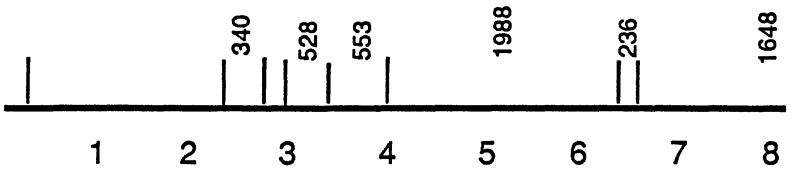
Cabb S



CM4-184



NY8153



W

nt. 1812 (2236) (Figure 30). This site produces fragments of 343 bp and 1688 bp (1255 bp in pLW76) (Figure 29, lane K and Figure 27, lane C, respectively). The absence of the site at 2236 in Cabb S produces a fragment of 2455 bp (pUM24, lane C of Figure 27 or lane F of Figure 28). As can be seen in lane G of Figure 28, pVR244-A displayed the 2455 bp fragment, indicating it did not contain the CM4-184 site at nt. 2236. Further confirming this conclusion was the absence of the 343 bp fragment in pVR244-A (lane G of Figure 29). As can be seen in lanes H and J and Figure 29, pVR244-B and pVR246, respectively, did display the 343 bp fragment, indicating they had the CM4-184 site at nt. 2236. However, the expected 1688 bp or 1255 bp fragment was not displayed by pVR244-B or by pVR246 (Figure 28, lane H and Figure 27, lane B, respec-tively). But, if the site at 2236 were present, and if the recombinants did not contain the 421 bp deletion that is characteristic of CM4-184, a 2109 bp fragment would result. As can be seen in lane H of Figure 28 and in lane B of Figure 27, pVR244-B and pVR246 (respectively) did display bands at 2.1 kbp.

Another site present in CM4-184 plasmids but absent in Cabb S plasmids is the MspI site at nt. 2936 (3360) (Figure 30). In pLW76, this site at 3360 produces two fragments of 528 and 553 bp (doubly-intense band in lane K of Figure 29). The absence of the site at 3360 in pUM24 yields a fragment of 1081 bp (lane A of Figure 27). pVR246 displayed the doubly-

intense band characteristic of the two bands of 528 bp and 553 bp (lane J of Figure 29) and did not display the 1081 bp fragment (lane B of Figure 27). Therefore, pVR246 had the CM4-184 site at 3360. Both pVR244-A and -B displayed the 1081 bp fragment (lanes G and H of Figure 28, respectively), indicating they both did not have the CM4-184 site at nt. 3360.

Two sites present in CM4-184 but not in Cabb S are at nts. 3725 (4149) and 4236 (4660). Three characteristic fragments result in CM4-184 plasmids: 236 bp, 511 bp, and 218 bp. (The 218 bp fragment contains some vector DNA). The absence of the two sites in Cabb S yields one fragment of 965 bp. If pVR244-A and -B were not to have the two CM4-184 sites at nts. 4149 and 4660, a 1040 bp fragment would result due to their opposite orientation. This 1040 bp fragment can be seen in lanes G and H of Figure 29. Also, the 511 bp fragment was not present in those lanes. While the 511 bp fragment was not present in pVR246 (lane J of Figure 29), the 236 bp fragment was. If the site at 4149 were to be present in pVR246, but the site at 4460 were to be absent, two fragments of 236 bp and 729 bp would result. As stated above, the 236 bp fragment was present, and it can be seen in lane J of Figure 29 that a fragment of 729 bp was present.

Since the sequence of pDLS19 is unknown, only obvious fragment differences from the gels could be used in diagnosing the restriction pattern for pVR1249. As can be seen in lane A of Figures 28 and 29, pDLS19 displayed a 1648 bp frag-

ment and a 236 bp fragment, indicating NY8153 had MspI sites at nts. 6509 and 6273. As described above, Cabb S plasmids lack these sites and have a unique site at nt. 7118 which yields fragments of 1030 bp and 846 bp. The 846 bp fragment and the 1030 bp fragment were both present in pVR1249 (Figure 28, lane B), indicating the Cabb S site at nt. 7118 was present and also indicating the NY8153 site at nt. 6509 was not present in pVR1249. A fragment of 2455 bp is found in both parents due to the common sites at nts. 124 and 2579. This fragment was not seen for pVR1249 (lane B of Figure 28), instead, a fragment of approximately 1.6 kbp was observed. This fragment was most likely the fragment common to both parents but contained the 800 to 900 bp deletion that is present in pVR1249.

The only other site differences between NY8153 and Cabb S that could be resolved from the gels is the presence of two sites at nts. 4149 and 4660 in pDLS19 that are not present in pUM124 (see Figure 30). These two sites produce three diagnostic fragments of 236 bp, 511 bp, and 218 bp in pDLS19, whereas a single fragment of 965 bp results in pUM124. Due to its opposite orientation, pVR1249 would have a fragment of 1040 bp if it did not have the two NY8153 sites at nts. 4149 and 4660. Lane B of Figure 28 did display such a fragment, indicating pVR1249 did not have the NY8153 sites at nts. 4149 and 4660.

An MspI site present in W DNA but not in Cabb S is the site at nt. 2235 (see Figure 30). This site produces a

fragment of 340 bp in pIC23 and another fragment of unknown size due to the presence of pACYC177 (vector) DNA. Lane D of Figure 29 did not display the 340 bp fragment, indicating that pVR1243 did not have the W site at nt. 2235.

A site at nt. 3356 is present in W and absent in Cabb S. This site yields two fragments of 528 and 553 bp in pIC23 (lane E of Figure 29) while only one fragment of 1081 bp is present in pUM124 (lane C of Figure 28). pVR1243 (Lane D of Figure 28) displayed the 1081 bp fragment while lane D of Figure 29 did not display the triply-intense band that would have indicated the presence of the 528 bp and 553 bp fragment. Instead, only the single-intesity band was present, which results from a vector fragment of 527 bp. Therefore, pVR1243 does not have the W site at nt. 3356.

The site at nt. 7118 which produces fragments of 846 and 1030 bp in pUM124 is not present in pIC23. pIC23 has a fragment of 1539 bp, which includes the pIC23 mutation. Lane D of Figure 28 revealed that pVR1243 had the 846 and 1030 bp fragments and therefore had the Cabb S site at nt. 7118.

TaqI. The TaqI-digested plasmids were electrophoresed through a 6% acrylamide gel (Figure 31). The TaqI restriction maps of the wild-type parental DNAs are shown in Figure 32.

A TaqI site that is present in Cabb S but not in CM4-184 is a site at nt. 1643 (see Figure 32). This site yields an 822 bp fragment in Cabb S, but has been deleted in CM4-184

Figure 31. Acrylamide gel electrophoresis of TaqI-digested plasmids. A) pDLS19, B) pVR1249, C) pUM124, D) pVR1243, E) pIC23, F) "pLW414", G) pVR244-A, H) pVR244-B, I) pUM24, J) pVR246, K) pLW76

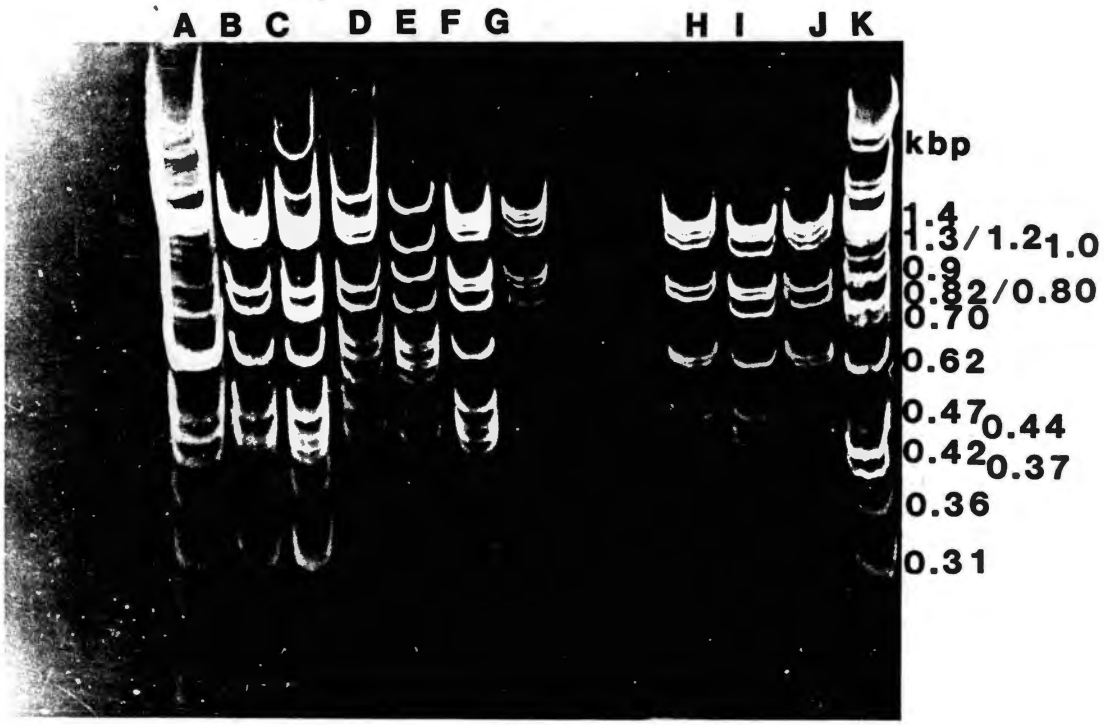
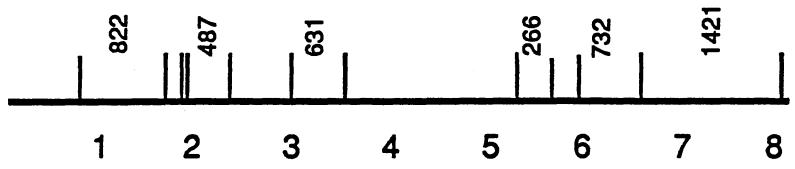
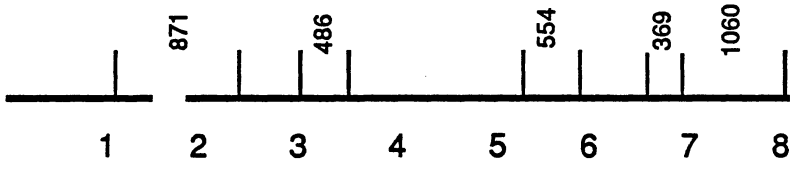

A B C D E F G H I J K
kbp
1.4
1.3/1.2
1.0
0.9
0.82/0.80
0.70
0.62
0.47
0.44
0.42
0.37
0.36
0.31

Figure 32. TaqI restriction maps for wild-type parental CaMV DNAs. Numbers indicate kbp.



Cabb S



CM4-184

due to the 421 bp deletion. Two sites present in CM4-184 but absent in Cabb S are the sites at nts. 1075 and 1946 (2367). These sites yield a fragment of 871 bp. However, due to the pLW76 mutation, the site at 1075 is deleted, cleavage occurs at a site common to both parents (nt. 613), and a 900 bp fragment results (lane K of Figure 31). Three more sites present in Cabb S but not in CM4-184 are the sites at nts. 1784, 1793, and 2280, which should yield fragments of 9 bp and 487 bp. However, cleavage does not occur at the sites at 1793 and 2280 as these are both dam sites. Instead, cleavage occurs at nts. 1784 and 2481 to produce one fragment of 697 bp (see pUM24, lane I of Figure 31). The site at 2481 is common to both parents. pVR244-A displayed the 697 bp fragment (lane G of Figure 31), indicating the Cabb S site at nt. 1784 was present. The presence of the 697 bp fragment in pVR244-A also indicated that the CM4-184 site at 2367 was not present. pVR244-B and pVR246 (lanes H and J of Figure 31, respectively) did not display the 697 bp fragment. They did, however, display a fragment of 583 bp that would result if the Cabb S site at nt. 1784 were present and the CM4-184 site 2367 were present.

A site that is found in Cabb S but not in CM4-184 is the site at nt. 5541. This site yields a fragment of 266 bp in pUM24 (lane I of Figure 31). The absence of this site in CM4-184 would produce a fragment of 554 bp, but a dam site occurs at nt. 5405 (5828), and cleavage instead occurs at nt.

6137 (6929). The result is a CM4-184-characteristic fragment of 1286 bp. The 266 bp fragment was displayed for all three recombinants: pVR244-A, pVR244-B, and pVR246 (Figure 31, lanes G, H, and J, respectively), therefore indicating that the Cabb S site at nt. 5541 was present.

Another site present in Cabb S but not in CM4-184 is the site at nt. 6560. This site yields fragments of 732 bp and 1421 bp.

No diagnostic fragments could be resolved between pDLS19 and pUM124 or between pIC23 and pUM124, therefore no information was learned about the TaqI restriction patterns for pVR1249 or pVR1243.

CHAPTER IV

SUMMARY AND DISCUSSION

Deletions in Recombinants

Deletions were detected in both recombinants pVR244-B and pVR1249. The deletion in pVR244-B was calculated to be 80 to 200 bp in length and was resolved to occur between nts. 3960 and 4663. The deletion in pVR1249 was calculated to be 800 to 900 bp in length and was resolved to occur between nts. 124 and 1514.

The deletion in pVR1249 currently appears similar to a frequent site-specific deletion reported occurring in the Japanese isolate CaMV-S (Hirochika et al., 1985). Analysis of the sequences around the deletion site revealed sequences similar to the donor and acceptor consensus sequences of RNA splicing. Point mutations of these sequences produced the activation of new (cryptic) donor sites. The deletion was found to be 856 bp in length and occurred between ORFs I and II, or more specifically, between nts. 652 and 1508. Hirochika et al., (1985) proposed that the site-specific deletion occurred by reverse transcription of spliced viral RNA, that a mechanism to regulate the rate of splicing would be required in a virus which replicates by reverse trans-

cription, and that their S strain had a mutation in the regulatory mechanism. While the exact location of the deletion that occurs in pVR1249 has not yet been located and the sequences around the deletion site have not been examined, pVR1249, like the CaMV-S strain, contains a deletion of 800 to 900 bp between nts. 124 and 1514.

Junction 1

Some junctions in pIC141, pIC143,and pIC148 found by restriction mapping (Choe et al., 1985) occurred between nts. 7228 and 14 (J1 in Figure 3). Nucleotide sequencing further resolved these junctions as occurring between nts. 8022 and 19 (Melcher, unpublished). The distance between these nucleotides is 22 bp, with gap 1 occurring between nts. 8024 and 1. For each recombinant, nucleotides like Cabb S were detected up to nt. 8022, then nucleotides like Cabb B-JI were detected from nt. 19. Restriction mapping of three of six of this study's new recombinants (pVR246, pVR244-A, and pVR244-B) revealed similar junctions occurring between nts. 7724 and 197. Nucleotide sequencing has further resolved the junction for pVR246, as well as revealing the presence of junctions in pVR1249 and pVR1243 (Melcher, unpublished), which had not been revealed by restriction mapping. Nucleotide differences between each recombinants' parental DNAs were detected with the apparent junction again near gap 1.

The presence of nucleotides from one parent and nucleotides from the other parent occurring on each side of gap 1

provides supporting evidence for the model of replication of CaMV by reverse transcription (Pfeifer and Hohn, 1983; Guilley et al., 1983; and Hull and Covey, 1983). In the model, gap 1 arises as a result of the mecha-nisms of reverse transcription (see Figure 2). Synthesis of the DNA minus-strand is initiated when the host cell $tRNA^{met}$ initiator base pairs with the fourteen nucleotides immediately to the right of gap 1 (as depicted in "C" of Figure 2). After reverse transcriptase has copied the 35S RNA to its 5' end, the enzyme switches templates by "jumping" to the 3' end of a 35S RNA and minus-strand synthesis continues. If RNAs from two isolates were used, the resulting DNA would be chimeric with one junction occurring near the position of the 5' end of the 35S RNA (~nt. 7435). Recombinants pVR244-A and pVR244-B contain a junction between nts. 7224 and 197. Sequencing data (Melcher, unpublished) has revealed that their junction does not occur at gap 1. It could be possible that the junction in these recombinants occurs near nt. 7435. For the above recombinants with junctions at gap 1, if no other, or an even number of subsequent template switches occurred, the resulting DNA would be chimeric with a junction also occurring at gap 1.

pVR1243 contains a junction between nts. 7118 and 7224. If this junction arose as a result of template switching, it was most likely due to an "illegal" template switch (described below).

Junction 2

Restriction mapping of recombinants pIC141, pIC143, and pIC148 revealed junctions between the parental alleles in four regions (Choe et al., 1985 and Figure 3). Regions considered to be near junction 2 (J2 of Figure 3) were sequenced to determine the junctions at the nucleotide level. Of the regions sequenced, junction 2 of the recombinants pIC143 and pIC148 was found to be between nts. 2100-2224 and 2224-2233, respectively (see Table 5). Junction 2 of pIC141 was found to be between nts. 2578 and 2623 (Melcher, unpublished). Restriction analysis of new recombinants revealed junctions near the junction 2 region for three of the five recombinants. A junction between parental alleles occurred between nts. 1784 and 2236 in both pVR246 and pVR244-B, while a junction occurred in pVR1243 between nts. 1218 and 2236 (Tables 7 and 9, respectively, and Table 12).

Several possible mechanisms could have produced junction 2 in the above-mentioned recombinants. In the general region encompassed, nts. 1218 through 2623, there could exist a region in the wild-type DNA sequences where recombination preferentially occurs; i.e. a "hot spot" for recombination.

Another possibility for preferential recombination in the junction 2 region could be the result of the mechanisms of reverse transcription. Condit and Meagher (1983) detected several genome-length 35S CaMV RNA transcripts. One minor transcript was characterized as having its 5' end lying with-

Table XII. Distances detected junctions occur from parental mutations.

TABLE XII

DISTANCES DETECTED JUNCTIONS OCCUR FROM PARENTAL MUTATIONS

Recombinant	Junction	Parental DNA	Position of Parental Mutation	Distance Between Junction and Parental Mutation (nts.)
pVR246	1784-2236	pLW76	1382-1802	~1-400
pVR244-B	1784-2236	pLW214	1382-1802	~1-400
pVR246	5943-6045	pUM24	6299-6338	~300
pVR244-A	5541-5943	pUM24	6299-6338	~300-700
pVR244-B	5541-5943	pUM24	6299-6338	~300-700
pVR1243	1218-2236	pUM124	886-1031	~200-1100
pVR1243	7118-7224	pIC23	6317-6664	~500-600

in 50 nts. of the AUG codon for ORF IV (nt. 2201). If template switching were to occur from one parental minor 35S RNA having its 5′ end near the ORF IV AUG codon region onto the other parent's major 35S RNA, the detected junction (J2) would result.

The recombinants pIC141, pIC143, and pIC148 arose from the coinoculation of pUM41 and pIC11. pUM41's mutation is a SmaI linker between nts. 2041 and 2044 (Choe et al., 1985). The altered sequence of the mutant parental allele may have caused preferential recombination in the region 2200 to 2600. Likewise, the sequences resulting from the presence of the CM4-184 421 bp deletion in pLW214 and pLW76, as well as their respective mutations, may have caused preferential recombination in the region 1784 to 2236 in the recombinants pVR244-B and pVR246.

A variation of the above-proposed mechanism of the mutated parental allele causing preferential recombination could support the idea of "illegal" template switching during reverse transcription, as postulated by Grimsley et. al., (1986). In support of a "strong stop DNA" theory, Grimsley et. al., (1986) reported an unpublished observation (of B. Gronenborn) of a DNA sequence that corresponded to within one nucleotide from the 5′ end of the 35S RNA transcript linked to a region further upstream in the genome, and suggested that an "illegal" template switch occurred. The sequences resulting from the mutated parental DNAs used in this study

could have created a sequence similar to the 5′ end of the 35S RNA and effected an "illegal" template switch.

Junction 2 occurs in pVR246 and pVR244-B, which is very near (from 1-400 bp) the position of the 421 bp deletion in the CM4-184-derived parental DNA (Table 12). Junctions were detected near a region of parental mutation in these (Table 12). Similarly, junctions were detected near (~200 to ~1100 bp) regions of much smaller parental mutations in the above recombinants as well as others (see Table 12). It should be noted that the above-mentioned recombinant's junctions (near parental mutations) occurred distal to the parental mutation and gap 1; i.e., the parental mutation was between the junction and gap 1 as depicted in a circular genome (as in Figure 1). This observation provides evidence in support of a recently proposed model (Melcher, unpublished) to be discussed below. These results are also consistent with the proposed mechanism for recombination by DNA repair of mismatches in a heteroduplex region formed during general recombination between duplex DNAs (Choe et al., 1985). In these cases (Table 12), the mismatches could have encompassed large deletions (~400 bp). Heteroduplex loops would have formed at these large mismatches (and the smaller mismatches), and repair of the DNA would have ensued, using either the mutant or wild-type allele as the template. CaMV DNA molecules that resulted from the use of the mutant template would not be detected, as these products would be non-infectious. Therefore, the resulting infectious

recombinants would possess junctions near the regions of parental alleles' mismatches.

The CM4-184 421 bp deletion does not render the virus nor the excised recombinant pLW414 (CM4-184 cloned in the pBR322 vector) non-infectious (Howarth et al., 1981 and Howell et al., 1980). The model for heteroduplex repair considers the use of either mutant or wild-type allele as a potential template in DNA repair (Choe et al., 1985). If so, then the naturally occurring 421 bp deletion of the CM4-184 isolate could theoretically be incorporated into the recombinant's genome and result in an infectious recombinant. Yet no recombinant that arose from a coinoculation with a CM4-184 derived-plasmid contained the CM4-184 421 bp deletion (recombinants pVR246, pVR244-A, and pVR244-B). One possible explanation for this observation could involve the proximity of the CM4-184 deletion to the mutation of the parental allele. pLW76 contains a 433 bp deletion from nts. 777 to 1208. The CM4-184 naturally-occurring deletion extends from nts. 1382 to 1802. Therefore, only 175 bp of the wild-type allele are present between nts. 776 and 1803 that are homologous with the 1026 wild-type nucleotides that are present between those positions in the other parent (pUM24). Likewise, the mutation in pLW214 is at nt. 1285, approximately 100 bp from the CM4-184 deletion. Approximately 50 bp of homology are required for assimilation (Singer et al., 1981 and Gonda and Radding, 1983). It could be possible that the region of homology in pLW76 and pLW214 with the wild-type

allele of pUM24 is too small to effectively allow the cross-over that would be required to incorporate the CM4-184 natural deletion but not the mutation of pLW76 or pUM24. Although these crossovers may happen, they could occur less frequently than the recombinations that utilized the non-mutated allele as the repair template across the entire region of DNA mismatch.

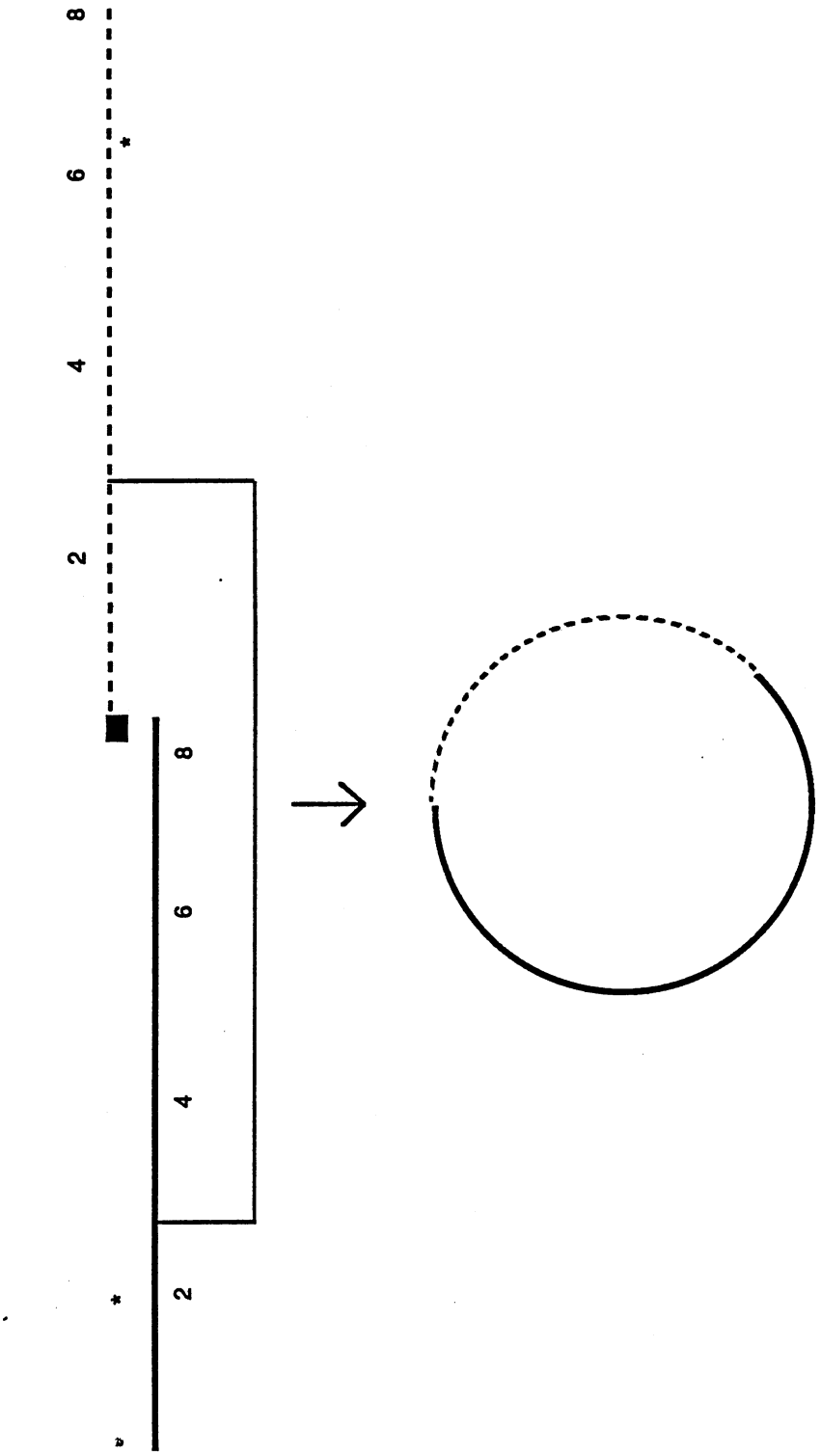
Infectious recombinants have been reported arising from the coinoculations of mutant parental DNAs with complementary sticky ends via two possible mechanisms (Walden and Howell, 1982 and Geldreich, 1986). One possible mechanism (Walden and Howell, 1982) would produce infectious recombinants by the formation of dimers (or concatamers) using complementary sticky ends which could be resolved into normal genomes by a single crossover event. The other mechanism produces infectious recombinants by dimerization of two heterologous CaMV DNA molecules, and subsequent transcription into a hybrid 35S RNA, which would be responsible for the replication of the recombinant genomes (Geldreich et al., 1986). In either situation, a junction between the parental DNAs would be detected occurring at the site of dimerization. All the coinoculations that produced new recombinants in this study were freed from the pBR322 vector DNA at the SalI site, except the combination of pUM124 and pIC23, which were cleaved at the SalI site and XhoI site, respectively. No recombinants obtained from the coinoculations utilizing only SalI sites revealed junctions occurring near the SalI site

(nt. 4836). A junction did occur between nts. 4149 and 4660 in pVR246 (see Table 7), but from nt. 4660 to 5943, only Cabb S-derived sites were detected.

Choe et al., (1985), reported the detection of allele interspersions near junction 2 of pIC141 and junction 4 of pIC143 (Figure 3). Junction 2's interspersion in pIC141 had been resolved as the presence of a HaeIII site, which was thought to be a Cabb S-unique site. However, sequencing data (Melcher, unpublished) has shown that the HaeIII site also exists in W DNA, and is therefore not different between the parents. Sequencing of the recombinants pIC143 and pIC148, as well as pIC141, also revealed the presence of the HaeIII site at nt. 2203. Therefore, no allele interspersions occurred at nt. 2203.

A model recently proposed (Melcher, unpublished) could account for the junctions detected at gap 1 and also for the junctions detected near the parental mutations. In this model (see Figure 33), reverse transcription produces the DNA minus-strand off of the 35S RNA template and the plus-strand is subsequently synthesized, as predicted by the model for reverse transcription (see pp. 7-11, and Figure 2). Instead of base pairing with the 14 $tRNA^{met}$ nucleotides newly-

Figure 33. Proposed model that accounts for junctions near gap 1 and mutations in parental DNAs. (Melcher, unpublished.)


8
6
4
2
8
6
4
2

synthesized in the DNA minus-strand (which would normally cirularize the molecule), the complementary sequences in the DNA plus-strand would base pair with the $tRNA^{met}$ nucleotides in another CaMV molecule, creating a DNA dimer molecule. At this point, intramolecular recombination, occurring so as not to incorporate the regions of mismatches due to mutations, could produce an infectious recombinant CaMV DNA molecule.

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VITA

Valerie Ray Vaden

Candidate for the Degree of

Master of Science

Thesis: PARENTAL JUNCTIONS IN RECOMBINANT CAULIFLOWER MOSAIC VIRUS DNAS

Major Field: Biochemistry

Biographical:

Personal Data: Born in Atoka, Oklahoma, July 15, 1961, the daughter of Thomas R. and Elma L. Ray. Married to David W. Vaden, April 24, 1982.

Education: Graduated from Atoka High School, Atoka, Oklahoma, in May, 1979; received Bachelor of Science Degree in Microbiology from Oklahoma State University in May, 1984; completed requirements for the Master of Science degree at Oklahoma State University in July, 1987.

Professional Experience: Research Technician, Department of Biochemistry, Oklahoma State University, September, 1986 to July, 1987; Graduate Research Assistant, January, 1985 to August, 1986.

Professional Organizations:
International Society for Plant Molecular Biologists
Phi Lambda Upsilon, Honorary Chemical Society
Phi Kappa Phi, National Honor Society