CONSTRUCTION AND CHARACTERIZATION OF DELETION AND INSERTION MUTANTS IN REGION VI OF CAULIFLOWER MOSAIC VIRUS DNA

By

IN SEONG CHOE

Bachelor of Science
Seoul National University
Seoul, Korea
1970

Master of Science in Agriculture
Seoul National University
Seoul, Korea
1978

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
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Thesis Approved:

Ulrich K. Melcher
Thesis Adviser

Margaret K. Essenberg

Richard C. Essenberg

Charles O. Gardner

John A. Bantle

Norman N. Durham
Dean of the Graduate College

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And finally, I wish to dedicate this thesis to my mother and elder sister whose love and encouragement have made it all possible.

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NOMENCLATURE

A	-	absorbance
amp^r	-	ampicillin resistant
ATP	-	adenosine triphosphate
bp	-	base pair
BSA	-	bovine serum albumin
CaMV	-	cauliflower mosaic virus
cpm	-	counts per minute
d	-	dalton
DNA	-	deoxyribonucleic acid
DTT	-	dithiothreitol
EDTA	-	ethylenediamine tetraacetic acid
M.W.	-	molecular weight
kd	-	kilodalton
LSH	-	leaf skeleton hybridization
RNA	-	ribonucleic acid
rpm	-	revolutions per minute
SDS	-	sodium dodecyl sulphate
tet^r	-	tetracycline resistant

CHAPTER I

INTRODUCTION

Cauliflower mosaic virus (CaMV) is the best studied of the group of double-stranded DNA-containing plant viruses, the caulimoviruses. CaMV provides a very useful model system for studying various aspects of DNA metabolism in plant cells. They can be easily manipulated *in vitro*, and can be easily gotten into plant cells, and later identified and isolated from them. Because the genetic material of CaMV is DNA, it is an attractive candidate for use as a vector in putting foreign genes in plants. The desire to create new and better crop species that can produce higher yields, are resistant to pathogens, and are of greater nutritional value, has long been a goal of plant breeders and scientists. Creating a plant vector is one possible way of accomplishing this goal.

However, there is not enough known about CaMV, its replication, transcription, translation and transfer in plants, to be able to use it intelligently yet. Much effort has been made to find out about the functions of the various parts of CaMV genome by studying infection by mutant DNAs formed in vitro. It is also known that mutant CaMV DNA genomes interact with other CaMV mutant or wild type genomes when coinoculated on leaves of plants. An understanding of this gene interaction mechanism is important to an understanding of mobile genetic elements in plants and to the design of successful gene

introduction strategies. Since CaMV DNA is infectious when excised from bacterial plasmids containing the entire CaMV genome, and it is fairly simple to make site specific mutations in the cloned DNA, CaMV is well suited to a study of the processes of genome interaction in plant cells.

Biological Properties

CaMV is classified within the taxonomic group, caulimovirus, which is the only group of plant viruses containing double-stranded DNA as their genetic material. Other less well studied members of this group include dahlia mosaic virus (DaMV), carnation etched ring virus (CERV), mirabilis mosaic virus and strawberry vein banding virus. The caulimovirus group contains these five well-established members plus two to three other viruses as tentative members (Harrison et al., 1971). Like the other caulimoviruses, CaMV was thought to have a relatively limited host range. In nature, it infects only members of the Cruciferae, which includes cauliflower (Brassica oleracea L.), turnip (Brassica rapa L.), mustard (Brassica campestris L.), and radish (Raphanus sativus L.) (Tompkins, 1937; Broadbent, 1957). It has been shown experimentally that some strains of CaMV can infect Nicotiana clevelandii (Hills and Campbell, 1968) or Datura stramonium (Lung and Pirone, 1973), both are members of Solanaceae. But there is some evidence that the host range of CaMV is not as limited as previously thought. It was demonstrated by leaf skeleton hybridization (LSH) that CaMV is capable of replicating in inoculated leaves of a wide variety of angiosperms (Melcher, unpublished data). It is important to note that in these plants the virus

is non-pathogenic, a desired property in a genetic engineering vector.

Disease symptoms exhibited by plants infected with CaMV usually resemble a mosaic-mottle type of disease which may be accompanied by vein clearing, vein banding, wrinkling of leaves, chlorotic local lesions or stunting (Broadbent, 1957; Shepherd, 1970). In turnips this disease is characterized by stunted leaves, chlorosis, and vein banding.

In nature the disease is spread from plant to plant by aphids. The mechanism of aphid transmission is considered to be stylet-born and nonpersistent (Shepherd, 1976). Aphid transmission of CaMV requires a helper component or an aphid acquisition factor present only in leaves infected with an aphid-transmissible isolate of the virus (Lung and Pirone, 1974). It was suggested that the putative protein-coding region II of CaMV DNA codes for a helper component polypeptide of 18,000 daltons required for aphid transmission (Armour, 1983; Woolston, 1983; Daubert et al., 1983). CaMV may also be transmitted by the mechanical inoculation of the virus or its DNA directly onto a plant but not through the seed of the infected plants (Shepherd et al., 1968).

Structure of CaMV

CaMV is an icosahedral virus of 50 nm in diameter consisting of 83% protein and 17% DNA with traces of RNA (Hull and Shepherd, 1977) and carbohydrate (DuPlessis and Smith, 1981). It appears to reside primarily in the granular matrix of the inclusion bodies of infected cells. Inclusion bodies are elliptical masses of electron-dense

material (mainly protein) that are produced within the cytoplasm of infected cells (Fujisawa, 1967). The particles of CaMV are penetrated by potassium phosphotungstate and appear empty with a diameter of 50 nm and a hollow center of 20 nm diameter. The virions of the CaMV appear to be devoid of any readily discernible external substructure. A variety of observations on the behavior of the viruses in solution suggests the exterior of the virion is very hydrophobic, a property that may account for their recalcitrant behavior to degradation. These features imply that the virions may have some unique structural properties. Using the Svedberg equation and values for the sedimentation and diffusion coefficients the molecular weight of the CaMV virion was calculated to be 22.8×10^6. The virions of CaMV have a buoyant density of 1.37 g/ml in cesium chloride (Shepherd, 1976).

Structure of CaMV DNA

The DNA of CaMV is double stranded and has a molecular weight of $4.5\text{-}5.0 \times 10^6$ daltons (d) (Shepherd et al., 1971; Russell et al., 1971). DNA isolated from virions consisted of two types; a nicked circular form and a linear form. The circular form makes up 80-90% of the virion DNA and is infectious whereas the linear form is not (Russell, 1971; Shepherd and Wakeman, 1971; Hull and Shepherd, 1977). It has been proposed that the linear form arises by accidental breakage of the circular form (Hull and Shepherd, 1977). Under the electron microscope, the circular form has a twisted or knotted appearance (Menissier et al., 1982) but is not supercoiled (Shepherd et al., 1971; Shepherd, 1970). However, the presence of a supercoiled

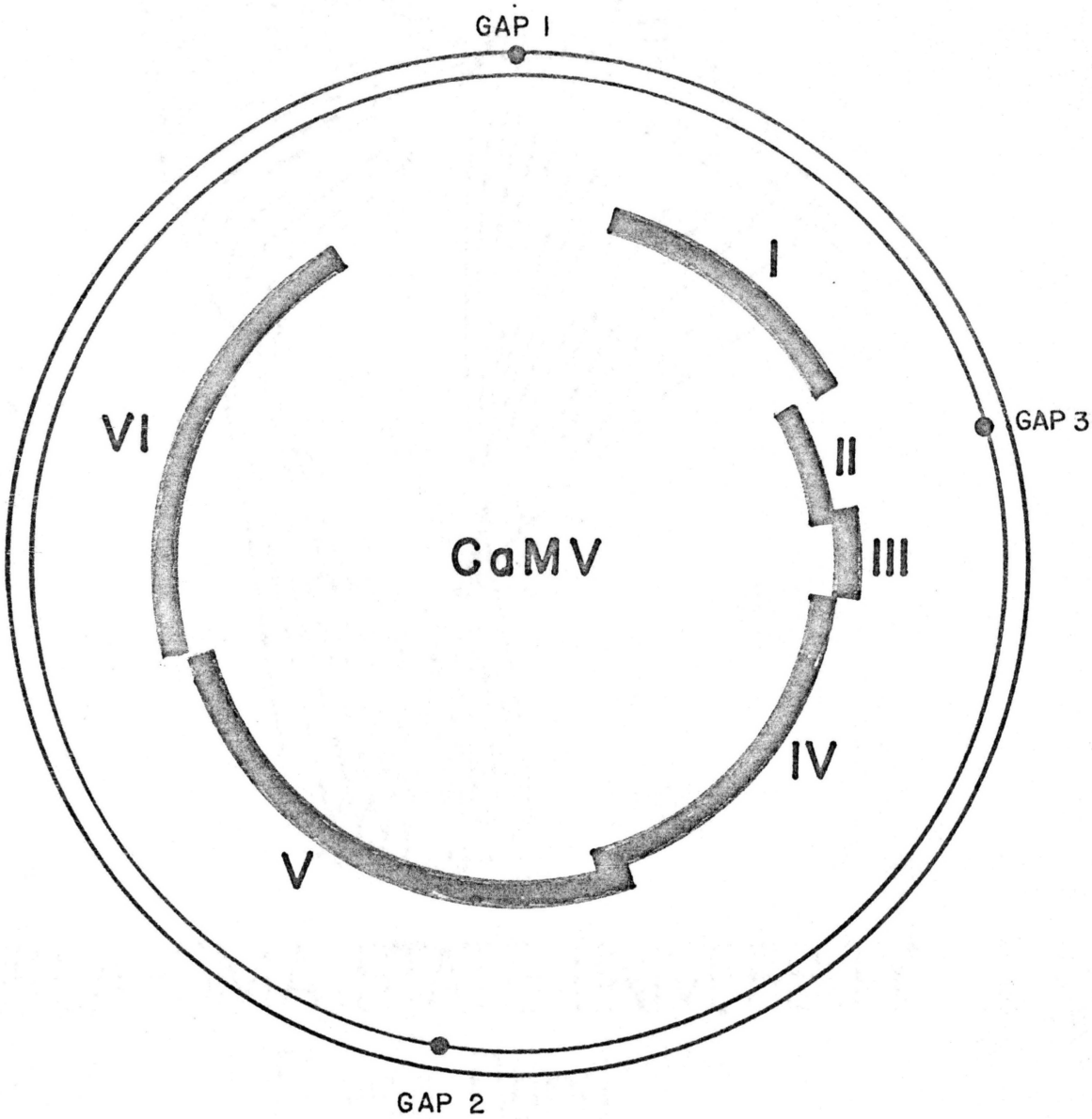
form that exists free in the nucleus of infected cells was reported (Menissier et al., 1982).

It has been shown that there are two or three single stranded discontinuities or gaps within the circular DNA. The gaps are at fixed sites within the DNA; two in the β strand and one in the α strand (Volovitch et al., 1978; Hull, 1980). Thus neither strand of DNA is covalently closed and this presumably explains the inability of the DNA to form a supercoiled configuration. The gaps are actually sites of single stranded sequence overlap (Franck et al., 1980; Richards et al., 1981). The function of the gaps is unknown but they may play some role in viral replication or transcription.

The complete nucleotide sequences of three isolates of CaMV Cabb-BS (Franck et al., 1980), CM1841 (Gardner et al., 1981) and Cabb-D/H (Balazs et al., 1982) have been determined. The sequences of these are about 8,000 base pairs long and each has at least six major open reading frames for protein synthesis in the α strand (Franck et al., 1980; Gardner et al., 1981; Balazs et al., 1982). Hohn et al. (1981) have shown that the Cabb-BS sequence contains two additional open reading frames, termed VII (96 triplets) and VIII (107 triplets), in a different reading frame. These are also present in Cabb-D/H and CM1841 sequences (Balazs et al., 1982). There are no open reading frames of more than 370 nucleotides in the corresponding β strand lending support to other evidence that only the α strand is transcribed (Howell and Hull, 1978; Guilfoyle, 1980). The six major open reading frames are designated regions I through VI (Figure 1) and are in different reading frames. Five of these (regions I to V) are organized with overlapping start and stop codons. There are two

Figure 1. Physical Map of CaMV Genome.

Inner segments give the positions of six open reading frames in the three phases (Franck et al., 1980). The outer circle gives the position of three gaps.


GAP 1
GAP 3
GAP 2
I
II
III
IV
V
VI
CaMV

intergenic regions which have no coding capacity. The large intergenic region is between regions VI and I and contains the only gap in the α strand (Franck et al., 1980; Gardner et al., 1981; Balzs et al., 1982).

RNAs and Proteins Coded by CaMV DNA

From infected leaf tissue it has been possible to isolate two polyadenylated RNA transcripts of the CaMV genome (Guilley et al., 1982; Dudley et al., 1982; Covey et al., 1981; Covey and Hull, 1981). One is a large transcript, 35S RNA, covering the entire genome. This is a transcript initiated at 7435 of the Cabb-BS DNA sequence and terminated at nucleotide 7615 (Guilley, 1982). This RNA has a terminal direct repeat of 180 base pairs. The other transcript, 19S RNA, has 5' terminus at nucleotide 5764 and is 3'-coterminal with 35S RNA at nucleotide 7615 (Guilley, 1982). Neither RNA appears to be a spliced transcript. One 8S RNA and four or five minor 35S RNAs present in small quantities have been reported (Condit, 1983; Guilley, 1982).

The 19S RNA codes for a 61 kilodalton (kd) protein and corresponds to region VI on the genetic map (Odell and Howell, 1980; Odell et al., 1981; Covey and Hull, 1981; Covey et al., 1981). The polypeptide of region VI is the major polypeptide of the inclusion body structures found inside infected cells (Xiong et al., 1982; Al Ani et al., 1980; Shockey et al., 1980). This protein has been convincingly demonstrated to be synthesized in vitro in response to polyadenylated CaMV-coded RNA extracted from infected tissue (Odell and Howell, 1980). Based on the comparison of the nucleotide sequence

with the amino acid composition of the viral coat protein, it is believed that only region IV could code for a precursor to the coat protein (Hahn and Shepherd, 1982; Franck et al., 1980). It was also reported that bacterial plasmids containing this region, but not those lacking it, cause the synthesis of protein in bacteria that is antigenically related to the capsid protein (Daubert et al., 1982). Toh et al. (1983) suggested that region V might code for a reverse transcriptase required in DNA replication since the putative region V polypeptide it codes for shows considerable amino acid sequence homology with retroviral reverse transcriptase. The region II polypeptide is thought to be the helper component for aphid transmission having molecular weight of 18,000 kilodaltons (Armour et al., 1983; Woolston et al, 1983). The functions of region I and III polypeptides are unknown. All viral transcripts are produced by RNA polymerase II and become polyadenylated and capped (Guilfoyle, 1980). 35S RNA is not translated in vitro, in contrast to the 19S transcripts.

It has been suggested that CaMV DNA is replicated via an RNA intermediate (Hull and Covey, 1983; Varmus, 1982). A model for CaMV DNA replication according to which the major transcript, 35S RNA, is reverse transcribed has been proposed (Pfeiffer and Hohn, 1983). Turner et al. (1984) found that a small minus-stranded DNA molecule is covalently linked to a 75 nucleotide RNA species. This RNA has a m^7G residue which is similarly located to a m^7G residue in $tRNA_i^{met}$ of both bean and wheat germ. The 5' end of the small DNA maps close to the gap 1 which itself is immediately adjacent to the 14 nucleotide CaMV DNA sequence homologous to the 3' end of plant $tRNA_i^{met}$ (Covey et al., 1983; Guilley et al., 1983; Hull and Covey, 1983; Pfeiffer and Hohn,

1983). This tRNA is thought to act as a primer for the first DNA strand synthesis by binding to a complementary sequence. Because of the terminal redundancy of 35S RNA, reverse transcription is thought to jump from the 5' end of the 35S RNA to the 3' end. Plus strand DNA synthesis starts predominantly at two major plus primer binding sites (gap 2 and gap 3). Intermediates consistent with this mechanism have been identified and characterized (Guilley et al., 1983; Hull and Covey, 1983; Covey et al., 1983). Various DNA polymerases isolated from uninfected plants have been characterized on the basis of their template specificity. One of these enzymes, DNA polymerase A, accepts RNA templates and is suggested to be the possible candidate to perform the synthesis of CaMV DNA (Pfeiffer and Hohn, 1983).

Potential as Plant Vector

CaMV has several features that make it an attractive genetic vector. It has a double stranded DNA genome which allows direct insertion of foreign genes. It has a small size which makes it easy to manipulate. It has several unique restriction enzyme sites for cloning and the capacity to spread systemically throughout the plant. CaMV has been cloned into a variety of bacterial plasmids and bacteriophages via one of its unique restriction enzyme sites (Howarth et al., 1981; Howell et al., 1980; Hohn et al., 1980; Szeto et al., 1977). In the cloned state, the gaps in the DNA are sealed and the recombinant molecules become super-coiled. The cloned CaMV DNA is not infectious unless released from its vector (Howell et al., 1980). Once released, the linear DNA is religated in vivo and the gaps

regenerated in the progeny virus (Howell et al., 1980; Lebeurier et al., 1980).

A series of insertions and deletions has been introduced into the various translational reading frames of several isolates of CaMV DNA (Dixon et al., 1983; Daubert et al., 1983; Melcher, unpublished data). Some insertions in the large intergenic region did not interfere with viral infectivity, while insertions within region II and in the amino-distal portion of region IV retarded the development of viral symptoms. Insertions in the amino-distal portion of region VI reduced the severity of symptoms in plants. All other insertion mutants analyzed were lethal. Region II has been found to be non-essential for infectivity, since it is almost entirely deleted in a naturally occurring mutant (Howarth et al., 1981; Hull and Howell, 1978) and insertions of up to 256 bp in this region did not abolish infectivity (Gronenborn et al., 1981). Thus, it appears that the intergenic region and region II can be modified to some extent and preserve infectivity. Regions I, III, IV, V and VI appear to be necessary to yield a symptomatic systemic infection. Preliminary evidence indicates that mutation in region III can be rescued by the wild type DNA coinoculated into leaves (Lebeurier et al., 1982; Melcher, unpublished data).

Genome Interaction

Mutant cauliflower mosaic virus DNA genomes interact with other CaMV mutant or wild type genomes when coinoculated on leaves of plants. Several observations support this conclusion. Interference is defined as the suppression of symptom induction by a virus or viral

DNA when that virus or DNA is inoculated together with another virus or viral DNA. Such interference has been observed between CaMV mutants and CaMV wild type DNA (Melcher, unpublished data). Recombinational rescue was observed when plants are inoculated with a mixture of two mutant DNAs whose mutations lie in non-overlapping regions of the DNA. Sometimes diseased plants result and the DNA recovered from virions from the diseased plants was shown to be wild type DNA. This DNA is thought to result from recombination between the two mutant DNAs (Howell et al., 1981; Lebeurier et al., 1982). Recombinational rescue makes the observation of complementation difficult. Despite this there are two instances of possible complementation. When plants were coinoculated with a deletion mutant in gene III and wild type DNA, some mutant DNA molecules were recovered from the diseased plants (Lebeurier et al., 1982). Using the smaller size of a Bgl II fragment in the mutated DNAs, it has been confirmed that the deleted DNA is indeed rescued by wild type DNA in some (1 of 3) plants (Melcher, unpublished data). Several hypotheses to explain CaMV genome interactions, all involving crossing-over events, have been proposed by Howell and colleagues (Walden and Howell, 1982; 1983; Lebeurier et al., 1982). Interaction between Region VI mutants constructed in this study and other mutants was also observed.

CHAPTER II

MATERIALS AND METHODS

Viral DNA

The plasmid, pLW303X is the kind gift from Dr. Howell. DNA from CaMV strain CM1841 which has been inserted into the Escherichia coli (E. coli) plasmid, pACYC177 via their unique XhoI restriction enzyme sites, was used as the starting material (Walden et al., 1982). The resulting plasmid, pLW303X transformed E. coli K-12 strain HB101. Because XhoI digestion of pACYC177 interrupts the kanamycin resistance gene of this plasmid but not the ampicillin resistance gene, bacteria containing pLW303X are kanamycin sensitive (kan^s) but remain ampicillin resistant (amp^r). The viral DNA in pLW303X is not infectious unless released from the plasmid vector by XhoI cleavage (Howell et al., 1980). DNA from CaMV strain CM4-184 had also been isolated from inoculated turnip leaves and was used after nick translation as probe DNA.

Plasmid DNA

pBR322 (Bolivar et al., 1977) was used as the source of the tetracycline resistance gene. pBR322 is a relaxed replicating plasmid, that does not produce and is sensitive to colicin El. It is an E. coli plasmid cloning vehicle. The molecule is a double stranded DNA circle and 4363 base pairs (bp) in length. pBR322 was constructed in

vitro and comprises the tetracyclin resistance determinant (Tc) from pSC101, the origin of DNA replication (Ori) from the colE1 derivative pBM1, and the ampicillin resistance determinant (Ap) from transposon Tn 3. The antibiotic resistance genes on pBR322 are not transposable (Sutcliffe, 1978; Bolivar et al., 1977).

Virus Isolation

CaMV was isolated according to the method of Hull et al. (1976). Infected leaves of turnip (Brassica rapa L. cv. Just Right) six weeks after inoculation were chilled and then blended at $4^{o}C$ in 0.5 M potassium phosphate buffer, pH 7.2, containing 0.75% sodium sulphite (1.5 ml/g leaf tissue). After filtration through cheese cloth, Triton X-100 and urea were added to the sap to give 2.5% and 1 M, respectively. The sap was then stirred overnight. The supernatant fluid from low speed centrifugation (10 min at 5,000 rpm in a Sorvall GSA rotor) was subjected to high speed centrifugation (3 hrs at 18,500 rpm in a Beckman 21 rotor) and the pellets dispersed in distilled water overnight. After low speed centrifugation (10 min at 7,000 rpm in a Sorvall SS-34 rotor) the virus suspension was sedimented in 10 to 40% sucrose gradients in 0.01M potassium phosphate buffer, pH 7.2. The virus containing band was collected using an ISCO density gradient collector. The virus in sucrose was diluted 1:1 with water and pelleted by high speed centrifugation (1 hr at 45,000 rpm in a Beckman 65 rotor). The final pellets were resuspended in sterile water.

Virus Isolation (Rapid Method)

Viral DNA isolation procedure of Gardner et al. (1980) was slightly modified and used in this study. Systemically infected leaves (1.0 - 2.0 g of tissue) were ground to a fine powder in liquid nitrogen using a mortar and pestle and suspended in 10 ml of TEU buffer (0.2M Tris-HCl, pH 7.5, 0.02M EDTA, 1.5M urea). The rest of the procedure of Gardner et al. was followed in this study.

Viral DNA Isolation

Viral DNA was isolated from virus in aqueous suspension according to the procedure of Gardner et al. (1980). CaMV particles were treated with proteinase K (Boehringer Mannheim, 0.5 mg/ml final concentration) in the presence of 1% SDS at 65^{o}C for 10 min. The viral DNA was purified by extraction with an equal volume of phenol and by precipitating twice with ethanol. DNA was dissolved in 10 mM Tris, 10 mM NaCl, 1 mM EDTA, pH 8.0 buffer.

Total Plant DNA Isolation

Total plant DNA was isolated according to the modified procedures of Heyn et al. (1973) as described in detail by Taylor and Powell (1982). The CsCl-ethidium bromide gradient centrifugation procedure was omitted.

Growth of Bacteria

E. coli K-12 strain HB101 (pLW303X), HB101 (pBR322), or HB101 containing a mutant plasmid were streaked out on nutrient agar plates

(1.0% w/v, tryptone, 0.5% w/v yeast extract, 1.0% w/v NaCl and 2.0% w/v agar; all obtained from Difco) containing 0.015 mg/ml ampicillin or 0.010 mg/ml tetracycline (Sigma Chemical Co.). Sometimes HB101 or HB101 (pBR322) were streaked out as control on the same plates. Plates were incubated at 37°C in a warm air incubator overnight.

Tubes containing 5 ml of nutrient broth (1.0% w/v tryptone, 0.5% w/v yeast extract and 1.0% w/v NaCl) were inoculated with single colonies obtained from the appropriate antibiotic nutrient agar plates and the cultures were grown overnight at 37°C. Flasks containing 250-500 ml of nutrient broth were inoculated with the 5 ml cultures. Flasks were placed in a 37°C gyrorotary shaker at 200-250 rpm. Cultures were grown overnight.

Plasmid Isolation

The procedure of Ish-Horowicz et al. (1981) was modified and used to isolate plasmid from bacterial culture. Plasmid containing E. coli cells are treated with lysozyme to weaken the cell wall. Cells harvested from 250 ml of a saturated culture were treated with 10 ml 50 mM glucose, 28 mM Tris pH 8.0, 10 mM EDTA, 0.5 mg/ml lysozyme (Sigma). All other procedures were the same as that of Ish-Horowicz et al. (1981).

Plasmid DNA Purification

Supercoiled plasmid DNA was purified according to Radloff et al. (1967). 3.3 g of cesium chloride (Var Lac Oid Chem. Co. Inc.) was measured into a ½ in. x 2 in. polyallomer centrifuge tube. Plasmid DNA solution from the plasmid isolation and water to total 3.0 ml were

added. Tubes were inverted several times to mix and dissolve the cesium chloride (CsCl). The density of this solution was calculated to be about 1.59 g/ml. 0.3 ml of ethidium bromide (10 mg/ml, Calbiochem Inc.) was added in the dark and tubes were again inverted. Paraffin oil was layered on to within 1-2 mm of the top of the tubes and was used to balance them. Tubes were placed in a Beckman SW 50.1 or SW 65 rotor and centrifuged for about 48 hours at 34,000 rpm, 20°C, in a Sorvall OTD-50 ultracentrifuge or a Beckman L5-65 ultracentrifuge.

The tubes were then removed from the rotor and illuminated in the dark with a long wave ultraviolet light (Blak-Ray UVL 22, Ultra Violet Product Inc.) to locate the band of plasmid DNA. The band was removed by piercing the tube with an 18G syringe needle and withdrawing the band into the barrel of the syringe. Ethidium bromide was removed by extracting ten times with an equal volume of 1-butanol saturated with 5M NaCl, 10 mM Tris-HCl, pH 8.6 and 1 mM EDTA. Two volumes of water were added followed by six volumes of 95% ethanol. The plasmid DNA precipitated at -20°C overnight. The DNA was pelleted at 17,140 x g for 10-15 minutes and the supernatant was discarded. The DNA was dried under vacuum and redissolved in 100 µl of DNA dissolving buffer (10 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA, 10 mM NaCl). Concentration of the plasmid DNA was determined by measuring the absorbance at 260 nm (A_{260}) and assuming that 50 µg/ml of DNA will have an $A_{260} = 1.0$.

Enzyme Reactions

Restriction enzymes, T_4 DNA ligase and double stranded exonuclease, Bal 31, were obtained from Boehringer Mannheim, New England Biolabs,

BRL and P-L Biochemicals and used according to the manufacturers' specifications.

Transformation

Transformation of E. coli K-12 strain HB101 with the mutant plasmid DNAs was performed according to Woods et al. (1980). The ligation reaction mixture containing 0.5 µg of DNA was mixed with two volumes of competent HB101 cells. The bacteria-DNA mixture was subjected to a cold shock treatment in an isopropanol-dry ice bath for 100 seconds, incubated at 42°C for 1 minute, and then at 37°C for 30 minutes in nutrient broth (1.0% w/v bacto-tryptone, 0.5% w/v yeast extract, 1.0% w/v NaCl). The mixture (0.2 ml) was spread on an appropriate antibiotic-nutrient agar plate and incubated overnight at 37°C. HB101 cells were also transformed using 0.01 µg pBR322 DNA as a positive control and with no DNA as a negative control to assure the procedure was working.

Determination of DNA Sequence

Ava I and Bgl II fragments of CaMV DNA were 3'-^{32}P-labelled by fill-in of the 3' recessed extremities with Klenow fragment of DNA polymerase I (BRL). After secondary restriction by Eco RI to separate the labeled extremities, the sequence of the first 70 - 210 nucleotides from 3' labeled terminus was determined by the limited chemical cleavage method of Maxam and Gilbert (1977) as outlined in detail by Franck et al. (1980).

Virus Propagation

Plasmid DNAs used for inoculation were first digested with XhoI or SalI enzymes as described to release the CaMV DNA. pBR322 or pACYC177 vector DNAs were not separated from the CaMV DNA nor was the CaMV DNA religated into a circular form prior to inoculation. The restriction enzyme mixtures were diluted in a solution of 60 mM potassium phosphate buffer, pH 8.5 to a DNA concentration of 10 µg/ml.

The third and fourth leaves of 3 week old plants were inoculated by gently rubbing 50 µl of the diluted DNA solution onto leaves dusted with celite. One or two circular holes were punched in the leaves to facilitate later identification. Inoculated turnip plants (Brassica rapa L. cv. Just Right) were maintained in a growth chamber set at 22°C for a 12 hour day period and at 18°C for a 12 hour night period.

Starch Lesion

Two weeks after inoculation of turnip plants, two leaves were removed from each plant and submerged in 2-methoxyethanol (Pierce Chem. Co.) at 37°C until the leaves lost their green pigment (4-8 hrs.). Leaves were rinsed once or twice in deionized water and potassium triiodide solution (0.06% w/v KI and 0.006% w/v I_2) was added to stain the leaves for detection of starch lesions. Stained leaves were photographed using transillumination.

Leaf Skeleton Hybridization

Starch stained leaves were processed for leaf skeleton

hybridization (LSH) according to Melcher et al. (1981) except that ethidium bromide staining was not done. Starch stained leaves (1-6 leaves) were incubated in 10 ml of 0.1 mM NaN_3, 0.1% (w/v) sodium dodecyl sulfate containing 0.1 mg/ml proteinase K to release DNA from viral particles. Leaves were then treated successively at room temperature for 15 min. each with 1.5 M NaCl, 0.5 M NaOH and then 3.0 M NaCl, 0.5 M Tris-HCl pH 7.0. They were then dipped in 2X SSC (SSC = 0.5 M NaCl, 0.015 M sodium citrate) and hung to air dry, before treatment in a vacuum oven at 80°C for 2 hr. For hybridization, dried leaves were carefully introduced into heat-sealable plastic bags (Seal N Save, Sears) containing 10 ml of hybridization solution (2X SSC, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 0.1% sodium dodecyl sulfate, 1 mg/ml heat denatured, sonicated calf thymus DNA, 5×10^5 cpm ^{32}P-labeled CaMV). The bags were heat sealed and immersed in water at 65°C for 24 hr. Leaves were washed after hybridization in 4 changes of 2X SSC each at 65°C for 10 min. They were then blotted dry and arranged on a piece of filter paper under plastic wrap (Handi Wrap). Autoradiography using Cronex Lightning Plus intensifying screens (Dupont) with XR-film (Kodak) was performed at room temperature for 1 to 3 days.

Systemic Symptoms

Development of systemic viral infection in turnip plants was determined by visual examination of the plants five weeks after inoculation. One or two inoculated leaves were left on each plant for five weeks for this study rather than being removed for starch staining and other experiments. The presence of typical systemic

symptoms such as mottling, crinkling of leaves, chlorotic lesions and stunting was the criterion for a positive result.

Nick Translation

CaMV DNA (0.2 µg) was nick translated in 20 µl 6.6 mM Tris-HCl pH 7.4, 6.6 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl containing 1.0 nmoles each of dATP, dGTP and dTTP and 0.15 nmole of $\alpha[^{32}P]$-dCTP (6 to 30 Ci/mmole; New England Nuclear). Incubation at 15°C was initiated after addition of 80 pg deoxyribonuclease I (Sigma) and 16 pg DNA polymerase I (New England Nuclear). The DNA (0.2 to 1.5 x 10^7 dpm/µg) was phenol extracted and ethanol precipitated with carrier DNA before use in hybridization.

Storage of Bacteria

Selected transformants were restreaked on tetracycline, ampicillin and kanamycin nutrient agar plates. Those colonies that had proper antibiotic resistance were incubated into 5 ml cultures of nutrient broth and grown overnight at 37°C. Cultures were retested for their antibiotic resistance characteristics. To a 1.5 ml cryo-vial, 1.0 ml of culture and 0.15 ml of sterile glycerol were added and the mixture vortexed. Cryo-vials were labeled and stored at -70°C. To obtain bacteria from the cryo-vial for experimental use, the surface of the frozen culture was scratched with a heat sterilized loop and streaked on the desired antibiotic nutrient agar plate.

CHAPTER III

RESULTS

Optimal Conditions for Inoculation of Turnip Plants

Turnip plants can be infected with CaMV by mechanical inoculation with either purified CaMV virions or DNA extracted from CaMV virions (Shepherd et al., 1968). CaMV DNA that has been synthesized by bacteria as part of recombinant plasmid DNA is also infectious if it is first excised from the plasmid vector with the restriction endonuclease that recognized the junction between vector and viral DNA (Howell et al., 1980; Lebeurier et al., 1980). Different conditions have been used by different investigators to inoculate turnip plants with DNA with apparently equal success (Daubert et al., 1983; Waden and Howell, 1983; Lebeurier et al., 1980). In general, conditions similar to those used for inoculation of leaves with CaMV virions have been used. Although successful infection is obtained with these methods, it is possible that other conditions may be more effective in infection of turnip leaves with CaMV DNA.

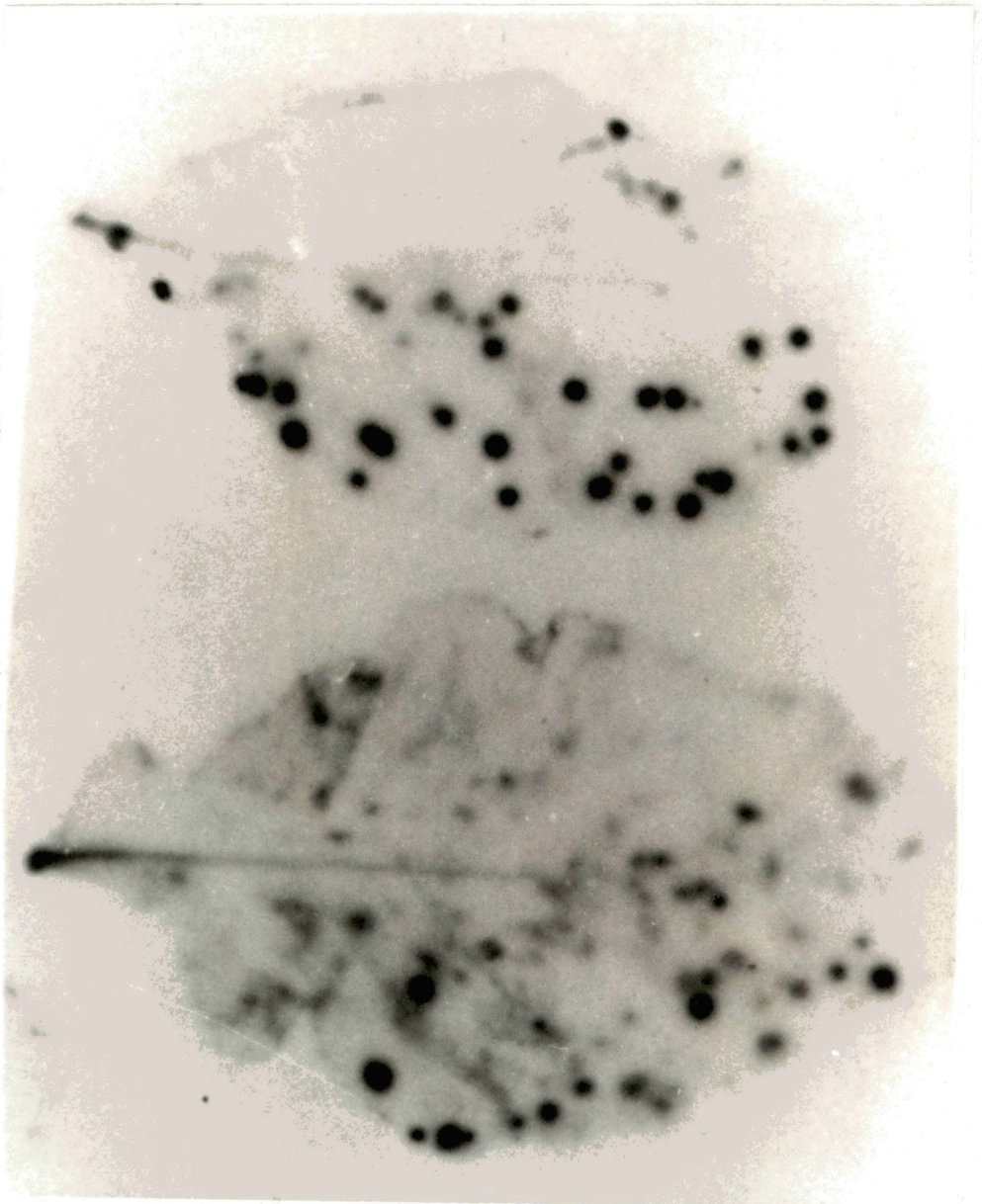
Some isolates of CaMV produce local lesions (Holmes, 1929) on the inoculated leaves of turnip. These lesions have also been detected in inoculated turnip leaves by staining for starch (Figure 2) and by leaf skeleton hybridization (Figure 3; Melcher et al., 1981). Comparisons of the optimum conditions used for inoculation of plants with

Figure 2. Turnip Leaves Stained with Potassium Triiodide for Detection of Starch Lesions.

Leaves were inoculated with CM4-184 CaMV DNA.

Figure 3. Autoradiographic Detection of Lesions Induced by CM4-184 DNA.

Leaves were treated as described in methods to produce leaf skeletons. These were hybridized with ^{32}P labeled DNA of CM4-184, washed and submitted to autoradiography.



RNA extracted from RNA containing plant viruses with those used with virions have been made (Sarkar, 1963; Break, 1962; Bawden and Pirie, 1959; Takahashi, 1956; Kado, 1964). Most variables tested (pH, buffer composition, abrasive used, temperature, dark treatment) affect the efficiency of inoculation with RNA and with virus differently. This suggests that the initial steps in plant RNA virus infection differ depending on whether naked RNA or virions are used for inoculation. Perhaps different mechanisms of uptake by the leaf are involved.

The effect of varying inoculation conditions on the efficiency of inoculation of turnip plants with CaMV DNA was examined. An efficient method of inoculation with DNA will enhance the utility of CaMV in two respects. CaMV has been considered a possible vector for the introduction of foreign genes into plant cells. CaMV can serve as a model for the genetic involvement of viral nucleic acid in the process of infection of a plant by a virus.

Optimization of Inoculation Conditions

When the leaves of three and one half week old turnip plants were inoculated with different concentrations (0.1 - 10 µg DNA/ml inoculum) of CaMV DNA (isolate CM4-184) and the resulting local lesions were assayed either by starch staining or leaf skeleton hybridization, no dependence of lesion number on the concentration of DNA was observed. Only the inocula of DNA concentration higher than 4 µg/ml of DNA solution have been used to infect turnip plants 1 µg of DNA/plant).

The effect of physiological state of the leaves, as reflected in leaf age, on infectivity was examined. When leaves of 3, 4, or 5 weeks

old plants were inoculated with 4 or 10 µg/ml CaMV DNA (50 µl/half leaf), the number of lesions on leaves of plants of various age were similar. The number of lesions per half leaf determined varied from 3 to 11 with an average of 6-8 lesions per half leaf. No significant difference was shown between different age groups.

In inoculating turnip leaves with virus, it had been determined (Melcher, unpublished observation) that carborundum was a more reliable abrasive than celite since leaf damage often resulted when celite was rubbed too vigorously on the leaves. Since the type of abrasive used affects the efficiency of inoculation of tobacco leaves with RNA of RNA containing plant viruses (Yarwood, 1968; Singer et al., 1961), the effect of abrasive on the number of lesions formed on turnip leaves by CaMV DNA was examined. The average number of lesions produced by inoculation with either celite or carborundum was not significantly different. However, each of the nine leaves inoculated using celite showed evidence of infection while this was not true of the carborundum inoculated leaves. Indeed when carborundum was added to celite only five leaves among nine inoculated leaves had detectable lesions. The addition of bentonite to the celite resulted in an increase in the number of lesions per half-leaf but this increase was not significant and was complicated by the observation of lesions on the uninoculated halves of some of the leaves.

The efficiency of infection of tobacco leaves with a TMV RNA inoculum is dependent on the pH of the inoculum buffer (Fraenkel-Conrat, 1957). Varying the pH from 6.5 to 8.5 had no significant effect on the mean lesion number obtained on turnip leaves using a CaMV DNA inoculum. Light deprivation of inoculated tobacco plants (Bawden and

Pirie, 1959) and the addition of sugar to inoculum (Kongsvik and Santilli, 1970) are reported to increase the efficiency of inoculation with RNA. The addition of various sugars to CaMV DNA inocula had no significant effect on the mean lesion number per leaf. Light grown plants were kept in the dark for up to 48 hours after inoculation. When these were later assayed for lesion frequency, it was found that a dark treatment of 36 hours or longer significantly increased the number of lesions (about 40% to 50%).

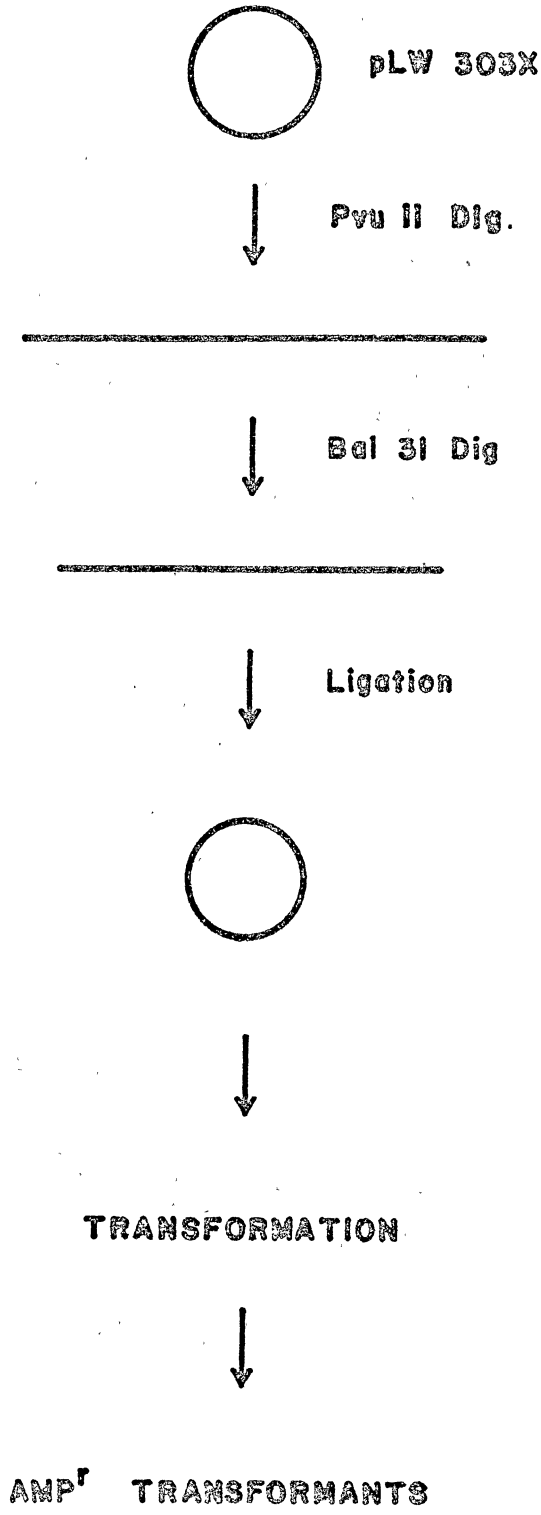
Among the variables tested (pH, age of plants, concentration of DNA in inoculum, abrasive used, light treatment and various sugars added to inocula), only light treatment had a significant effect on the efficiency of inoculation with CaMV DNA.

Unfortunately, the present results indicate that quantitation of local lesions either by starch staining or leaf skeleton hybridization can not be used to quantitate the infectivity of a DNA preparation. Not only is the number of lesions not dependent on DNA concentration over the range tested, but the lesion number is also highly variable and probably reflects more the physiological state of the leaf than the infectivity of the DNA preparation. This made statistical analysis difficult.

Construction of Deletion Mutants

The experimental scheme for construction of deletions in cloned CaMV DNA is shown in Figure 4. Purified pLW303X DNA was digested with the restriction enzyme, PvuII. PvuII cuts once in the pLW303X plasmid producing a linear molecule. Analysis of PvuII digested pLW303X by agarose gel electrophoresis revealed that all detectable

Figure 4. Experimental Scheme for Construction of Deletion Mutants.


pLW 303X
Pvu II Dig.
Bal 31 Dig
Ligation
TRANSFORMATION
AMP^r TRANSFORMANTS

DNA was in the linear form and that the reaction was thus complete. The site of PvuII cleavage is in region VI of the CaMV DNA portion of the plasmid. Therefore, after digesting with PvuII, deletions in region VI can be made by digestion with Bal 31 enzyme.

Mapping of Restriction Enzyme Sites

pLW303X was the kind gift from Dr. Howell. It was claimed that pLW303X had been constructed by inserting CaMV isolate Cabb B-JI into plasmid pACYC177 through their unique XhoI sites (Walden et al., 1982). The sites of various restriction endonucleases were mapped on the DNA of CaMV isolate Cabb B-JI (Hull and Howell, 1978; Hull, 1980) and on the DNA of plasmid pACYC177 (Chang and Cohen, 1978; Walden and Howell, 1983). The sites of several restriction enzymes, AccI, AvaI, BglII, EcoRI, HhaI, HindIII, PvuII, SacI and XhoI, were mapped again in this study. In most experiments 1.0-2.0 µg DNA was digested using 1-5 units of enzyme for 1 hour at 37°C. For double digests the DNA was digested with one enzyme and then, after phenol extraction and ethanol precipitation, was digested with the other enzyme. Restriction fragments were separated by electrophoresis in 1.2-1.4% agarose slab gel and 3.5-8.0% polyacrylamide slab gel. DNA size markers consisted of CM4-184 DNA digested with HindIII (number of base pairs = 3753, 1096, 824, 592, 541, 491, 452, 229 and 222; Gardner et al., 1980). When the restriction patterns obtained were compared with fragment sizes expected for pLW303X, it was found that some restriction patterns did not agree with the published ones (Hull and Howell, 1978; Hull, 1980). One of two PvuII sites reported by Hull at 0.75 map unit was not found in my restriction digests

(Figure 5). pLW303X DNA was digested with HindIII and double digested with HindIII and EcoRI and restriction fragments were electrophoresed in 3.5% polyacrylamide gel. Electrophoretic mobility of each fragment was plotted versus log of the number of base pairs of expected DNA fragment for pLW303X (Figure 6; open circle). Closed circles represented the electrophoretic mobilities of HindIII and HindIII-EcoRI double digested fragments versus log of chain length of expected DNA fragments for putative plasmid consisted of pACYC177 and CaMV isolate CM1841 (Gardner et al., 1981). The positions of HindIII digested CaMV DNA fragments used as size marker molecules were represented by a solid line. The electrophoretic mobilities of the CaMV DNA-HindIII fragments were linearly related to log of their chain length. From Figure 6, it can be proposed that pLW303X consists of pACYC177 and CaMV isolate CM1841 DNA sequences. All other restriction enzyme fragment patterns agreed with those of a putative plasmid consisting of CM1841 and pACYC177 DNAs.

The orientation of the CaMV DNA insert in pACYC177 was determined by comparing the resulting fragments of restriction enzyme digestions with fragment sizes expected for each of the two possible orientations of CaMV DNA relative to pACYC177. A map of five restriction enzyme sites in the DNA of pLW303X was constructed (Figure 7). The DNA sequence of CaMV isolate CM1841 was used to construct the restriction enzyme map.

Strategy

The experimental scheme for construction of deletions in cloned CaMV is shown in Figure 4. pLW303X DNA was linearized by digesting

Figure 5. Gel Electrophoresis of pLW303X DNA Digested with Various Restriction Enzymes.

A) Intact pLW303X DNA, B) Xho I digestion, C) Xho I and Pvu II double digestion, D) Pvu II digestion, E) EcoRI digestion, F) EcoRI and Pvu II double digestion.

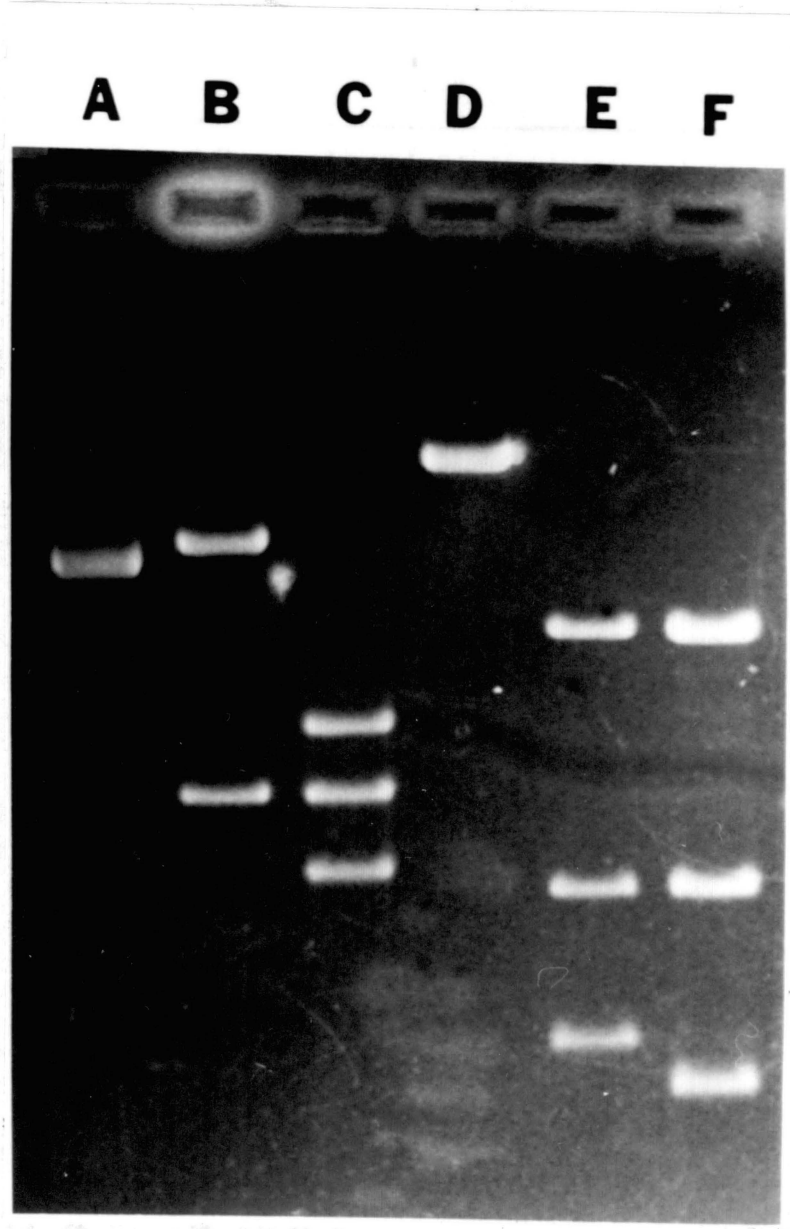

A
B
C
D
E
F

Figure 6. Plot of Electrophoretic Mobility vs. Log of Chain Length for DNA Fragments.

The open circles represent the fragments for pLW303X. DNA fragments for putative plasmid consisted of pACYC177 and CaMV isolate CM1841 were represented by solid circles. The positions of HindIII digested CM4-184 DNA fragments used as size marker molecules were represented by a solid line.

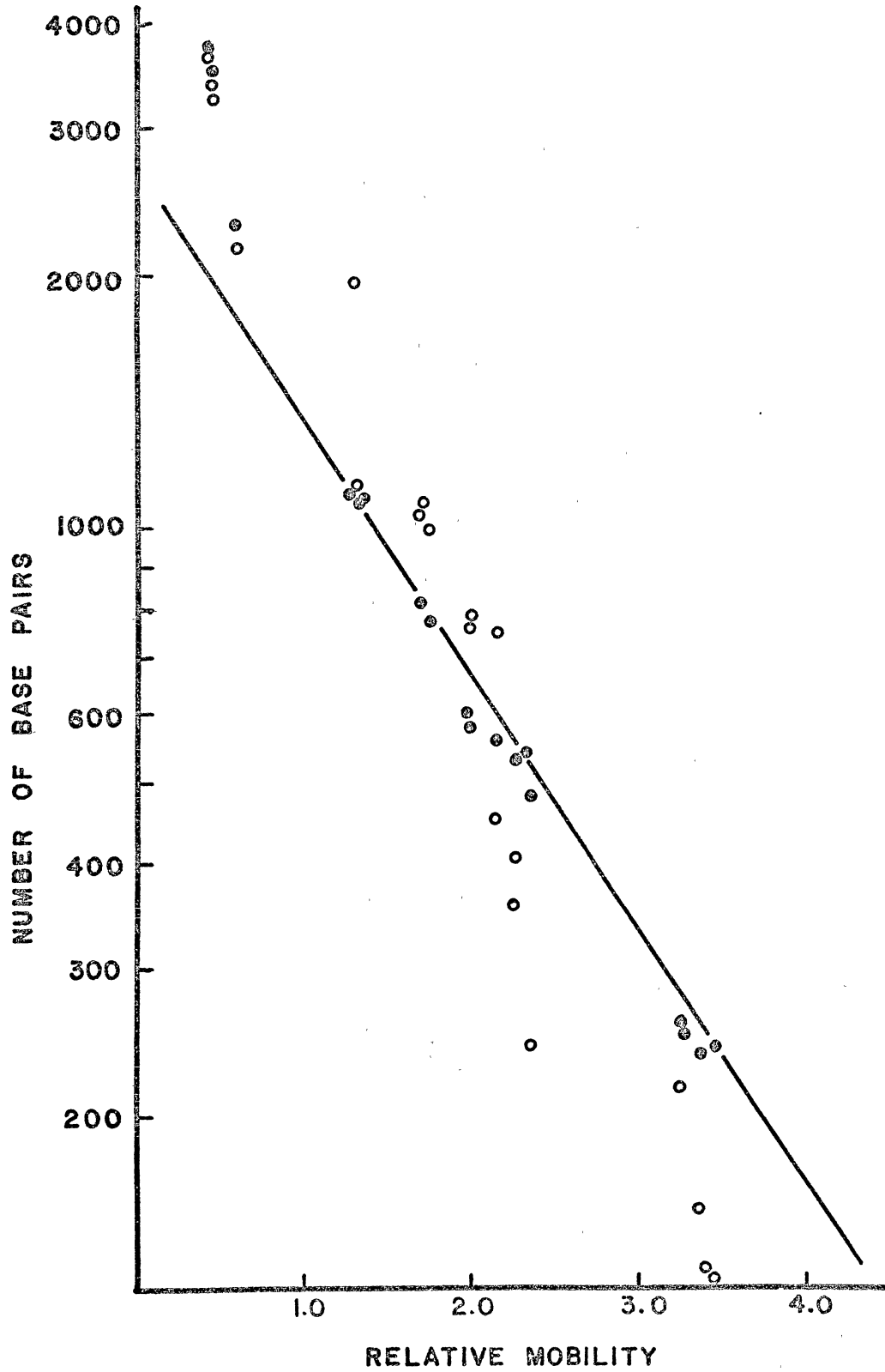
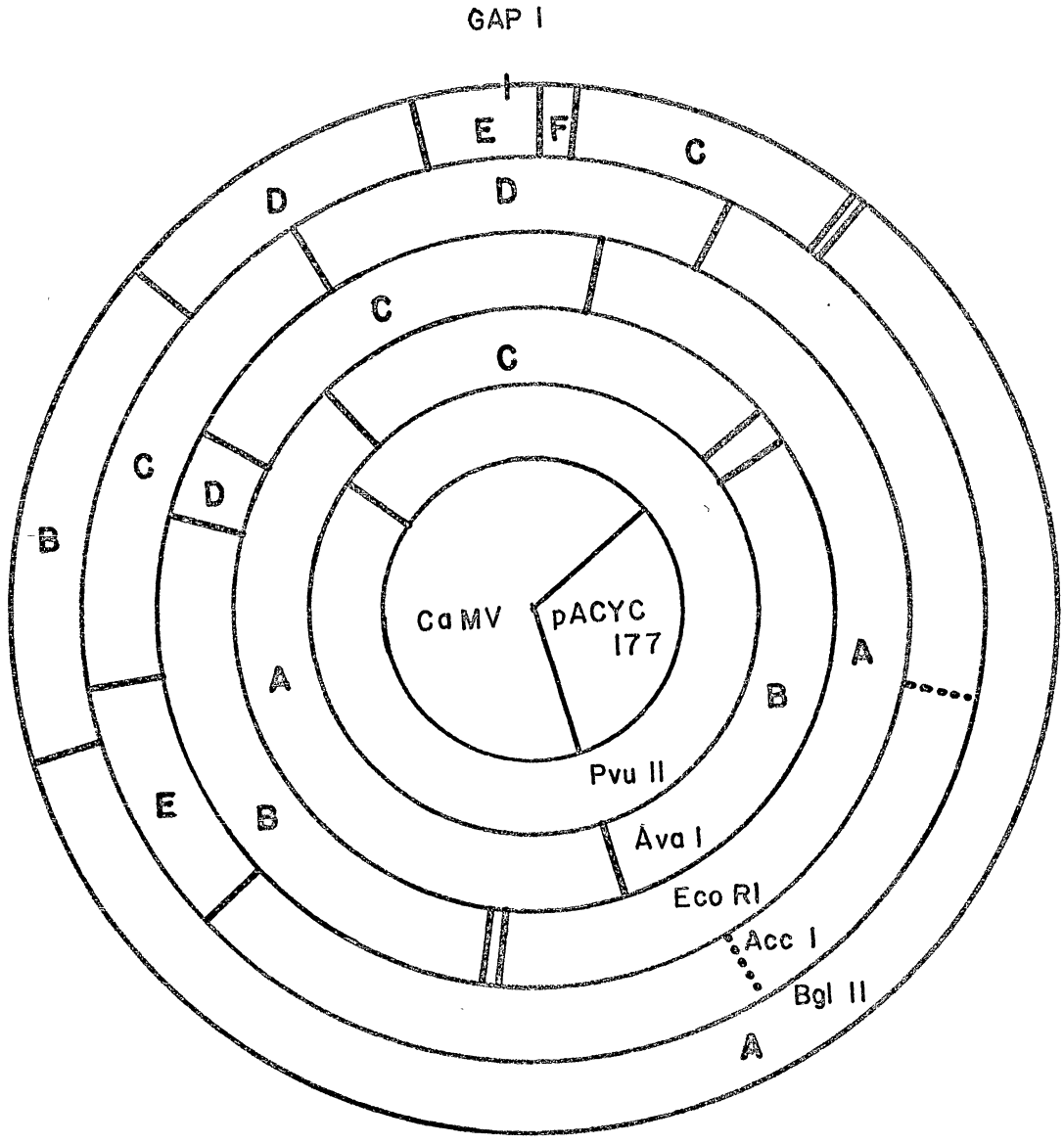

4000
3000
2000
1000
600
400
300
200
NUMBER OF BASE PAIRS
1.0
2.0
3.0
4.0
RELATIVE MOBILITY

Figure 7. Map of pLW303X.

Restriction enzyme sites in CaMV DNA (Gardner et al., 1981; Hull, 1980; Hull and Howell, 1978; this study) and in pACYC177 sequence (Chang and Cohen, 1978; Walden and Howell, 1983; this study) were mapped. Acc I sites shown within dotted lines were not determined clearly. Both positions give identical fragment pattern.


GAP I
E
F
C
D
D
C
C
C
D
B
A
B
E
B
A
B
A
CaMV
pACYC
177
Pvu II
Ava I
Eco RI
Acc I
Bgl II
A

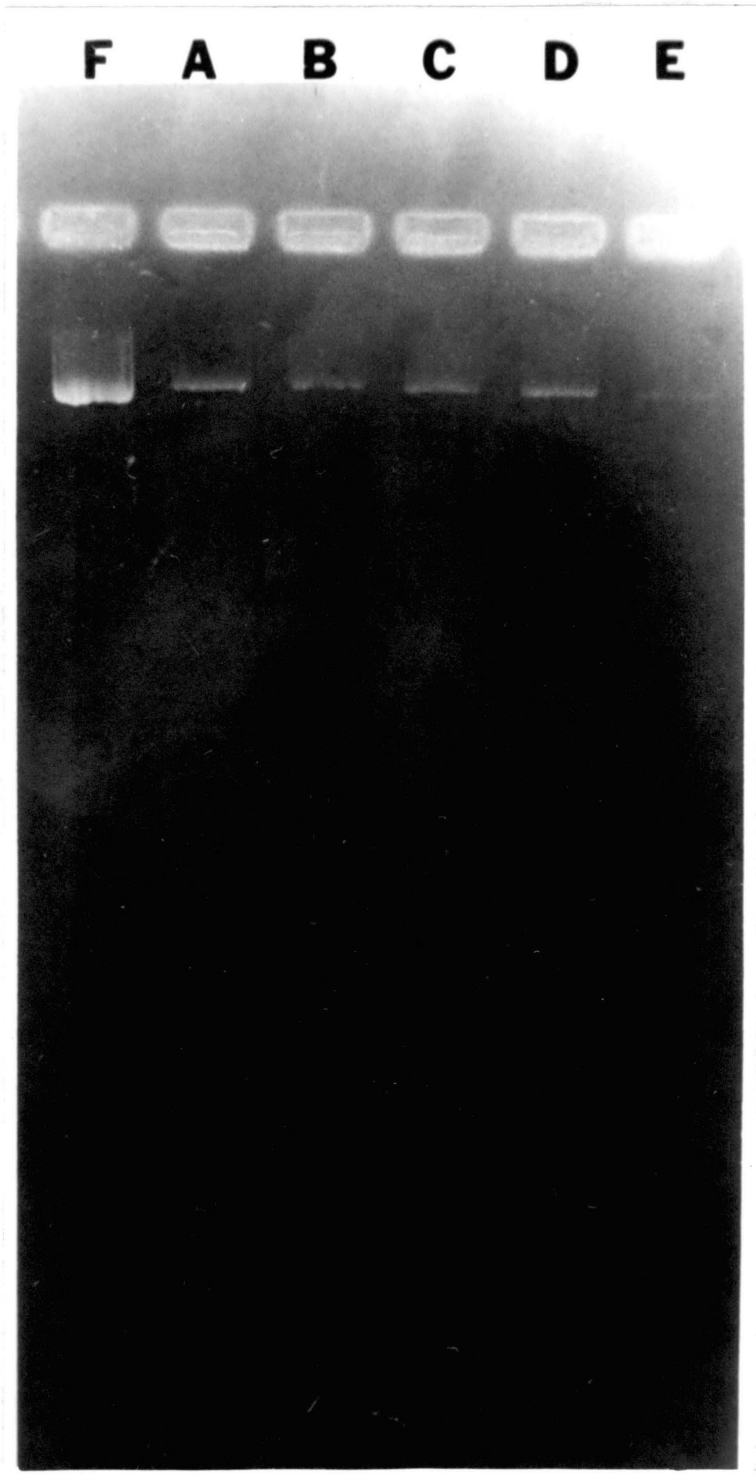
with PvuII and then treated with Bal31 (BRL). When a DNA concentration of 0.02 µg/µl and 1 unit of Bal31 per µg DNA was used for 0.5, 1, 2, 3 and 5 min, gel electrophoresis of reaction mixtures showed a definite decrease in the size of the DNA molecules with time (Figure 8). The rate of Bal31 digestion determined using DNA sequencing was 320-340 bp/min.

Blunt end ligations were done using Bal31 digested DNA at a concentration of 0.007 µg/µl of reaction mixture and 50 units of T_4 DNA ligase per µg of DNA. When analyzed on an 0.8% agarose gel, only DNA molecules in the linear form were seen. Transformation of HB101 with these reaction mixtures was successful. The number of transformants obtained for these reaction mixtures (20 - 30 colonies/0.5 µg of DNA) was small compared to the standard (>300 colonies/0.1 µg of pBR322 DNA). It is possible that the ligation reaction was so inefficient that only a small proportion of DNA was religated and thus could not be detected on the gel.

Single colony lysates were prepared from transformants and analyzed on an 0.8% agarose gel and twenty colonies having plasmids with small deletions were selected. Plasmid DNAs from 5 ml cultures of selected colonies were prepared using alkaline-SDS method and double digested with EcoRI and PvuII enzyme. Four plasmids having a smaller EcoRI C fragment than that of pLW303X and lacking the PvuII site in the EcoRI C fragment were selected for further experiments. The four plasmids were designated pIC11, pIC21, pIC22 and pIC23. The fragmentation patterns of double digestion indicated that most of plasmid DNAs from transformants were wild type. pIC11 was obtained by digesting linear pLW303X molecule with Bal31 for 30 seconds and the

Figure 8. Gel Electrophoresis of Linear pLW303X DNA Treated with Bal 31 Enzyme for Various Times.

A) Standard linear pLW303X, B) 0.5 min, C) 1.0 min, D) 2.0 min, E) 3.0 min, F) 5.0 min.


F
A
B
C
D
E

other three were created by digesting for 60 seconds.

DNA Sequencing

The restriction enzymes, AccI, AvaI, BglII and EcoRI, were chosen to estimate the size of deletion in each of the selected plasmids. These enzymes cleave at several sites in pLW303X and give fragments in a size range that can easily be separated on agarose gels. A restriction enzyme map of these enzyme sites was constructed (Figure 7). Deletions created by Bal31 digestion would be expected to produce an altered restriction enzyme pattern for those fragments flanking the PvuII site. All four plasmids and wild type, pLW303X DNA, were digested with EcoRI and PvuII (Figure 9). EcoRI digested plasmid gave a fragment pattern similar to the control (EcoRI digested pLW303X) except that fragment C was smaller. The fragment pattern of double digestion with EcoRI and PvuII showed no difference from that of EcoRI digestion except that fragment C of wild type became smaller. This indicated that the four deletion mutants lost their PvuII site and the deletion was limited within EcoRI fragment C. The EcoRI C fragment has one AccI site at nucleotide 7040, one AvaI site at nucleotide 6688 and four BglII sites at nucleotides 6492, 7670, 91 and 220 (Figure 7). These restriction enzyme sites were used for further determination of deletion size.

When the four mutant and wild type DNAs were digested with BglII (Figure 10), pIC11 and wild type showed similar fragmentation patterns except fragment B of pIC11 was smaller than that of pLW303X. In the other three mutant plasmids, fragments B and D were fused to give one new large fragment. This result indicated that pIC11 had its deletion

Figure 9. Gel Electrophoresis of EcoRI Digestion and EcoRI-PvuII Double Digestion Products of pLW303X and Mutant DNAs.

A) pLW303X digested with EcoRI, B) pLW303X digested with EcoRI and PvuII, C) pIC11 digested with EcoRI, D) pIC11 digested with EcoRI and PvuII, E) pIC21 digested with EcoRI, F) pIC21 digested with EcoRI and PvuII, G) pIC22 digested with EcoRI, H) pIC22 digested with EcoRI and PvuII, I) pIC23 digested with EcoRI, J) pIC23 digested with EcoRI and PvuII.

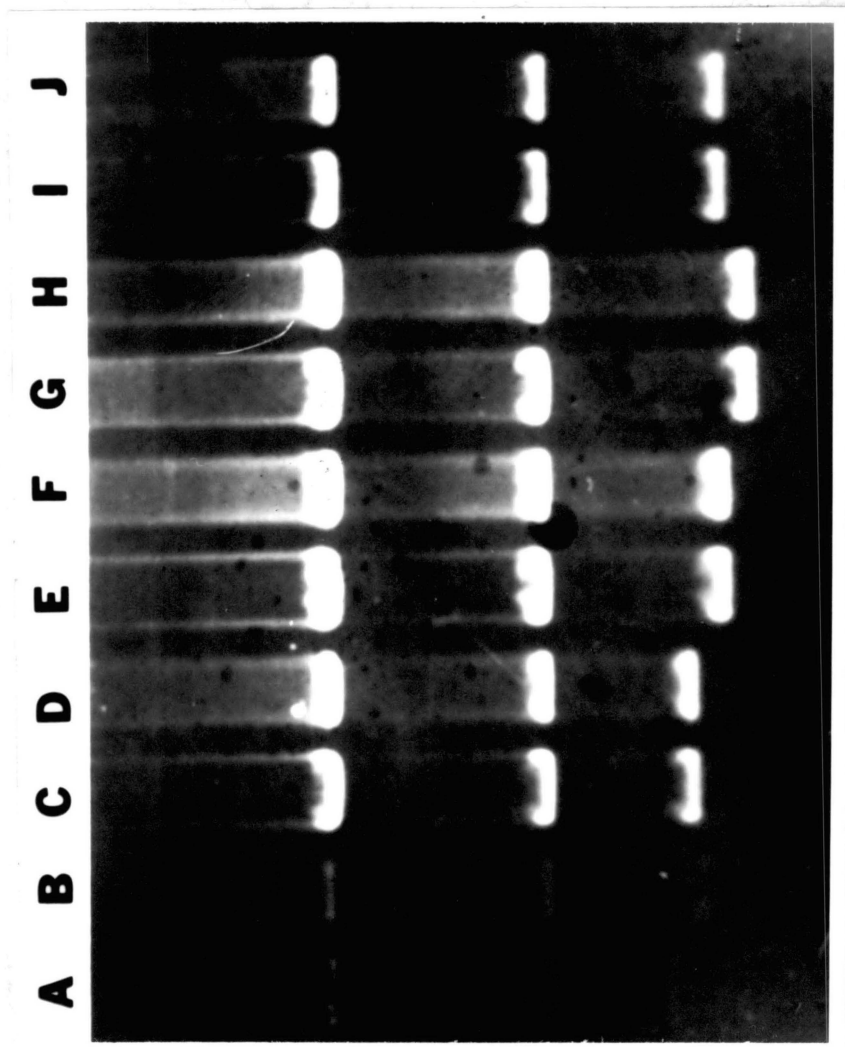
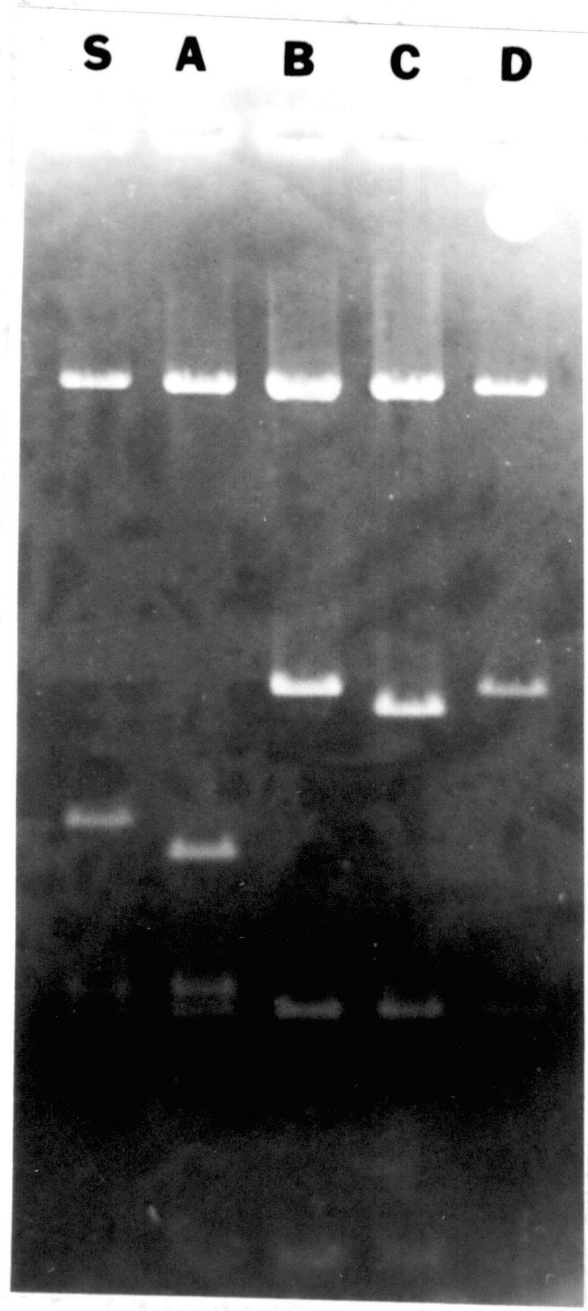

A
B
C
D
E
F
G
H
I
J

Figure 10. Gel Electrophoresis of Deletion Mutants Digested with BglII Restriction Enzyme.

S) pLW303X, A) pIC11, B) pIC21, C) pIC22, D) pIC23.


S
A
B
C
D

between the EcoRI site at nucleotide 6105 and the BglII site at nucleotide 6492.

When the deletion mutants and pLW303X DNA were digested with AvaI (Figure 11), pIC11, pIC21 and pIC23 gave a fragment pattern similar to the control except that fragment A was smaller in both plasmids. Fragment A and C of pIC22 were fused indicating the lack of an AvaI site in this mutant. The deletion in pIC21 and pIC23 plasmid DNAs were located between EcoRI site at nucleotide 6105 and AvaI site at nucleotide 6688.

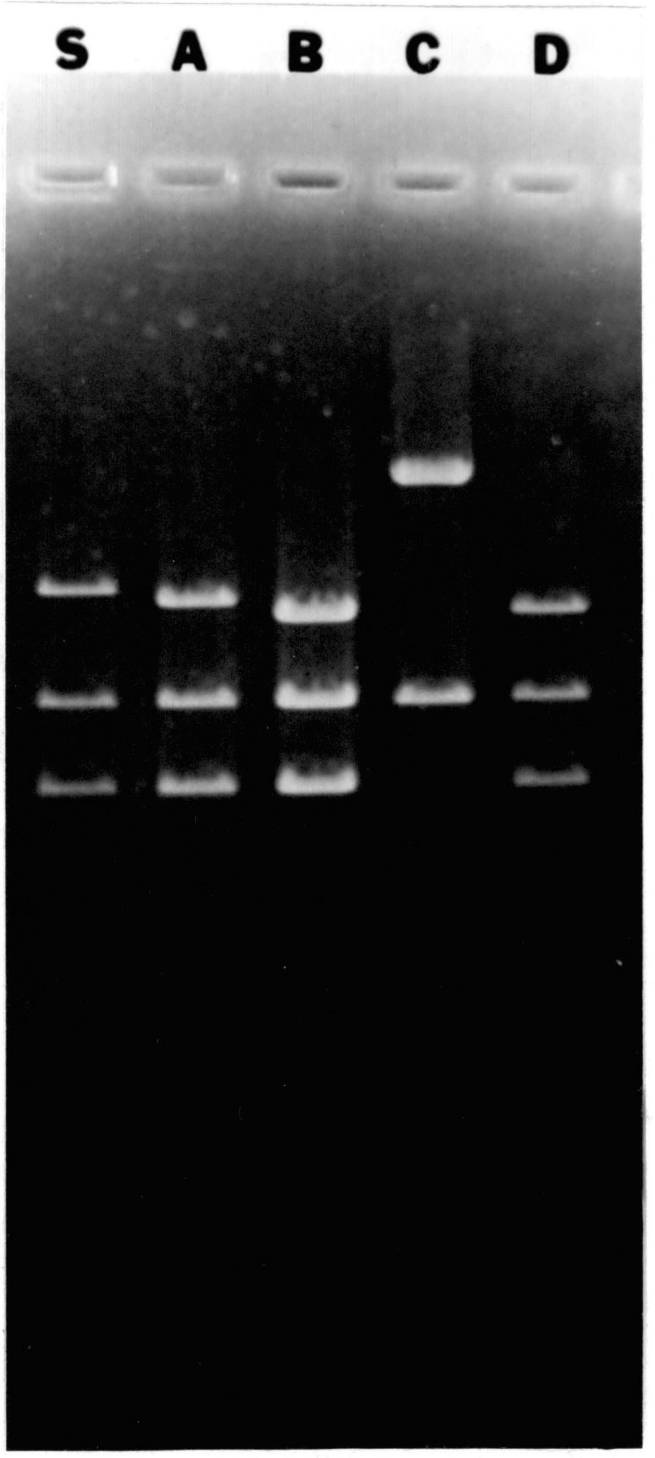
pIC22 and wild type DNAs were digested with AccI (Figure 12). The fragment pattern of both DNAs were similar except that AccI fragment C of pIC22 disappeared and a new smaller fragment appeared between fragment D and E. Thus the deletion in pIC22 was located between the EcoRI site at nucleotide 6105 and the AccI site at nucleotide 7040.

The migration distance of each fragment was plotted on semilog paper and used to estimate the size of deletion. The estimated sizes of deletions were 100 - 150 bp in pIC11, 260 - 340 bp in pIC21 and pIC23 and 420 - 680 bp in pIC22.

The DNA sequence of the mutated regions of three plasmids, pIC11, pIC21 and pIC23, were determined using the Maxam and Gilbert method. pIC11 was digested with BglII and pIC21 and pIC23 were digested with AvaI, respectively. All fragments were labeled with ^{32}P-dCTP by filling their 3' ends with the Klenow fragment of DNA polymerase I. After second enzyme (EcoRI) digestion, desired fragments were recovered from 6% acrylamide gels and were subjected to sequencing reactions. The reaction mixtures were applied on 8% sequencing gels

Figure 11. Gel Electrophoresis of Deletion Mutants Treated with AvaI Enzyme.

S) pLW303X, A) pIC11, B) pIC21, C) pIC22, D) pIC23.


S
A
B
C
D

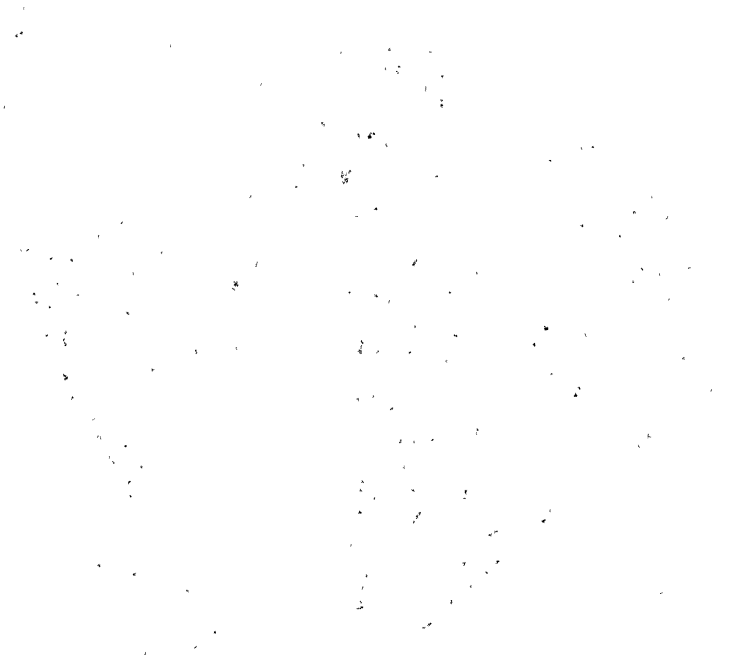
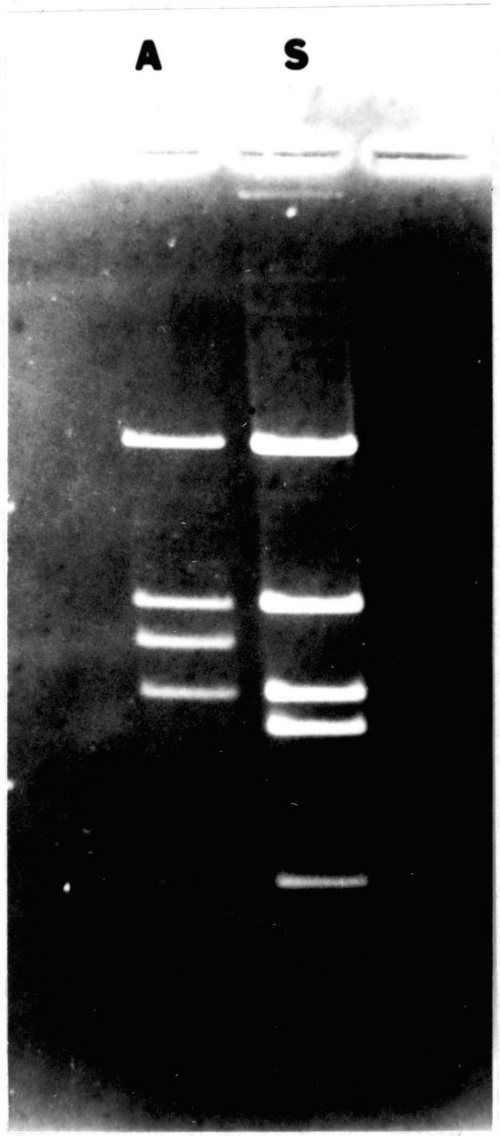


Figure 12. Gel Electrophoresis of AccI Digestion Products of pLW303X and pIC22.

S) pLW303X, A) pIC22.


A
S

and electrophoresed at 1000 volts for the desired length of time.

It was found that pIC11 has a 160 bp deletion between nucleotide at 6261 and nucleotide at 6420 (or nucleotide at 6260 and nucleotide at 6419). pIC21 and pIC23 have 348 bp deletions between nucleotide at 6317 and nucleotide 6664 (or nucleotide 6321 and nucleotide 6668). During the DNA sequencing process, a total of 320 bp in region VI was sequenced. The comparison of this sequence with the previously published sequence of CaMV isolate CM1841 (Gardner et al., 1981) shows that both DNA sequences are identical. The location of deletions is shown in Figure 13.

Construction of Insertion Mutants

An insertion mutant was constructed by introducing the tetracycline resistance (tet^r) gene into pLW303X through the PvuII site. 1656 bp DdeI fragment A from pBR322 containing the entire gene for tetracycline resistance was used as a marker gene. This gene has at least two restriction enzyme sites which are not present in pLW303X DNA and the size of insertion can be reduced easily when needed using these sites.

Strategy

The experimental scheme for construction of the insertion in cloned CaMV DNA is shown in Figure 14. Purified pLW303X DNA was digested with PvuII resulting in blunt ended linear molecules. pBR322 was digested with DdeI and fragment A was recovered after 1.2% agarose gel electrophoresis using electro-elution. This recovered fragment containing the tetracycline resistance gene was polished

Figure 13. Deletion Map of Mutants.

Solid arrows represent the deleted regions determined by DNA sequencing. The DNA sequence of the mutated region of pIC22 was not determined.

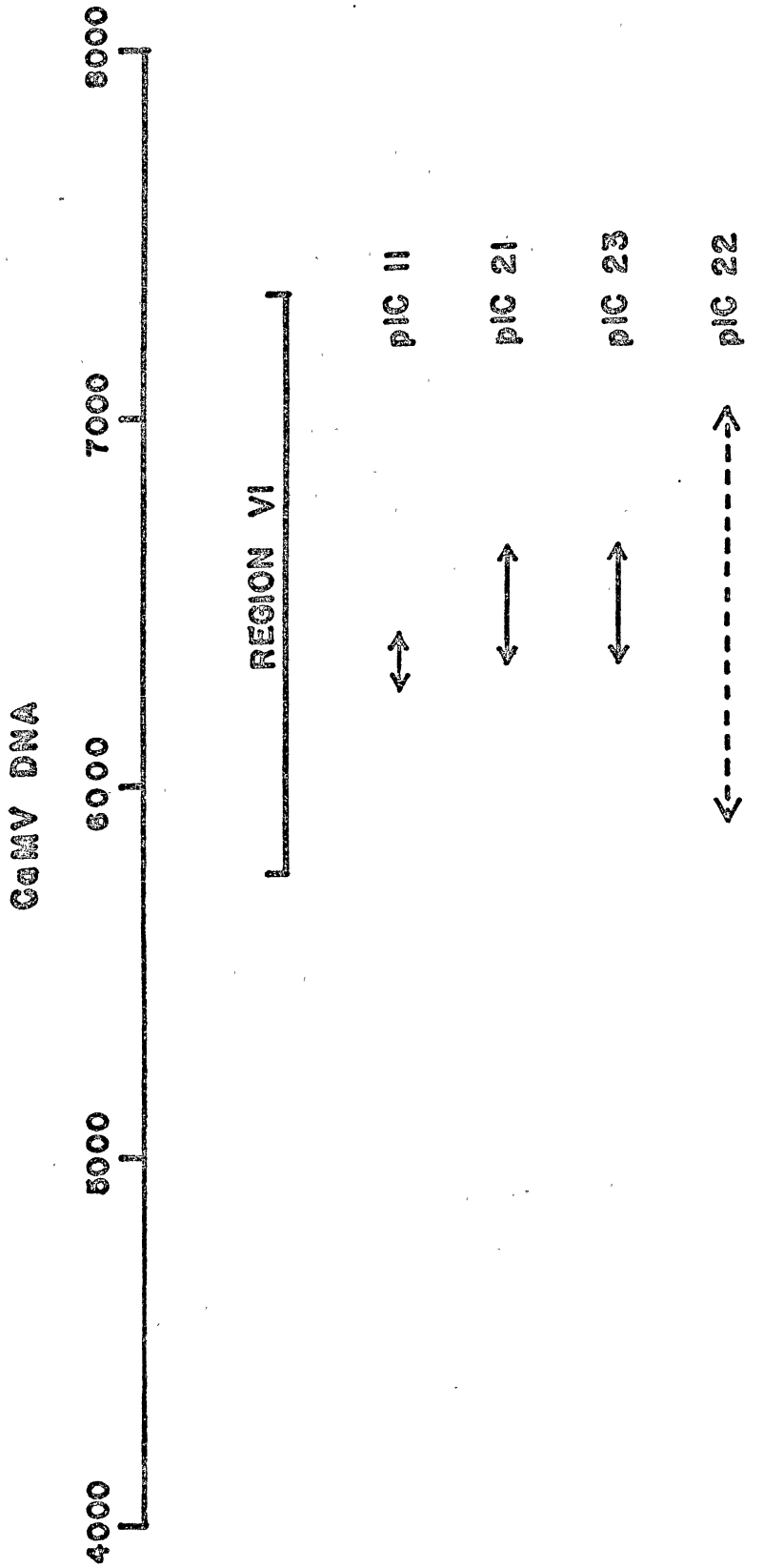
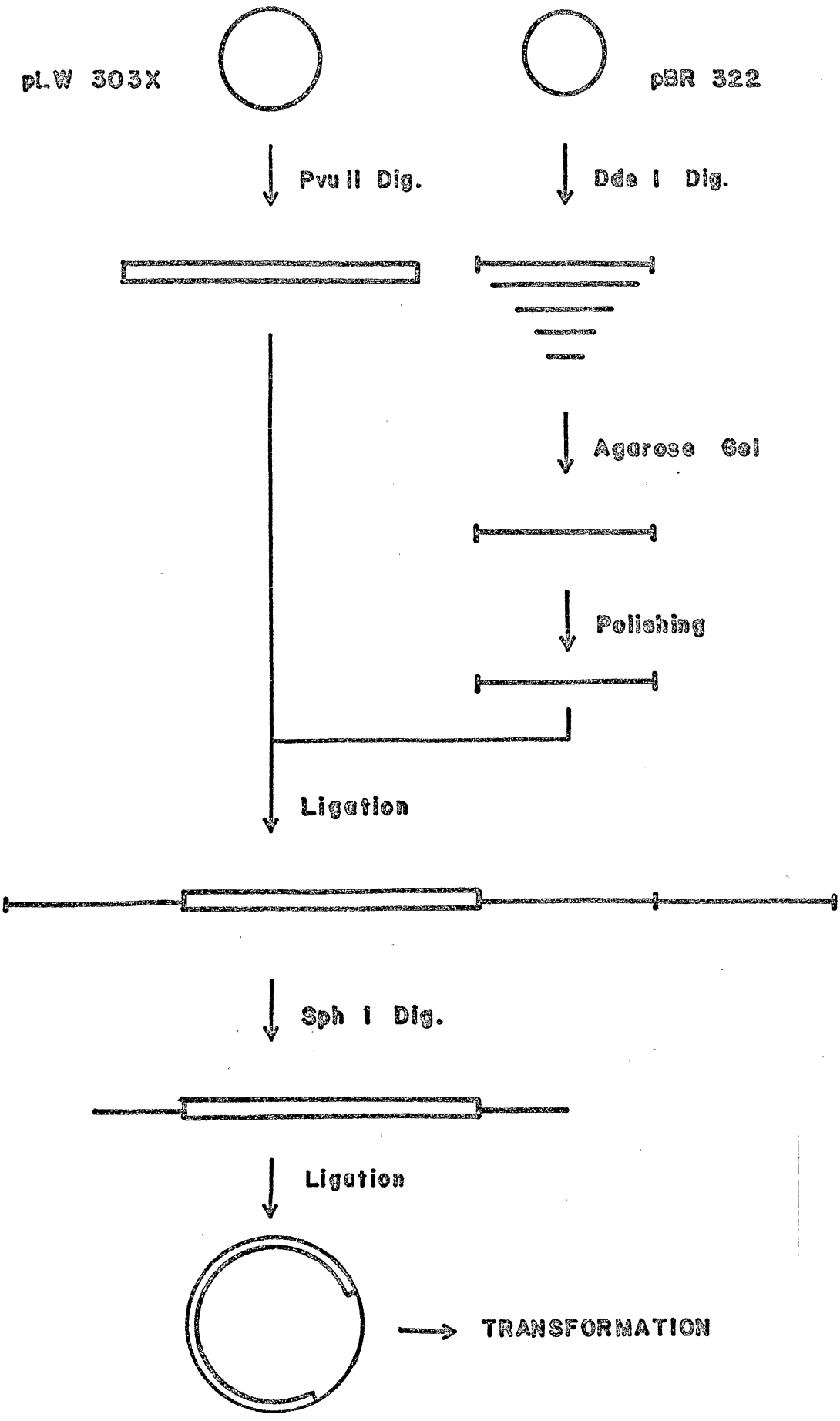

CaMV DNA
4000
5000
6000
7000
8000
REGION VI
pIC 11
pIC 21
pIC 23
pIC 22

Figure 14. Experimental Scheme for Construction of Insertion Mutant.


pL.W 303X
pBR 322
Pvu II Dig.
Dde I Dig.
Agarose Gel
Polishing
Ligation
Sph I Dig.
Ligation
TRANSFORMATION

with the large fragment of DNA polymerase I. An excess amount (ten times higher DNA end concentration than that of PvuII-pLW303X fragment) of the resulting blunt ended molecules were ligated to linear PvuII-pLW303X molecules. The ligation product showed various sizes of DNA fragments containing one to several tetracycline resistance genes. Also the presence of several very high molecular weight bands suggested that concatamers were formed. Part of this ligation mixture was used to transform HB101 cells.

The rest of the ligation product was digested with SphI enzyme (Bio Labs) which cut once in the tetracycline resistance gene and the reaction mixture was applied on an 0.8% agarose gel and electrophoresed at 40 volts overnight. The DNA molecules of desired size (about 14,000 bp long), PvuII-pLW303X fragments harboring SphI-cut tet^r gene half-molecules at both its ends, were recovered from the gel and religated at a dilute DNA concentration (0.01 µg DNA per µl reaction mixture) to form open circles. This ligation mixture was used to transform HB101 cells. More than one hundred tetracycline and ampicillin resistant transformants were screened. Single colony lysates were prepared from 40 transformants and analyzed on 0.8% agarose gel and the nine colonies having the smallest plasmid were selected. The size of these plasmids estimated from the agarose gel and the EcoRI restriction patterns of these plasmids were identical. One of these plasmids was designated pIC51 and used for further study.

Orientation of Insert

DdeI digestion of CaMV DNA produced thirty-five fragments of known size and location as determined from nucleotide sequence analysis

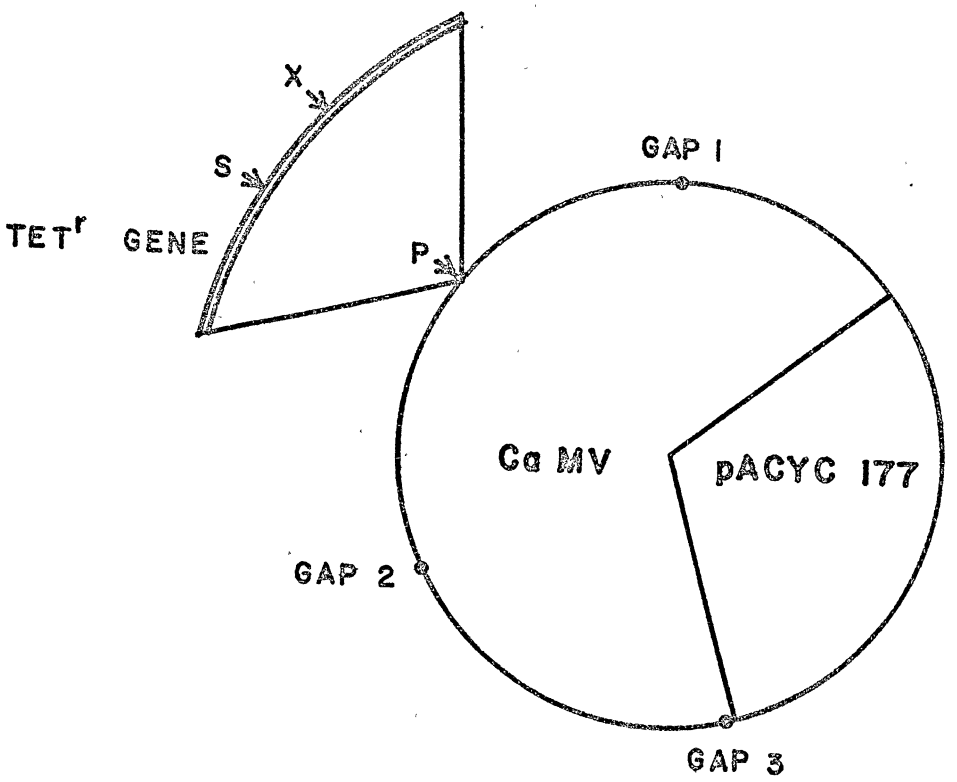
of isolate CM1841 (Gardner et al., 1981). Insertion of tet^r gene (1656 bp) affects only DdeI fragment C (724 bp long) and the newly created 2380 bp fragment has one SphI site originating from the tet^r gene. It has been confirmed that the newly created insertion mutant has one SphI site and lacks the PvuII site through which the gene was inserted. The orientation of the tet^r gene insert in pLW303X was determined by double digesting with DdeI and SphI and comparing the resulting fragments with fragment sizes expected for each of the two possible orientations of tet^r gene relative to pLW303X. An insertion map was constructed (Figure 15).

Analysis of the Ability of Deletion and Insertion Mutants to Replicate and Induce Infection in Turnip Plants

Each of the five deletion and insertion mutants of CaMV DNA was tested for its ability to replicate and produce infection in turnip plants. Controls used in each experiment were CaMV (isolate CM4-184), CaMV DNA (isolate CM4-184) and pBR322 DNA. After recombinant plasmids were digested with XhoI to release the CaMV DNA portion, the reaction mixtures were diluted to a final DNA concentration of 10 µg/ml. Each DNA or virus sample was inoculated onto two 3½ week old turnip plants. Each experiment was repeated and a total of four plants were inoculated for each DNA or virus sample. Leaves were rinsed with water from a spray bottle after inoculation to prevent DNA sticking to the leaves. Disposable gloves were worn and changed between each sample inoculation to prevent cross-contamination. After two weeks, two inoculated leaves were removed from each plant and processed for starch staining and LSH

Figure 15. Location and Orientation of Insert.

DdeI A fragment of pBR322 (1656 bp, open bar) was introduced to pLW303X (about 12,000 bp). Three restriction enzyme sites are represented by arrows (S, SphI; X, XmaI; P, PvuII).


X
S
TET^r GENE
P
GAP 1
Ca MV
pACYC 177
GAP 2
GAP 3

(Melcher and Essenberg, 1981).

Starch staining allows for detection of starch lesions which are analogous to the chlorotic local lesions produced by infection with CaMV. Starch lesions are identified as small unstained circles in the dark purple background of the stained leaf (Figure 2).

LSH is a method whereby viral nucleic acid can be detected in leaves by molecular hybridization with radioactive homologous DNA. The viral nucleic acid in infected leaves produces dark circles on autoradiographs of leaf skeletons. The autoradiographic spots correspond closely to starch lesions. The presence of one or two small isolated spots or large irregular areas of darkening on leaf autoradiographs was not taken as positive evidence of viral nucleic acid replication. The criterion for a positive result in this study was the presence of more than two dark circular areas on the inoculated leaf (Figure 3).

Total plant DNA preparations were obtained from small amounts of fresh plant tissue by extraction with phenol (Heyn et al., 1973). Using this procedure, 5-40 µg of plant DNA can be obtained from a gram of fresh tissue (Taylor and Powell, 1982). This method allows for the simultaneous isolation of plant DNA and naked viral DNA which is not protected by a protein coat from plant tissue and is called the total plant DNA preparation method. It is believed that the conditions of the phenol extraction procedure in this method (the concentration of phenol and SDS, and pH) are not sufficient to remove the coat protein from the virus (Melcher, personal communication).

Viral DNA was prepared by the modified method of Gardner et al. (1980). This is a method whereby only the DNA encapsulated in a

protein coat can be isolated from leaf tissue. Plant DNA and CaMV DNA which was not protected by a protein coat becomes digested with deoxyribonuclease I. If there were no CaMV DNA in virions in the plant tissue, no DNA would be detected by this method.

To analyze the ability of mutants to replicate, total plant DNA and viral DNA preparations were made from inoculated leaves. Both total plant DNA preparations and viral DNA preparations were electrophoresed on an 0.8% agarose gel, transferred onto nitrocellulose sheets and hybridized with ^{32}P-labeled CaMV DNA.

The results of starch staining and LSH in this experiment are summarized in Table I. The absence of starch lesions and of any evidence for hybridization on skeletons of leaves inoculated with mutant CaMV DNAs except the leaves inoculated with pIC23 in experiment 2 indicated that the replication of these mutant viral DNAs had not occurred or had occurred to only a small extent. No plants inoculated with mutant DNAs were systemically infected. To test the possibility that replication of mutant DNAs occurred to an extent too low to be detected by starch lesions or LSH, total plant DNA was prepared from the leaves harvested ten to twelve days after inoculation. Viral DNA preparations were also made from the leaves harvested fifteen to twenty days after inoculation.

When total plant DNA and viral DNA preparations isolated from leaves inoculated with wild type DNA (pLW303X) and mutant DNAs were probed with CaMV sequences using molecular hybridization techniques, none of these preparations showed any evidence of hybridization.

TABLE I

RESULTS OF LSH AND STARCH STAINING ON TURNIP LEAVES

Inoculum	Leaves Positive/Total	
	Expt. 1	Expt. 2
CM4-184 Virus	4/4	4/4
CM4-184 DNA	4/4	3/4
pLW303X DNA	4/4	4/4
pIC11	0/4	0/4
pIC21	0/4	0/4
pIC22	0/4	0/4
pIC23	0/4	3/4
pIC51	0/4	0/4

Experiment 1 shows the result of starch staining and Experiment 2 shows the result of LSH.

Interaction Between Region VI Mutants and Other Mutants

It has been reported that plants can be coinfected with appropriate pairs of modified viral genomes which are not infective when inoculated on their own (Howell et al., 1981; Walden and Howell, 1982).

The ability of mutant DNAs inoculated on different parts of the same leaves to coinfect plants was tested. Twenty plants were divided into five groups. The control group was inoculated with a mixture of pUM24 DNA (region VI mutant) and pUM41 DNA (region III mutant). For the second group, pUM24 DNA was inoculated on the right halves of the leaves while the left halves of the same leaves were inoculated with pUM41 DNA. Both areas were at least 1 cm apart. The third group of plants were inoculated in a similar way except pUM24 was inoculated on the left side and pUM41 was inoculated on the right side. The leaves of the fourth group of plants were inoculated with pUM24 on their basal half and with pUM41 on the apical half. The inoculated areas did not overlap and were at least 1 cm apart. The fifth group was inoculated similarly to the fourth group but with basal and apical inocula reversed. Only the control treatment produced systemic symptoms in all four plants inoculated.

In repeating such an experiment done by Howell et al. (1981) and Walden and Howell (1982), it has been found that certain mutants lead at high frequency to recombinational rescue (Melcher, unpublished). As the mutated viral DNAs in plasmids constructed in this study did not cause systemic symptoms on their own, pairwise inoculations of one of these plasmids with other plasmid containing mutations in different

regions of the CaMV genome were performed. The plasmids used in this study are listed in Table II. The construction of the following plasmids has been previously described; pSA103 (Armour et al., 1983), pUM24, pUM124, pUM41, pUM37 and pUM133 (Melcher et al., 1983). Viral DNA was released from plasmid DNA by restriction enzyme digestion prior to inoculation.

Most of the inoculation combinations of region VI mutants (pIC23 or pIC51) with wild type or one of the listed mutants resulted in systemic symptoms (14 of 17; Table III). Three inoculation combinations which did not show symptoms were combinations between region VI mutants whose mutated regions overlapped with each other. Two inoculation combinations between two different regions (pIC23 and pUM133, and pIC51 and pUM124) showed delayed symptom induction. Plants coinoculated with the above two combinations produced detectable systemic symptoms 28 days after inoculation. Viral DNAs were prepared from plants coinfected with the pairs of mutants 15 days after inoculation and digested with the appropriate restriction enzyme and analyzed using the Southern blotting technique (Figure 16). The concentration of DNA in the viral DNA preparation from the leaves inoculated with a mixture of pIC51 and pUM124 was very low when compared to the concentration of DNA of other viral DNA preparations (lane C and D, Figure 16). Since the DNA concentration in the viral DNA preparation from the plants coinoculated with the combination of pIC23 and pUM133 was similar to the DNA concentration of viral DNA preparations from other plants (Figure 16) and the plants coinoculated with pIC23 and pUM133 did not show any symptoms until 28 days after inoculation, it is possible that the two mutants

TABLE II

CaMV PLASMID MUTANTS

Plasmid	Insertion at	Insertion bp	Deletion from	Deletion to	Strain
Region I					
pUM124	-	-	ca. 400	ca. 1100	Cabb BS
Region II					
pSA103	-	-	ca. 1600	ca. 1690	NY8153
Region III					
pUM41	2040	8	2041	2044	Cabb BS
Region IV					
pUM37	-	-	2579	3517	Cabb BS
Region V					
pUM133	-	-	3906	5270	Cabb BS
Region VI					
pIC11	-	-	6261	6420	CM1841
pIC21	-	-	6317	6664	CM1841
pIC22	-	-	ca. 6200	ca. 6800	CM1841
pIC23	-	-	6317	6664	CM1841
pIC51	6318	1656	-	-	CM1841
pUM24	6299	16	6299	6338	Cabb BS

TABLE III

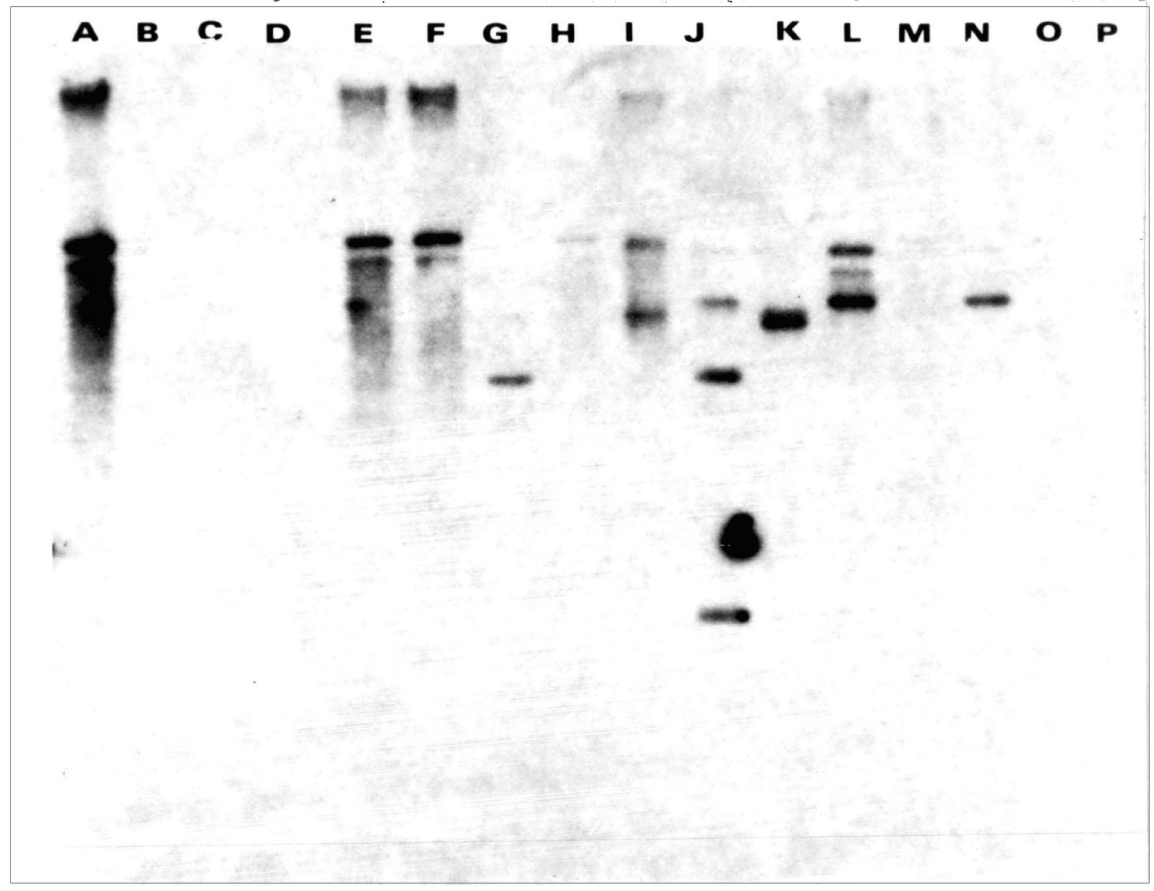
PAIRWISE INFECTIVITY GRID

	pLW303X	pUM124	pSA103	pUM41	pUM37	pUM133	pIC11	pIC23
pIC11	POS	NT	NT	POS	NT	NT	NT	NT
pIC23	POS	POS	POS	POS	POS	POS	NEG	NT
pIC51	POS	POS	POS	POS	POS	POS	NEG	NEG

Each of four plants was inoculated with 1 microgram of each plasmid. A test was scored positive (POS) if at least one test plant showed symptoms. NEG, negative. NT, not tested.

Figure 16. CaMV DNA Detected by Molecular Hybridization.

Viral DNA preparations from plants inoculated with wild type and mutant DNAs were electrophoresed on an 1.0% agarose gel and transferred to a nitrocellulose sheet for molecular hybridization. Viral DNA preparations were from plants inoculated with A) pLW303X, B) pIC51, C) and D) pIC51 and pUM124, E) and F) pIC51 and pUM37, G) and H) pIC51 and pUM133, I) and J) pIC23 and pUM124, K) and L) pIC23 and pUM37, M) and N) pIC23 and pUM133, O) pIC23 and pUM41, P) 60 mM potassium phosphate buffer, pH 8.5. DNA preparations in lane B, D, F and H were digested with SphI prior to electrophoresis. DNA preparations in lanes J, L and N were digested with PvuII before electrophoresis.


A B C D E F G H I J K L M N O P

coinoculated recombined to give hybrid molecules having altered region VI or region V genomes. These altered genomes may be responsible for the delayed symptom induction in plants. It has been reported that insertions in the amino-distal portion of region VI reduced the severity of symptoms (Daubert et al., 1983). The delayed symptom induction observed in these studies and the reduction of the severity of symptoms observed by Daubert et al. (1983) may be related to each other. The delayed symptom induction in plants could be observed as the reduction of the severity of symptoms during the short observation period.

When plants were coinfected with region VI mutants and wild type DNA, symptoms appeared about 5 days later than infection with wild type DNA alone. In the cases of coinfection with region VI mutants and other mutants, symptom development was delayed about 11 to 15 days relative to plants inoculated with wild type DNA alone. This delay in symptom induction occurred even in the plants coinoculated with region VI mutants and pUM41 which can lead at high frequency to recombinational rescue (Melcher, 1983). It is known that some region VI mutants interfered with symptom production when coinoculated with other infectious CaMV DNAs (Melcher, unpublished). The delay of symptom production in plants coinfected with two defective mutants may be related to recombinational rescue.

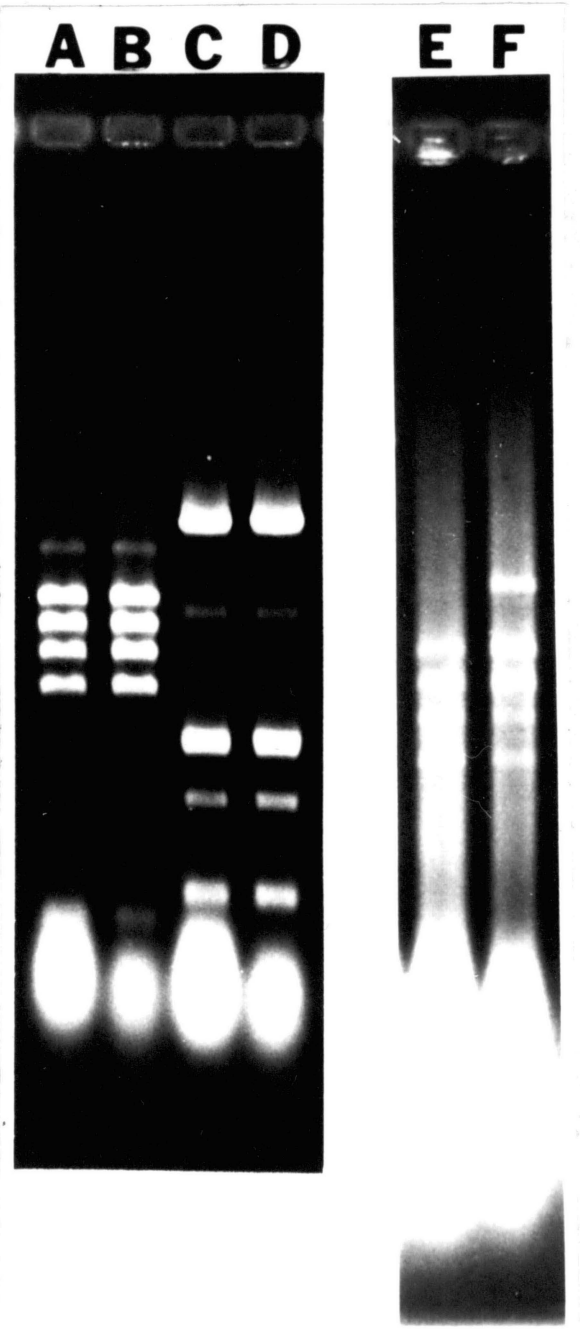
Howell et al. (1981) reported that viral DNAs, resulting from coinfection with two defective genomes, are indistinguishable from the wild type genomes from which they were derived. In this study, the viral DNAs which resulted when rescue had taken place between region VI mutants (pIC11 and pIC51) and pUM41 were isolated and

studied. Region VI mutants and pUM41 were derived from different viral isolates (Table II). I was able to use the differences in specific restriction sites which characterize each isolate in order to examine the recombinant viral DNAs. At least two different viral DNAs were identified from each plant.

Two viral DNA preparations from two different plants inoculated with the combination of pIC11 and pUM41 were digested with EcoRI or HindIII and electrophoresed in an 1.4% agarose gel (Lanes A, B, C and D, Figure 17). The four major bands in lane A and B showed the characteristic EcoRI digestion pattern of CaBB BS DNA. The size of the fragments are 2325 bp, 2009 bp, 1721 bp and 1451 bp. CaMV isolate CM1841 lacks one EcoRI site at nucleotide 3928 which is present in CaBB BS DNA and gives three characteristic large fragments (3172 bp, 2334 bp and 2006 bp). EcoRI C and D fragments of Cabb BS are fused to give one large fragment (3172 bp) in the EcoRI restriction pattern of CM1841. In the lane A and B of Figure 17, there is a minor band whose size is estimated to be about 3200 bp from the distance of its migration. This is the same size as the EcoRI A fragment of CM1841. Also upon HindIII digestion (lane C and D), major and minor bands were present. These minor bands were present even in the digests treated with ten times more restriction enzymes than the amount of the enzymes used in this experiment (data not shown). This indicates that the minor bands are not partial digestion products. These results show that the viral DNA preparations tested are the mixture of at least two different viral DNAs. The results of several other restriction enzyme digestion patterns (Bgl I, Hae III, Hha I, HindIII, Kpn I and PvuII) also support the conclusion that the viral

Figure 17. Gel Electrophoresis of EcoRI and HindIII Digests of the Viral DNAs Isolated from Plants Coinoculated with pIC11 and pUM41 or pIC51 and pUM41.

Four viral DNA preparations were from four different plants. A) and B) DNAs digested with EcoRI (pIC11 and pUM41), C) and D) DNAs digested with HindIII (pIC11 and pUM41). E) and F) DNAs digested with EcoRI (pIC51 and pUM41).


A B C D
E F

DNA preparations are heterogeneous mixtures (data not shown).

Viral DNA preparations from two different plants inoculated with the combination of pIC51 and pUM41 were digested with EcoRI and electrophoresed on an 1:2% agarose gel (lane E and F, Figure 17). The fragmentation patterns of the digests are basically identical to those of the viral DNA preparations from plants inoculated with pIC11 and pUM41. As shown in lane F, there are four DNA bands which are characteristic in EcoRI restriction pattern of Cabb BS DNA as in the lane A and B of Figure 17 and there is one larger band which has the same size as EcoRI A fragment of CM1841 DNA. Another viral DNA preparation (lane E) showed an EcoRI restriction pattern identical to that of lane F. But the intensity of the EcoRI A fragment of CM1841 DNA in this sample was very low compared to that of the fragment in lane F. This shows that the concentration of two different viral DNAs was not in the same proportion in all viral DNA preparations. Moreover, the intensity of EcoRI A and B fragments of Cabb BS DNA in lane E is much higher than that of EcoRI C and D fragments. This may suggest that fragments at 2330 bp and 2010 bp correspond to those expected from both Cabb BS (EcoRI A and B fragments) and CM1841 (EcoRI B and C fragments) DNAs. This observation also supports the idea that the DNA molecules in viral DNA preparations are not homogeneous. Analysis of the EcoRI fragmentation pattern shows that the viral DNA isolated from plants inoculated with pIC51 and pUM41 lost the insert in pIC51 DNA. If the insert is present in progeny DNA, the EcoRI A fragment of Cabb BS DNA or EcoRI B fragment of CM1841 DNA should disappear and a new large fragment (about 4000 bp) should appear on the gel. The size of progeny DNA is

similar to the wild type DNA. In summary, analysis of the four viral DNA preparations from plants inoculated with combination of pIC11 and pUM41 (2 plants) and from plants inoculated with combination of pIC51 and pUM41 (2 plants) showed that those were the mixtures of at least two different viral DNA molecules. The viral DNA preparation isolated from the plant coinoculated with pIC11 and pUM41 was digested with SalI and viral DNAs were cloned into pBR322 through its unique SalI site. After ligation by T4 DNA ligase, the reaction mixture was used to transform HB101 cells. Eleven transformants which were ampicillin resistant and tetracycline sensitive were selected and the fragmentation patterns of HindIII digestion and EcoRI digestion were analyzed. From the restriction analysis data it has been confirmed that two different viral DNA molecules are present in the viral DNA preparation. There was one major form of viral DNA cloned into pBR322 DNA (10 colonies) and one minor form cloned into pBR322 (1 colony) among the eleven transformants screened. The two plasmids were designated pIC141 (containing the major form of viral DNA) and pIC143 (containing the minor form). The location and orientation of viral DNA in pIC141 and pIC143 was determined by digesting with EcoRI and HindIII and comparing the resulting fragments with fragment sizes expected for each of the two possible orientation of viral DNAs relative to pBR322 DNA (Figure 18).

The restriction analysis of the major form was done using various enzymes (Figure 19). The hybrid DNA contains two EcoRI sites (at nucleotide 3928 and 6045), two PvuII sites (at nucleotide 3990 and 6320), one HaeIII site (at nucleotide 7224) and one HpaI site (at nucleotide 3094) which are characteristic of the Cabb BS isolate.

Figure 18. Orientation of Hybrid CaMV DNAs.

Major and minor form of hybrid CaMV DNA were cloned into pBR322 plasmid through their unique SalI sites. The orientations of both forms were the same.

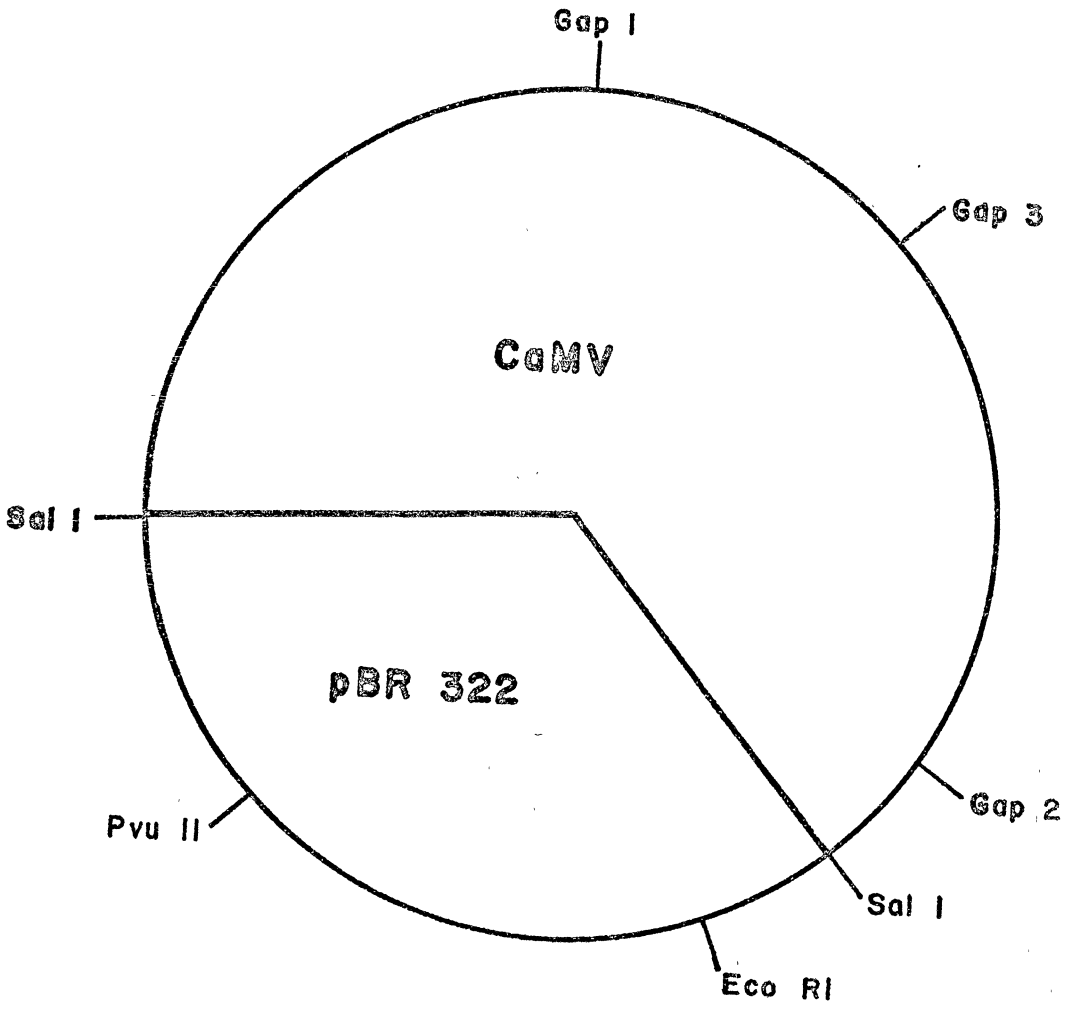
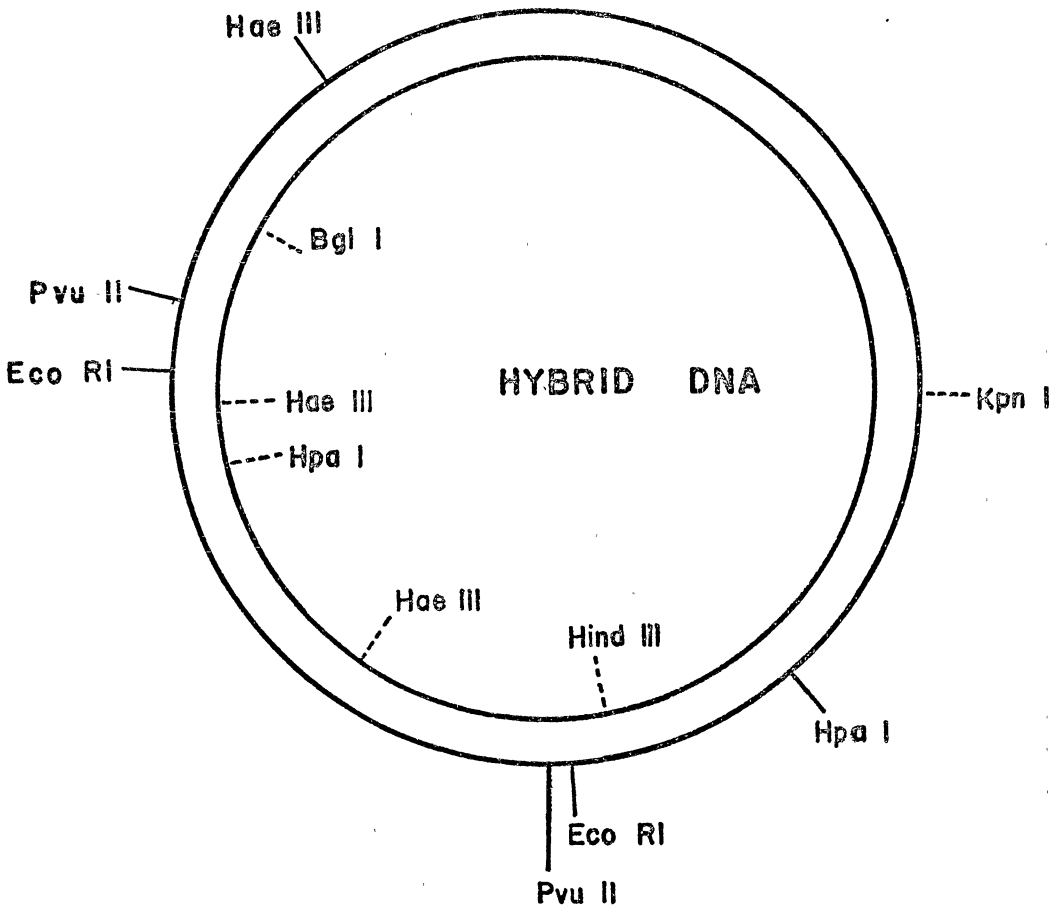

Gap 1
Gap 3
CaMV
Sal I
pBR 322
Pvu II
Gap 2
Sal I
Eco RI

Figure 19. Restriction Map of Major Hybrid DNA.

Inner circle gives some restriction enzyme sites characteristic of CM1841. The outer circle gives the enzyme sites characteristic of Cabb BS. Solid lines represent the sites present in hybrid DNA and dotted lines represent the sites absent in hybrid DNA.


Hae III
Bgl I
Pvu II
Eco RI
HYBRID DNA
Hae III
Kpn I
Hpa I
Hae III
Hind III
Hpa I
Eco RI
Pvu II

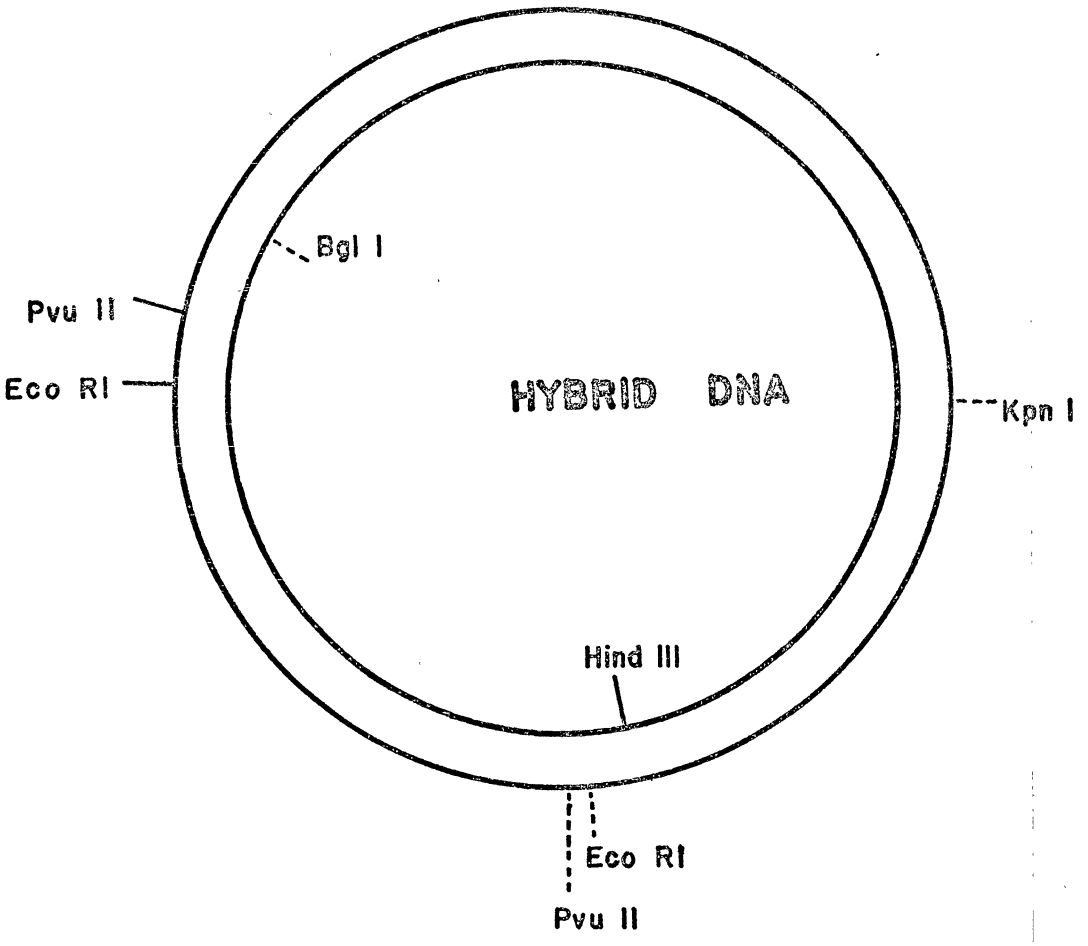
Several restriction enzyme sites (two HaeIII sites at nucleotide 4751 and 5941, one HindIII site at nucleotide 3773, one HpaI site at nucleotide 5514 and one BglI site at nucleotide 6656) which are characteristic of CM1841 were lost in the hybrid DNA molecule. There is no direct evidence for the presence of CM1841 specific restriction sites in the hybrid DNA. However, the mutation in region III of pUM41 containing Cabb BS DNA was repaired and the functional gene should have originated from CM1841 DNA.

The restriction analysis of the minor form which is present in all four viral preparations but in small quantity showed that the minor form was also a hybrid molecule between CM1841 and Cabb BS (Figure 20). The hybrid DNA contains one PvuII site at nucleotide 6320 and one EcoRI site at nucleotide 6045 which are characteristic of Cabb BS and one HindIII site at nucleotide 3773 which is characteristic of CM1841. One BglI site at nucleotide 6656 which is characteristic of CM1841, and one PvuII site at nucleotide 3990 and one EcoRI site at nucleotide 3928 which are characteristic of Cabb BS were lost in the minor form. The restriction analysis of major and minor forms of hybrid DNA molecules showed that recombination or gene conversion between two defective parent molecules occurred at different sites in both forms. The major and minor form of DNA lost their Kpn I site (at nucleotide 2040) which is characteristic of pUM41, the absence of the Kpn I site in the hybrid DNA only means that region III of pUM41 was not converted to the original region III genome of Cabb BS DNA. The infectivity of both forms is being tested.

Seven viral DNA preparations were isolated 15 days after inocu-

Figure 20. Restriction Map of Minor Hybrid DNA.

Inner circle gives some restriction enzyme sites characteristic of CM1841. The outer circle gives the enzyme sites characteristic of Cabb BS. Solid lines represent the sites present in hybrid DNA and dotted lines represent the sites absent in hybrid DNA.


Bgl I
Pvu II
Eco RI
HYBRID DNA
Kpn I
Hind III
Eco RI
Pvu II

lation from the leaves coinoculated with the combination of one of the region VI mutants and another mutant of the different regions of the CaMV genome. Half of each viral DNA preparation was digested with Sph I (pIC51-pUM124, pIC51-pUM37 and pIC51-pUM133), PvuII (pIC23-pUM124, pIC23-pUM37 and pIC23-pUM133) or SmaI (pIC23-pUM41). The digested and undigested viral DNAs were electrophoresed on an 0.8% agarose gel and transferred onto nitrocellulose sheet for molecular hybridization. The results of restriction patterns were not easy to interpret. However, one viral DNA preparation isolated from the coinoculated leaves with pIC23 and pUM124 (region I mutant) shows that there are two different viral DNA molecules in the preparation. One type of DNA molecule has one PvuII site and the other has two PvuII sites (data not shown). They may be the two different hybrid DNA molecules similar to those observed in the plants coinoculated with the region VI mutant (pIC11 or pIC51) and pUM41.

CHAPTER IV

DISCUSSION

Four deletion mutants and one insertion mutant of CaMV DNA were isolated and characterized (Table II, Figure 13 and Figure 15). Of the five mutants isolated, none could produce systemic symptoms in turnip plants. pIC23 which showed evidence for replication of viral DNA in inoculated leaves detected by LSH, pIC11 which has smallest deletion among deletion mutants and pIC51 which has a 1656 bp insert at nucleotide 6318 were selected for further studies.

pIC11 has a 160 bp deletion (at nucleotide 6261 - at nucleotide 6420). This deletion enables a new stop codon (TAG) at nucleotide 6453 in the same reading frame as that of the gene VI protein. The size of the protein which can be coded by mutated region VI of pIC11 is a 173 amino acids long and is about 33% of the size of the protein which can be coded by wild type region VI (520 bp; Gardner et al., 1981). pIC23 has a 348 bp deletion (at nucleotide 6317 - at nucleotide 6164). This deletion does not interrupt the reading frame and the mutated region VI of pIC23 can code for a polypeptide of 404 amino acids. The size of this polypeptide is about 78% of that of wild type. The insertion of the tetracycline resistance gene into pLW303X for the construction of pIC51 does not alter the reading frame of region VI of CaMV genome. But the insert itself has two stop codons (TAA and TAG) in the same phase as that of region VI of

CaMV DNA at 33 and 39 nucleotides downstream from the site of insertion. The mutated region VI of pIC51 can code for a polypeptide of 192 amino acids. The size is about 37% of the wild type protein.

It has been reported that region VI can be altered and still function with the insertions in the genome at nucleotides 6491 (12 bp, CM1841 DNA), 6923 (12 bp, CM1841 DNA) and 6921 (22 bp or longer, CabbB-JI) (Daubert et al., 1983; Dixon et al., 1983). The longer insertions at positions 6923 (65 bp, CM1841 DNA) and 6106 (30 bp, CM1841 DNA) or the insertion of 10 bp at position 6109 (CabbB-JI DNA) destroy virus infectivity. The results showed that CaMV DNA could tolerate a short insert (12 bp - about 22 bp) in the amino-distal portion of its region VI genome. The mutated regions of mutant plasmids constructed in this study include the amino-proximal portion of region VI and the resulting polypeptides which can be coded by mutated region VI genomes are smaller than the wild type protein by 20-70%. The results of Daubert et al. (1983) and Dixon et al. (1983) are thus consistent with the observation of this study that region VI mutants made for this study are not infectious.

When the ability of the region VI mutant plasmids to replicate in plant tissue was tested using various methods, none of the mutants gave positive results. These results showed that the region VI mutants constructed in this study could not replicate or could not replicate to the extent which can be detected by the methods used.

From the inference that the replication of CaMV DNA can not occur in inoculated leaves, it may be supposed that the presence of inclusion body protein is essential for the replication of viral DNA. It was also possible that CaMV DNA might replicate to a small extent which

could not be detected by the methods used in these studies.

Inclusion bodies are the structures which have been shown to be the intracellular site of virus accumulation (Fujisawa et al., 1967). These are elliptical masses of electron dense material (mainly protein) that are produced within the cytoplasm of infected cells. Most inclusion bodies have one or more vacuole-like spaces and virus particles found in cytoplasm tend to crowd near or in the vacuole-like spaces. It has been suggested that intracytoplasmic virus particles are presumed to be few in number, if they occur at all. No virus-like particles were found in the nuclei, chloroplasts, mitochondria or spherosomes. A model for CaMV DNA replication according to which the major transcripts (35S RNA) is reverse transcribed in cytoplasm has been suggested (Pfeiffer and Hohn, 1983). The localization of virus particles in inclusion bodies may indicate that inclusion bodies provide a stable environment for the replication of CaMV DNA.

It has been reported that CaMV DNA can tolerate small insertions in the amino-distal portion of region VI and can retain its infectivity (Dixon et al., 1983; Walden and Howell, 1983). But same or smaller insertions in the amino-proximal portion destroyed the viral infectivity. Inclusion body is rich in proline, as indicated by the DNA sequence (Gardner et al., 1981), and is the most variable region of the three sequenced strains (Balazs et al., 1982). It may be more plastic than the other viral gene products and more tolerant of conformational changes induced by small insertions. But another explanation is also possible. Inclusion body protein may have some vital role in the life cycle of CaMV other than to be a simple

building block of inclusion body and the insertions in the specific portion of CaMV genome may destroy this function. If this is the case, replication of the region VI mutants which can not produce normal inclusion body protein would not be possible without this protein. Since some mutations in region VI reduced the severity of disease symptoms (Daubert et al., 1983), it is possible that the inclusion body protein functions both in symptom production and viral DNA replication. Another possible explanation for the observation by Daubert et al. is that the slower spread of virus caused by mutated inclusion body protein may be the reason for less severe symptoms. It has been suggested that symptom induction in plants by CaMV may be caused by a nucleic acid (Melcher et al., 1983).

Pairs of one region VI mutant with another mutant of a different region of CaMV genome coinoculated in plants are rescued at high frequency (Table III). Three pairs (pIC23-pUM37, pIC23-pUM133 and pIC51-pUM124) showed delayed symptom production. The plants inoculated with the combination of pIC23 and pUM37 showed only mild chlorotic lesions on the inoculated leaves until 20 days after inoculation but the replication of DNA was confirmed by analyzing the viral DNA preparation isolated from the leaf harvested 15 days after inoculation (lane K and L, Figure 16). The systemic symptoms in the plants appeared 22 days after inoculation. The plants coinoculated with pIC23 and pUM133 or pIC51 and pUM124 did not show any detectable systemic symptoms until 25 days after inoculation but the replication of DNAs was confirmed by analyzing the viral DNA preparations isolated from the inoculated leaves harvested 15 days after inoculation. The systemic symptoms in the plants appeared 28

days after inoculation (lanes C, D, M and N, Figure 16). The other eleven pairs including three pairs with wild type DNA caused systemic symptoms in plants between 6 to 18 days after inoculation. The delayed symptom induction observed in these studies or the slower spread of virus may be responsible for the reduced severity of symptoms by the insertion in region VI of the CaMV genome reported by Daubert et al. (1983). Analysis of viral DNA preparations isolated from the plants coinfected with the pairs of defective mutants indicates that the viral DNA preparations are heterogeneous mixtures of viruses.

To study the characteristics of CaMV DNAs resulting from the coinfection with two defective mutant DNAs, viral DNA was prepared from four independently inoculated plants, two coinfected with pIC11 and pUM41 and the other two coinfected with pIC51 and pUM41. The analysis of EcoRI and HindIII restriction enzyme digestion patterns of four viral DNA preparations shows that there are at least two different types of DNA molecules in each viral DNA preparation (Figure 17). The major form of DNA molecule is present in all four viral DNA preparations and is more abundant than the other form. The relative amount of minor form to that of the major form varies from one viral DNA preparation to the other.

The major and minor form of DNA molecules were cloned into bacterial plasmid pBR322 through their unique SalI sites. Both forms of DNA molecules were subject to restriction mapping using various restriction enzymes (Figures 19 and 20). Almost half of the DNA molecule of the major form shows the characteristics of the Cabb BS isolate which is the parent of pUM41. The characteristics of the

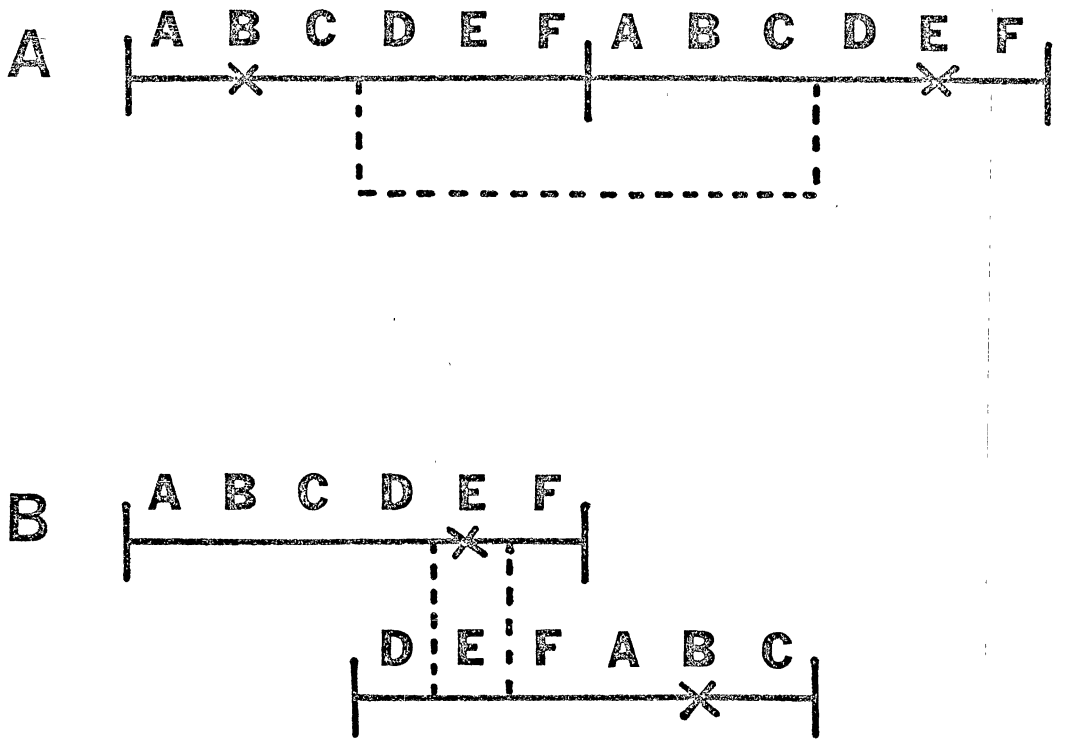
other half were not tested. Since this DNA can cause systemic symptoms, this molecule should have a functional gene III originating from the CM1841 isolate (the parent of pIC11 and pIC51) and should thus be hybrid molecule between CaMV isolate Cabb BS and CaMV isolate CM1841. Walden and Howell (1982) also reported that coinfection of plants with pairs of defective genomes derived from different viral isolates results in the formation of hybrid viral genomes. The restriction sites which are characteristic of Cabb BS and some other enzyme sites which are characteristic of CM1841 showing that the minor form is also a hybrid molecule.

It has been suggested that the recombination between a pair of defective CaMV genomes could take place at different sites in the viral genome (Walden and Howell, 1982). Based on the observations that there are one major and one minor form of hybrid DNAs, when judged from restriction enzyme patterns, in all four viral DNA preparations isolated from plants which are independently coinfected with a region VI mutant (pIC11 or pIC51) and a region III Mutant (pUM41), it may be suggested that there are some sites in viral genomes at which recombination or gene conversion could occur more readily than at other sites or some types of hybrid DNAs among the hybrid DNAs which were formed randomly through recombinational events may have a selective advantage over others which grow slowly.

Walden and Howell (1982) have proposed two general mechanisms that promote recombination among defective viral genomes coinoculated onto plants (Figure 21). The first mechanism involves recombination events that can be resolved by a single crossover event (Figure 21A). By such a mechanism, it is presumed that input viral genomes join

Figure 21. Proposed Mechanism of Recombination Taking Place Between Mutant Viral Genomes in vitro (Walden and Howell, 1982).

A) Single recombination event, B) Double recombination or internal gene conversion event. X, virus-lethal modification sites.


A
A B C D E F A B C D E F
B
A B C D E F
D E F A B C

end-to-end by virtue of their homologous sticky ends to form mixed dimers or higher order concatamers. Head-to-tail mixed dimers of input viral DNA molecules cut at the same restriction site are circularly permuted and out of certain of these dimers normal DNA molecules can be derived by a single crossover event. The second type of recombination takes place among viral DNA molecules which do not have complementary sticky ends. This mechanism resolves mixed defective pairs into normal viral genomes by a double crossover event or an internal gene conversion event equivalent to a double crossover (Figure 21B). However, they observed that the second mechanism did not act upon all parental DNA molecules coinoculated. They described a possible constraint on the operation of the second mechanism that double crossover events occur among progeny DNA molecules. The inoculation combinations tested in these studies did not have complementary sticky ends. This may mean that the inoculation combinations tested in these studies are rescued by the second mechanism described by Walden and Howell.

CHAPTER V

SUMMARY

Mutants of CaMV DNA were constructed that had deletions or insertion in coding region VI. The mutants were created in an attempt to determine the amount of DNA that could be eliminated or inserted and still retain an infective viral DNA and how these mutants interact with other mutants or wild type DNA.

pLW303X is a recombinant plasmid containing a full length copy of the viral DNA (isolate CM1841) cloned into the bacterial plasmid (pACYC177) via their unique Xho I sites. Deletion mutants of CaMV DNA were made by digesting the PvuII treated linear molecules of pLW303X with Bal 31 exonuclease and then religating the deleted plasmids. Insertion mutants were created by inserting the largest fragment of DdeI digested pBR322 plasmid containing the tetracycline resistance gene into pLW303X DNA at the PvuII site using blunt end ligation. Four deletion mutants and one insertion mutant were selected and tested by starch lesions and LSH for the ability to infect plants. None of these mutants except pIC23 showed evidence of replication or infection when inoculated onto turnip plants. Some, but not all, of the leaves inoculated with pIC23 showed a few LSH spots.

Viral DNAs were isolated from the leaves inoculated with these mutants (pIC11, pIC23 and pIC51). The presence of CaMV DNA was not

detected in any inoculated leaf by using the Southern blotting technique and molecular hybridization with ^{32}P-labeled CaMV DNA. A conclusion to be drawn from these results is that region VI mutants can not replicate in inoculated leaf tissue. It may be speculated that the presence of inclusion body protein is essential for the replication of viral DNA. The localization of virus particles in inclusion bodies was reported by Fujisawa et al. (1967). This may indicate that inclusion bodies provide a stable environment for the replication of CaMV DNA. It is also possible that inclusion body protein plays some direct role in the replication. All the hypotheses remain to be tested.

Coinoculation of plants with a region VI mutant and another mutant of different region of CaMV genome resulted in rescue at high frequency (Table III). All of fourteen pairs tested resulted in systemic symptoms. Most of the pairs (12 of 14) produced systemic symptoms within fifteen days after inoculation. Three pairs (pIC23 and pUM133, pIC23 and pUM37, and pIC51 and pUM124) showed delayed systemic symptom development. It is possible that the resulting hybrid DNAs have altered region VI or V genomes and that caused the delay of symptom induction. At least two of the mutants constructed in these studies showed the ability to recombine at high frequency with mutants from different regions of the CaMV genome.

Two different types of hybrid DNA molecules were detected in the viral DNA preparations from the plants coinoculated with the combination of a region VI mutant (pIC11 or pIC51) and a region III mutant (pUM41). Both forms of DNA molecules were cloned into pBR322

through their unique Sal I sites. The restriction analysis of the two newly created plasmids shows that these are the hybrid DNA molecules between the region VI mutants and the region III mutant. Based on the observations that there are two forms of hybrid DNAs, when judged from restriction enzyme patterns, in four viral DNA preparations isolated from plants which are independently coinfected with a region VI mutant and a region III mutant, it may be suggested that there are some sites in viral genomes at which recombination or gene conversion could occur more readily than at other sites or some types of hybrid DNAs among the hybrid DNAs occurred randomly through recombinational events may have a selective advantage over others which grow slowly.

A SELECTED BIBLIOGRAPHY

Armour, S. L., Melcher, U., Pirone, T. P., Lyttle, D. J., and Essenberg, R. C. (1983) Virology, 129, 25-30.

Al Ani, R., Pfeiffer, P., Lebeurier, G., and Hirth, L. (1979) Virology, 93, 175-187.

Balazs, E., Guilley, H., Jonard, G., and Richards, K. (1982) Gene, 19, 239-249.

Bawden, F. C., and Pirie, N. W. (1959) J. Gen. Microbiol., 21, 438-456.

Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., and Boyer, H. W. (1977) Gene, 2, 95-113.

Broadbent, L. (1957) Investigations of Virus Diseases of Brassica Crops, Cambridge Univ. Press, London and New York.

Brcak, J., Ulrychova, M., and Cech, M. (1962) Virology, 16, 105-109.

Condit, C., and Meagher, R. B. (1983) J. Mol. Appl. Genetics, 2, 301-314.

Covey, S. N., and Hull, R. (1981) Virology, 111, 463-474.

Covey, S. N., Lomonossoff, G. P., and Hull, R. (1981) Nucleic Acids Res., 9, 6735-6747.

Covey, S. N., Turner, D., and Mulder, G. (1983) Nucleic Acids Res., 11, 257-264.

Daubert, S., Richins, R., Shepherd, R. J., and Gardner, R. C. (1982) Virology, 122, 444-449.

Daubert, S., Shepherd, R. J., and Gardner, R. C. (1983) Gene, 25, 201-208.

Dixon, L. K., Koenig, I., and Hohn, T. (1983) Gene, 25, 189-199.

Dudley, R. K., Odell, J. T., and Howell, S. H. (1982) Virology, 117, 19-28.

DuPlessis, D. H., and Smith, P. (1981) Virology, 109, 403-408.

DuPlessis, D. H., and von Wechmar, M. B. (1981) Phytopathol. Z., 100, 270-278.

Fraenkel-Conrat, H. (1957) Virology, 4, 1-4.

Franck, A., Jonard, G., Richards, K., Hirth, L., and Guiley, H. (1980) Cell, 21, 285-294.

Fujisawa, I., Rubio-Huertos, M., Matsiu, C., and Yamaguichi, A. (1967) Phytopathology, 57, 1130-1132.

Gardner, C. O., Jr., Melcher, U., Shockey, M. W., and Essenberg, R. C. (1980) Virology, 103, 250-254.

Gardner, R. C., and Shepherd, R. J. (1980) Virology, 106, 159-161.

Gardner, R. C., Howarth, A. J., Hahn, P., Brown-Luedi, M., Shepherd, R. J., and Messing, J. (1981) Nucleic Acids Res., 9, 2872-2888.

Gronenborn, B., Gardner, R. C., Schaefer, S., and Shepherd, R. J. (1981) Nature, 294, 773-776.

Guilley, H., Dudley, R. K., Jonard, G., Balazs, E., and Richards, K. E. (1982) Cell, 30, 763-773.

Guilley, H., Richards, K. E., and Jonard, G. (1983) EMBO J., 2, 277-282.

Guilfoyle, T. J. (1980) Virology, 107, 71-80.

Hahn, P., and Shepherd, R. J. (1980) Phytopathol. Abst., 156.

Harrison, B. D., Finch, J. T., Gibbs, A. J., Hollings, M., Shepherd, R. J., Valenta, V., and Wetter, C. (1971) Virology, 45, 356-363.

Heyn, R. F., Hermans, A. K., and Schilperoort, R. A. (1973) Plant Sci. Letters, 2, 73-78.

Hills, G. J., and Campbell, R. N. (1968) J. Ultrastruct. Res., 24, 134-144.

Holmes, H. O. (1929) Bot. Gaz., 87, 39-55.

Hohn, T., Hohn, B., Lesot, A., and Lebeurier, G. (1980) Gene, 11, 21-31.

Howarth, A. J., Gardner, R. C., Messing, J., and Shepherd, R. J. (1981) Virology, 112, 678-685.

Howell, S. H., and Hull, R. (1978) Virology, 86, 468-481.

Howell, S. H., Walker, L. L., and Dudley, R. K. (1980) Science, 208, 1265-1267.

Howell, S. H., Walker, L. L., and Walden, R. M. (1981) Nature, 203, 483-486.

Hull, R. (1980) Virology, 100, 76-90.

Hull, R., and Covey, S. N. (1983) Trends. Biochem. Sci., 8, 119-121.

Hull, R., and Howell, S. H. (1978) Virology, 86, 482-493.

Hull, R., and Shepherd, R. J. (1977) Virology, 79, 216-230.

Hull, R., Shepherd, R. J., and Harvey, R. D. (1976) J. Gen. Virol., 31, 93-100.

Ish-Horowicz, D., and Burke, J. F. (1981) Nucleic Acids Res., 9, 2989-2998.

Kado, C. I. (1964) Virology, 24, 660-665.

Kongsvik, J. R., and Santilli, V. (1970) Virology, 40, 655-664.

Lebeurier, G., Hirth, L., Hohn, T., and Hohn, B. (1980) Gene, 12, 139-146.

Lebeurier, G., Hirth, L., Hohn, B., and Hohn, T. (1982) Proc. Nat. Acad. Sci., U.S.A., 79, 2932-2936.

Lung, M. C. Y., and Pirone, T. P. (1973) Phytopathology, 63, 910-914.

Lung, M. C. Y., and Pirone, T. P. (1974) Virology, 60, 260-264.

Maxam, A. M., and Gilbert, W. (1977) Proc. Natl. Acad. Sci., U.S.A., 74, 560-564.

Melcher, U., Gardner, C. O., Jr., and Essenberg, R. (1981) Plant Mol. Biol., 1, 63-73.

Melcher, U., Richards, K., Lebeurier, G., and Essenberg, R. C. (1983) Phytopathology; in press.

Menissier, J., Lebeurier, G., and Hirth, L. (1982) Virology, 117, 322-328.

Odell, J. T., and Howell, S. H. (1980) Virology, 102, 349-359.

Pfeiffer, P., and Hohn, T. (1983) Cell, 33, 781-789.

Radloff, R., Bauer, W., and Vinograd, J. (1967) Proc. Nat. Acad. Sci., U.S.A., 57, 1514-1521.

Richards, K. E., Guilley, H., and Jonard, G. (1981) FEBS Letters, 134, 67-70.

Russell, G. J., Follett, E. A. C., Subak-Sharpe, J. H., and Harrison, B. D. (1971) J. Gen. Virol., 11, 129-138.

Surkar, S. (1963) Virology, 20, 185-193.

Shepherd, R. J. (1970) Commonw. Mycol. Inst. Descriptions Plant Viruses, 24.

Shepherd, R. J. (1976) Adv. Virus Res., 20, 305-339.

Shepherd, R. J., and Wakeman, R. J. (1971) Phytopathology, 61, 188-193.

Shepherd, R. J., Wakeman, R. J., and Romanko, R. R. (1968) Virology, 36, 150-152.

Singer, B., and Fraenkel-Conrat, H. (1961) Virology, 14, 59-65.

Sutcliffe, J. G. (1978) Proc. Natl. Acad. Sci., U.S.A., 75, 3737-3741.

Szeto, W. W., Hamer, D. H., Carlson, P. S., and Thomas, C. A., Jr. (1977) Science, 196, 210-212.

Takahashi, W. N. (1956) Phytopathology, 46, 654-656.

Taylor, B., and Powell, A. (1982) Focus (BRL), 4, 4-6.

Toh, H., Hayashida, H., and Miyata, T. (1983) Nature, 305, 827-829.

Tompkins, C. M. (1937) J. Agric. Res., 55, 33-46.

Turner, D. S., and Covey, S. N. (1984) FEBS Letters, 165, 285-289.

Varmus, H. (1982) Science, 216, 812-820.

Volovitch, M., Drugeon, G., and Yot, P. (1978) Nucleic Acids Res., 5, 2913-2925.

Walden, R. M., and Howell, S. H. (1982) J. Mol. Appl. Genetics, 1, 447-456.

Walden, R. M., and Howell, S. H. (1983) Plant Mol. Biol., 2, 27-31.

Woods, D., Crampton, J., Clarke, B., and Williamson, R. (1980) Nucleic Acids Res., 8, 5157-5168.

Woolston, C. J., Covey, S. N., Penswick, J. R., and Davies, J. W. (1983) Gene, 23, 15-23.

Xiong, C., Muller, S., Lebeurier, G., and Hirth, L. (1982) EMBO J., 1, 971-976.

Yarwood, C. E. (1968) Phytopathology, 58, 132-136.

VITA 2

In Seong Choe

Candidate for the Degree of

Doctor of Philosophy

Thesis: CONSTRUCTION AND CHARACTERIZATION OF DELETION AND INSERTION MUTANTS IN REGION VI OF CAULIFLOWER MOSAIC VIRUS DNA

Major Field: Biochemistry

Biographical:

Personal Data: Born in Seoul, Korea, May 7, 1947, the son of Mr. and Mrs. Soon C. Choe; married Seong M. Kim in 1977; father of two children: Ann Sunah and Thomas Jayoung.

Education: Graduated from Kyunggi High School, Seoul, Korea, in 1966; received the Bachelor of Science degree in Agricultural Chemistry from Seoul National University in February, 1970; trained in the post-graduate course in Microbiology sponsored by Tokyo University, Tokyo, Japan, from 1976-1977; received the Master of Science degree in Agriculture from Seoul National University in February, 1978; fulfilled requirements for the Doctor of Philosophy degree in Biochemistry at Oklahoma State University in May 1984.

Professional Experience: Laboratory Assistant, Department of Agricultural Chemistry, Seoul National University, 1968-1970; Research Scientist, Applied Biochemistry Laboratory, Korea Institute of Science and Technology, 1970-1980; Graduate Research Assistant, Department of Biochemistry, Oklahoma State University, 1980-1984; Teaching Assistant, Department of Biochemistry, Oklahoma State University, September 1983-December 1983.