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STUDY OF ALLELE DOMINANCE IN CAULIFLOWER MOSAIC VIRUS DNA

BY

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STUDY OF ALLELE DOMINANCE IN CAULIFLOWER

MOSAIC VIRUS DNA

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I would like to dedicate this thesis to my parents for their love.

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ABBREVIATIONS

ATP - adenosine triphosphate

bp - base pair

BSA - bovine serum albumin

CabbS - Cabbage-S strain

CaMV - cauliflower mosaic virus

CIP - calf intestinal alkaline phosphatase

CTP - cytosine triphosphate

DNA - deoxyribonucleic acid

DTT - dithiothreitol

EDTA - ethylenediaminetetraacetic acid

GTP - guanosine triphosphate

2-Me - 2-mercaptoethanol

mins - minutes

mRNA - messenger RNA

RNA - ribonucleic acid

rpm - revolutions per minute

SDS - sodium dodecyl sulfate

SSC - saline sodium citrate

Tris - Tris(hydroxymethyl) aminomethane

tRNA - transfer RNA

TTP - thymidine triphosphate

CHAPTER I

INTRODUCTION

Cauliflower Mosaic Virus

Biological Properties

Cauliflower mosaic virus (CaMV) is a plant virus with isometric particles about 50 nm in diameter (Shepherd, 1970). It usually infects members of the *Cruciferae* family. Some strains also infect members of the Solanaceae or induce local necrotic lesions on *Datura stramonium* (Lung and Pirone, 1972). Recently, a strain of CaMV that causes chlorotic local lesions on *D. stramonium* has been found (Schoelz, 1986).

In general, inoculated leaves show mild symptoms consisting of chlorotic local lesions. Symptoms on the systemic leaves are more severe and include chlorotic mottling, vein-clearing, stunting of growth, and wrinkling of young emerging leaves (Shepherd, 1970).

In the cytoplasm of infected cells, virus particles are associated with granular, electron-dense structures called inclusion bodies (Shepherd, 1970).

CaMV is transmitted from plant to plant in nature by aphids. The virus can also be mechanically transmitted by rubbing the virus on to the plant leaves (Shepherd et al., 1968).

CaMV Genome and Its Products

The genome of CaMV consists of a circular, double-stranded DNA molecule (Shepherd et al., 1977), which is slightly over 8000 bp (Hull and Shepherd, 1977; Balazs et al.,1982).

CaMV DNA is unusual in that it contains three single-strand discontinuities which are refered to as 'gaps': G1, G2, and G3 (Volovitch et al., 1978; Hull and Howell, 1978). G1, which is located in the minus strand, is taken as the zero point of the conventional restriction map (Hohn et al., 1980). The two breaks in the plus strand, G2 and G3 are situated at 0.20 and 0.53 map units, respectively. The nucleotide sequence of CaMV reveals that these gaps are not single strand breaks, but rather that the 3' and 5' extremities of the interrupted strand overlap from 8 to 43 residues (Franck et al., 1980; Richards et al., 1981). Both 3' and 5' -termini of the gaps have free hydroxyl groups (Volovitch et al., 1978; Hull et al., 1979).

Eight open reading frames have been discovered in the plus strand (Franck et al., 1980; Hohn et al., 1982). Regions I through VI are the major open reading frames and are considered most likely to produce functional viral proteins. Regions VII and VIII are minor ones and are not thought to code for any important viral proteins. Each of these coding regions has a start codon close to its beginning and ends with a stop codon. The coding regions are packed closely together and many of them overlap (Balazs et al., 1982; Howell, 1982).

Little is known about the functions of regions I and III and their products. Both of these coding regions seem to be essential. The product of region II is the helper component for aphid transmissibility (Armour et al., 1983; Woolston et al., 1983). It is not essential for infection, as it can be either deleted or expanded by small insertions (Howell et al., 1981; Gronenborn et al., 1981; Dixon et al., 1983). Both deletion and insertion at

ORF II retard infectivity. The ORF II product , an 18Kd polypeptide, was found associated with viroplasms (Woolston et. al., 1983). This peptide was absent from the viroplasm preparations of leaves infected with the CM4-184 isolate of CaMV which lacks ORFII. Mutant SΔII with deletion of 105 bp at ORFII had a shorter peptide in the viroplasms. Both CM4-184 and SΔII were lose bound within the viroplasms (Givord et. al., 1984).

The viral coat protein is produced by region IV. This was confirmed by identification of coat protein antigens in lysates of *Escherichia coli* cells which contain plasmids with the region IV gene (Daubert et al., 1982).

Region V is thought to code for the viral reverse transcriptase. A portion of the predicted region V gene product is homologous to the amino acid sequence of the polymerase of Moloney murine leukemia virus, a retrovirus (Toh et al., 1983). Homology between CaMV gene V product and the protease and polymerase domains of retrovirus reverse transciptase is also described (Toh et al., 1983). There was no homology with the retroviral endonuclease region which has been implicated in integration (Panganiban and Temin, 1984). Analysis by activity gels of the proteins in replication complexes has shown that there are two polypeptides, 110K and 75K, with DNA polymerase activities (Pfeiffer and Hohn, 1984). The 110K polypeptide was identified as a host DNA polymerase. The 75K product might be the CaMV-specific reverse transcriptase. The strongest evidence that CaMV DNA replication involves reverse transcription comes from the cloning expression of the ORF V gene in the yeast *Saccharomyces cerevisiae* The reverse transcriptase accumulated to significant levels in yeast that have ORF V gene (Takatusuji et al., 1986).

In vitro translation studies showed that gene VI coded for a 62 Kd polypeptide, which was very similar by peptide finger print analysis to the

major protein component of virus inclusion bodies (Odell and Howell, 1980; Covey and Hull, 1981). Recent studies on CaMV genomic hybrids suggest that the ORF VI product is also involved in disease expression, including local symptoms of chlorosis and necrosis or systemic infection of the virus in *Solanaceous* hosts (Daubert et al, 1984). This host range determinant is controlled only by a 496-bp DNA segment of the first half of ORF VI (Schoelz et al., 1986).

Little is known about the functions of regions VII and VIII. Insertion or deletion within ORFVII did not interfere with viral infectivity (Dixon et al., 1983). Infectivity was retained only when the AUG initiation codon of ORFVII was in-phase with a termination codon upstream of the initiation codon of ORFI (Dixon and Hohn, 1984). A "relay race" mechanism for translation of ORFs VII and I from a polycistronic mRNA was proposed to interpret this fact.

CaMV Transcripts

Only the minus strand of the viral DNA is transcribed (Guilfoyle, 1980). This gives RNA sequences equivalent to those of the plus strand, which contains all of the potential coding regions.

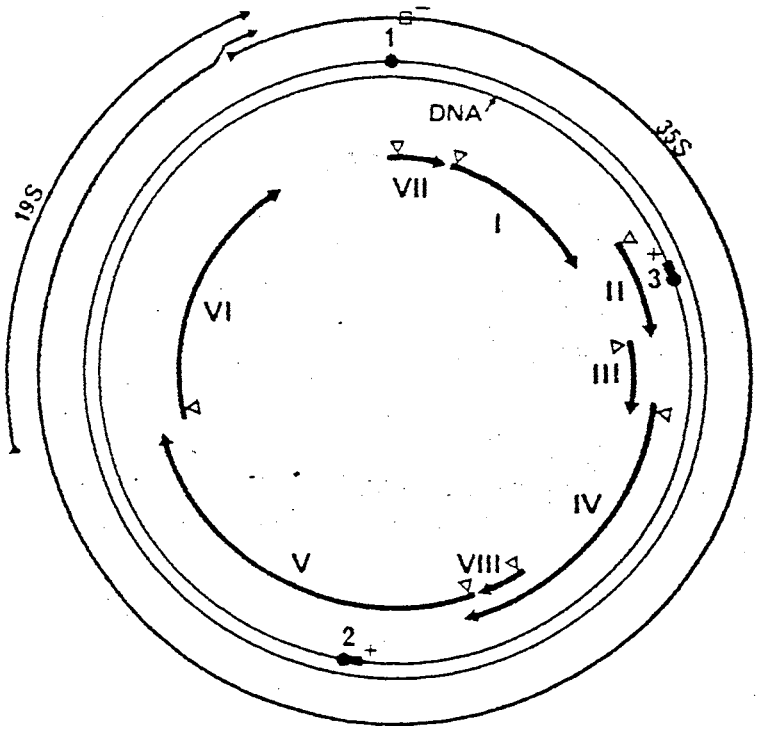
Two major polyadenylated transcripts termed the 19S and 35S RNA have been found. 19S RNA has an eleven nucleotide leader sequence transcribed from minus-strand DNA located immediately upstream of the AUG initiation codon of ORF VI. The 3'- end of the 19S RNA terminates about 400 nucleotides upstream of the DNA minus-strand discontinuity, G1 (Covey and Hull, 1981). So 19S RNA encompasses ORF VI and codes for the synthesis of the inclusion body protein in an in vitro system (Covey and Hull, 1981).

The 35S transcript begins at 0.93 map units 600 nucleotides upstream from G1, counterclockwise to G1, and ends at 0.95 map units (Covey et al, 1981). So 35S RNA is a transcript of the entire viral genome plus a direct repeat of 180 nucleotides at the 3' and 5' ends, thus resembling retroviral genomic RNA. The 35S RNA is considered to be the template for DNA synthesis.

CaMV Replication

A model for the replication of CaMV DNA has been proposed (Hull and Covey, 1983; Pfeiffer and Hohn, 1983; Hohn et al., 1985). When a DNA molecule gets into a cell, it becomes associated with nuclear proteins to form a minichromosome (Olszewski, 1982; Menissier et al., 1984) from which the 35S RNA is transcribed. This transcript is transported into the cytoplasm where it is reverse transcribed. A tRNA is thought to bind 600 bp downstream of the promoter of 35S RNA synthesis and act as a primer for DNA synthesis. Reverse transcriptase then synthesizes a DNA strand using 35S RNA as a template. Synthesis is towards the 5' end of the RNA and stops when it reaches the end of the molecule and RNase H degrades the transcribed part of the template. The directly repeated sequence at the 3' end of the DNA strand reanneals to the free analogous sequence at the 3' end of the original or another RNA template molecule and continues reverse transcription until the original minus primer binding site is passed . Plus strand DNA synthesis starts predominantly at two major plus primer binding sites (G2 and G3). Synthesis from G2 proceeds undisturbed to the 5' end of the minus strand DNA template and synthesis from G3 proceeds to region G2. This model explains pretty well the formation of gaps and various subcellular DNA forms found in infected plants, particularly molecules which

Figure 1. Genome structure of cauliflower mosaic virus. Viron DNA has one gap(1) in the (-) strand and two (2, 3) in the (+) strand. Open reading frames I-VIII are overlapped or packed together. 19S and 35S transcripts are also indicated.
from Covey, S.N., and Hull, R. 1985 Oxford Surveys of Plant Mol. and Cell Biol. 2:340


1
DNA
35S
19S
VII
I
II
3
VI
III
IV
V
VIII
2

are double-stranded with single-strand extensions of the plus-strand (Hull and Covey, 1983). A host cell $tRNA^{met}$ has been isolated from a CaMV preparation, where it was covalently linked to the 5'-end of the CaMV DNA fragment which is complementary to the CaMV 35S RNA transcript (Turner and Covey, 1984). A 14 nucleotide sequence of perfect homolgy with the 3'-end of plant $tRNA^{met}$ is present in the DNA minus-strand adjacent to G1(Pfeiffer and Hohn, 1983). The presence of a mixture of RNA and DNA templates is suggested by the partial inhibition of CaMV DNA synthesis by both RNAse and actinomycin D (Pfeiffer and Hohn, 1983).

Gene Conversion

Gene conversion is the nonreciprocal transfer of information from a donor to a recipient DNA duplex. Two major mechanisms have been proposed for gene conversion: heteroduplex repair (Meselson and Radding, 1975) and double strand gap repair (Orr-Weaver and Szostak, 1983). Both are thought to occur as a consequence of the mechanisms of genetic recombination (Radding, 1982; Orr-Weaver and Szostak, 1985). A brief description of the heteroduplex repair model follows(see Fig.2).

1. A single-strand break in one DNA molecule becomes the site of strand displacement by a DNA polymerase.

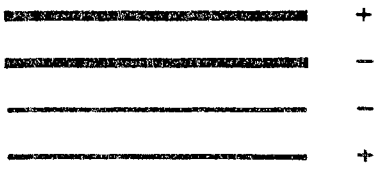
2. The displaced single-strand pairs with the complementary sequence in another molecule of DNA.

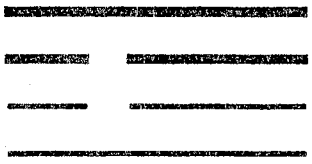
3. Branch migration extends the exchange region.

4. A one-strand crossover becomes a two-strand crossover by isomerization.

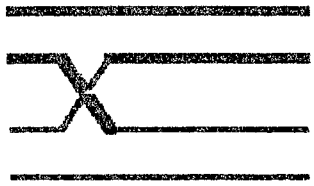
5. The joint molecule formed by strand exchange is resolved into two separate duplex molecules by a further pair of nicks.

Figure 2. Gene conversion model proposed by Meselson and Radding

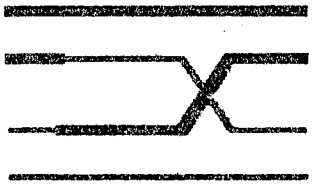




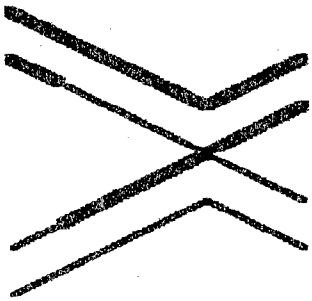
nicking



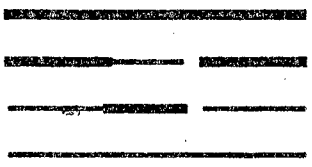
assimilation



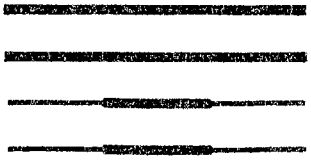
branch migration



isomerization



heteroduplex formation



mismatch repairing

6. Heteroduplex mismatch repair leads to gene conversion.

Evidence is accumulating favoring this model. *E.coli* polymerase could facilitate the creation of single strand breaks(nicks) with a 3'-hydroxyl terminus (Kelly, 1970). RecA protein is able to assimilate 3'-hydroxyl terminated strands (single or double) onto a complementary double strand (West et al., 1981). The experiment used a mixture of a single-stranded circle with a small fragment annealed to it and a duplex rod that was homologous to the double-stranded area on the circle but was slightly longer. When recA protein and ATP were added, the two duplex areas exchanged strands.

Recombination intermediates of strand crossover have been illustrated by Thompson et al. (1975). They observed figure-8 structures, which were formed when two circular molecules of DNA were linked by a two-strand crossover. Using conditions that cause partial denaturation, Valenzuela and Inman (1975) demonstrated that the two-strand crossover was joined at a homologous site. Benbow et al. (1975) also observed about a tenfold reduction of figure-8 formation in recA$^-$ *E.coli*. The existence of branch migration was concluded from the work of Thompson et al. (1976). They used a restriction endonuclease to convert figure-8 molecules of phage G4 DNA into X-Shaped structures. Migration of the crossover to the end of the X-form produced two linear monomers. By this way, they also measured the rate of branch migration. It appears that the crossover will move 850 nucleotides from its starting point in 8 mins.

The formation of heteroduplexes is not a stringent homologous pairing process. DasGupta and Radding (1981) showed that a few single base pair mismatches were not enough to inhibit the formation of heteroduplex DNA by the recA protein. However, three mismatches in a row were sufficient to

stop strand transfer (as was gross nonhomology). The mismatches in the heteroduplex region lead to gene conversion, if the mismatches are repaired before replication.

Mismatch repair consists of both excision and resynthesis of DNA by using one of the strands as template. Different directions of gene conversion has been observed in different systems. In *Ascobolus immersus* the longer strand is copied (Leblon, 1972), while in bacteriophage of *E. coli* the mismatched base in the longer strand is excised (Benz and Berger, 1973). The same occurs in pneumococcal transformation (Claverys et al., 1980).

In general, the strand-breaking event in the initiation of recombination may be random. However, it has been proposed that inverted repeats or palindromes are targets for cleavage and lead to initiation of strand exchange (Sobell, 1975; Wagner and Radman, 1975). Certain DNA sequences termed chi, naturally present in *E. coli* actually enchance recombination, both at regions neighbouring the sequence and at a distance (Stahl, et al.,1975). The existence of specific sites for initiation of strand exchanges is also implied by the polarities in gene conversion seen in *A. immersus* (Lissouba, 1961; Hamza et al., 1981), and the existance of mutants altered in the regulation of initiation of gene conversion (Angel et al., 1970).

In the gap repair model, a double-stranded gap spanning the non-homologous region of one duplex is created. The gap is filled using information from the intact double helix. Gene conversion in yeast transformation is probably associated with gap repair (Orr-Weaver et al., 1981).

Gene conversion has been observed both in prokaryotes and eukaryotes. During the transformation of *Diplococcus pneumoniae* by a strand of donor DNA that is heteroduplex and heterozygous, some markers transform

efficiently and others inefficiently (Tiraby and Sicard, 1973). Gene conversion happens in a number of transfections by heteroduplex DNAs made in vitro. Examples of correction of heteroduplex DNA are øX174 (Bass and Jansz, 1975) in *E. coli*, phage SPP1 DNA in *Bacillus subtilis* (Spatz and Trautner, 1970), SV40 in African green monkey cells (Lai and Nathans, 1975) and polyoma DNA in mouse embryo cells (Miller et al., 1976).

Allele Dominance in CaMV

CaMV produces virion DNA by reverse transcription as described above. It is widely held that reverse transcription can account for genetic interactions observed between CaMV DNAs. Examination of the DNA sequence of CM4-184 revealed that this strain is a chimera of CM1841 and a CabbS-like strain and probably arose by strand switch between the RNA templates of two virus strains during reverse transcription(Dixon et al., 1986). Grimsley et al. (1986) used a hybrid plasmid, containing tandemly arranged pieces of two different but well-defined CaMV DNAs to study the mechanism by which infectious viral DNA can escape from transforming DNA. They showed that the majority of viral progeny were probably descendants of DNA produced by intramolecular template switching during viral reverse transcription. The hot spot for template switching is very close to the 5' end of the terminal repeat on the 35S RNA molecule. They also detected some minority descendants arising from products of recombination between homologous regions of CaMV DNA.

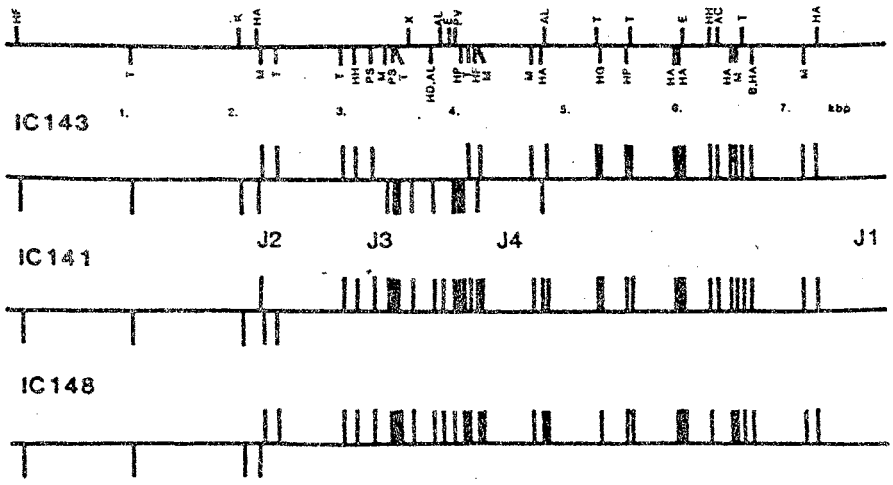
Other evidence of CaMV DNA undergoing homologous recombination came from studies of the infectivity of some partial nested dimers. Partial nested dimer pLW113D-A, which can not be directly transcribed to a full length 35S RNA, is infectious (Walden and Howell, 1983), while partial

dimer pUM122, which can be transcribed to 35S RNA, is only weakly infectious (Melcher et al., 1986). Thus the infectivity of partial nested dimers must be due to intramolecular recombination to produce an entire monomer.

Virion DNAs extracted from plants inoculated with a pair of mutant DNAs of different CaMV isolates have been characterized (Choe et al., 1985). Each recombinant analyzed had two (the minimum expected) or four junctions between parental alleles. One of the junctions between parental sequences occurred near the 35S RNA start site, consistent with recombination having occurred during reverse transcription. However, two junctions (J2 in IC141 and J4 in IC143) exhibited a mixture of parental alleles in small stretches of sequence (fig.3). If the alternating alleles were due to strand switching during reverse transcription, the enzyme would have to switch strands several times at a hot spot for switching. The random junctions between parental sequences in retroviral recombinants (Coffin, 1979) argue against hot spots in switching. The alternation pattern of parental alleles in the CaMV recombinants can be explained better by the repair in small patches of heteroduplexes created during recombination.

Melcher and Essenberg (1985) have observed allele dominance at the KpnI site, which is quite near hot strand switching junction J2. UM130 DNA is a variant of CabbS DNA obtained by putting an extra 12bp containing an EcoRI site in the KpnI site. Since the reading frame is preserved, UM130 is as infectious as CabbS DNA as judged by the time of appearance of symptoms on turnip plants, the severity of symptoms and the yield of virus from infected plants. The additional EcoRI site is maintained during growth of the virus in the plants. But when it was coinoculated with CabbS on turnip leaves, all progeny virus had the CabbS EcoRI pattern . It happened even when three fold more UM130 than CabbS DNA was inoculated and was

Figure 3. CabbS-W sequence junctions in CaMV DNA recombinants. from Choe, I.S., Melcher, U., Richards, K., Lebeurier, G., and Essenberg, R.C. 1985 Plant Mol. Biol. 5: 281-289


IC143
IC141
IC148
J2
J3
J4
J1
1.
2.
3.
4.
5.
6.
7.
kbp

highly reproducible (20/20 plants). Most strikingly, allele dominance occurred even when the CabbS DNA was given a competitive disadvantage. When pUM24, a CabbS DNA derivative which is not infectious due to a mutation in ORF VI, was mixed with pUM130, complete conversion to the CabbS pattern was observed in 8 of 10 plants.

The goals of the present study were to construct other infectious mutants by linker insertion and to test the effect of sequence and site of heterologies on their susceptibility to gene conversion.

CHAPTER II

MATERIALS AND METHODS

Linker Preparation

Linker Phosphorylation

XhoI and KpnI linkers purchased from Collaborative Research, Inc., were obtained nonphosphorylated. Linkers were heated to 90°C for 5 mins and cooled slowly to room temperature. 30 pmol XhoI linker and 33 pmol KpnI linker were mixed separately with 0.02 µmol ATP, 15 pmol ^{32}P-ATP with 20 µCi (from Dupont), and 10 units polynucleotide kinase in Linker kinase buffer (Table I) at volume of 20 µl, and reacted at 37°C for 15 mins. 0.01 µmol ATP and 10 units kinase in 10 µl of Linker kinase buffer were added before a second incubation for 15 mins. The preparation was stored at -20°C. The final concentration was 11.3 pmol/µl for the XhoI linker and 10.0 pmol/µl for the KpnI linker.

Test for ligatability of linkers.

57 pmol and 50 pmol of the above phosphorylated XhoI and KpnI linkers were used in a self ligation reaction with 10 units T4 DNA ligase in ligation buffer (table I) at 37°C for 4 hours.

28 pmol or 25 pmol of the above ligated phosphorylated XhoI or KpnI linker was digested with 10 units XhoI or KpnI restriction enzyme at 37°C for 1 hour.

TABLE I

ENZYME BUFFER

CIP buffer	Ligation buffer
0.05 mM Tris-HCl, pH 9.0 1 mM $MgCl_2$ 0.1 mM $ZnCl_2$ 1 mM spermidine	66 mM Tris-HCl, pH 7.6 5 mM $MgCl_2$ 5 mM DTT 1 mM ATP
Linker kinase buffer	**Reverse Transcriptase buffer**
70 mM Tris-HCl, pH 7.6 10 mM $MgCl_2$ 5 mM DTT	40 mM Tris-HCl, pH 8.0 50 mM KCl 5 mM $MgCl_2$ 5 mM DTT

40 pmol phosphorylated linker, 25 pmol ligated linker and 25 pmol digested linkers were electrophoresed on 10% acrylamide gel in 0.5 X TBE buffer (Table II). The electrophoresis was terminated when the bromophenol blue marker moved to the middle of the gel. X-Ray film (Kodak) was exposed to the gel for 10 hours. On developed film, a ladder of bands shows the linker was phosphorylated and ligatable.

Parental plasmids

pCS101 is the plasmid which has CabbS DNA cloned at SalI site of pBR322. pUM 41 was constructed by digestion of pCS101 at the KpnI site, flushing the ends with T4 exonuclease-polymerase followed by ligation of an 8bp SmaII linker. The CaMV DNA in pUM41 is non-infectious probably due to frame shift.

SΔII, pCa-BB1 and Ca-NB2 are the other plasmids I used to check allele dominance in ORFII. SΔII has a deletion within the ORFII from nucleotide 1537 to 1643bp of CabbS DNA (Givord et al., 1984). Ca-BB1 is derived from CM4-184 with whole ORFII deleted and XhoI(9bp) linker inserted in its place. Ca-NB2 has a 234bp dihydrofolate reductase gene inserted at the XhoI site of Ca-BB1 (Brisson et al., 1984). SΔII was obtained from G. Lebeurier and pCa-BB1 and Ca-NB2 from T. Hohn.

Mutant Construction

Restriction enzyme digestion

Restriction enzyme digestion of DNA was carried out in the appropriate buffer (Table III). All reaction mixtures were supplemented with 100µg/ml BSA.

TABLE II

ELECTROPHORESIS BUFFER

Alkaline electrophoresis buffer	Loening buffer
30 mN NaOH 1 mM EDTA	36 mM Tris base 1 mM EDTA free acid 30 mM NaH_2PO_4
TAE buffer	**TBE buffer**
40 mM Tris Acetate 2 mM EDTA	10.8 g/l Tris base 5.5 g/l Boric acid 0.2 mM EDTA

Table III

RESTRICTION ENZYME ASSAY BUFFERS

Asp718	ClaI
100 mM NaCl 6 mM Tris-HCl, pH 8.0 6 mM $MgCl_2$ 6 mM 2-Me	50 mM NaCl 6 mM Tris-HCl, pH 7.9 6 mM $MgCl_2$ 6 mM 2-Me
EcoRI	**KpnI**
50 mM NaCl 100 mM Tris-HCl, pH 7.2 5 mM $MgCl_2$ 6 mM 2-Me	6 mM NaCl 6 mM Tris-HCl, pH 7.4 6 mM $MgCl_2$ 6 mM 2-Me
SalI	**SmaI**
150 mM NaCl 6 mM Tris-HCl, pH 7.0 6 mM MgCl2 6 mM 2-Me	25 mM KCl 25 mM Tris-HCl, pH 7.8 10 mM $MgCl_2$ 2 mM 2-Me
XbaI	
100 mM NaCl 6 mM Tris-HCl, pH 7.4 6 mM $MgCl_2$	

All buffers were supplemented with 100µg/ml BSA.

Plasmid pXZF is derived from pCS101 and pXZG from pUM41. 10µg pCS101 DNA was digested with 40 units XhoI enzyme in 200µl volume under standard condition (SalI assay buffer) at 37°C for more than 4 hours. 10 µg pUM41 DNA was digested with 40 units of SmaI in 200 µl volume. 2.5 µl of each reaction mixture was mixed with stop reagent (50% w/v glycerol, 1 mM EDTA, and 1% w/v bromphenol blue, pH7.0) and electrophoresed on a 1.0% agarose gel in Loening buffer (Table II)to check whether digestion was complete.

Phenol extraction and ethanol precipitation

1/50 volume of 5 M NaCl, 1/10 volume of of 1.0 M Tris pH8.0, and 1 volume of water saturated phenol were added to each digestion mixture. The mixtures were vortexed and micro-centrifuged 5 mins to separate two phases. The aqueous phases were transferred to other eppendorf tubes and traces of phenol in them were extracted by ether. After removal of the ether, 2.5 volumes of 95% ethanol was added to precipitate the DNA overnight at -20°C or one hour at -70°C. After 5 mins micro-centrifugation to separate the DNA pellet from solution, the DNA pellet was washed by adding 0.5 ml of 75% ethanol and micro-centrifuging for 5 mins. The pellet was dried under vacuum.

End filling

The dry pCS101 DNA pellet was dissolved in 20 µl of reverse transcriptase buffer (Table I) with each 0.02 µmol of dATP, dGTP, dCTP and dTTP, and10 units reverse transcriptase added.The reaction was allowed to proceed for 30 mins at 37°C. H_2O was added to make 200 µl total volume. Phenol

extraction, ethanol preciptation, and drying was done as above. The dry pellet was dissolved in 40 µl H_2O .

Dephosphorylation

Dephosphorylation was done as described by Maniatis et al., (1982). 5 unit of CIP in 50 µl CIP buffer (Table I) was added to dephosphorylate DNA at 37°C for 15 mins and 56°C for another 15 mins. 5 unit of CIP was added and the mixture incubated for a second time as above. The solution was phenol extracted as described above. During ethanol precipitation, 25 µl of 7M NaOAc was added to increase efficiency of ligation in the next step.

Ligation

Dry DNA (2.5 pmol ends) was combined with 50 pmol of phosphorylated KpnI linker (1:20 ratio) and 10 units T_4DNA ligase in ligase buffer at volume of 20 µl and incubated at 4°C overnight. 2 µl of the ligation mixture was electrophoresed on an agarose gel to check whether the ligation reaction had worked.

Redigestion

The pCS101 and KpnI linker ligation mixture was diluted to 200 µl and digested with XhoI at 37°C for 2 hours, while pUM41 and XhoI linker ligation mixture was digested with SmaI. 20 µl of aliquots each were electrophoresed on a 1.0% agarose gel.

The DNA preparations went through phenol extraction, ethanol precipitation, and drying. The final DNA was stored in 100 µl TEN buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, and 10 mM NaCl), ready for transformation.

Transformation

Competent cell(*E.coli* K-12 strain HB101) for transformation were prepared by the method of Morrison(1979). After deep-frozen competent cells were thawed in ice water for 10 mins, 1 µl or 10 µl DNA was added to 80 µl of the cell suspension. The mixture stood in ice water for 30 mins, was transferred to 42°C for 2 mins of heat shock, cooled in ice for 2 mins, and incubated 30 mins at 37°C with 200 µl nutrient broth (1.0% tryptone, 0.5% yeast extract, and 1.5% NaCl) added. Finally , 200 µl of the mixture was spread onto an ampicillin antibiotic plate (20 µg/ml) for overnight growth at 37°C. The parental plasmids and ligated dephosphorylated plasmid were used as controls for each transformation to test that both transformation and dephosphorylation worked properly.

Small scale plasmid isolation suitable for DNA digestion

Transformant colonies were streaked to another antibiotic plate and grown overnight at 37°C. Colonies were inoculated in 5ml nutrient broth with 20µg/ml ampicillin. After shaking at 37°C overnight, each1ml culture was harvested in a 1.5ml Eppendorf tube and used for isolating plasmid DNA as described by Ish-Horowicz and Burke (1981). Resuspended in 100 µl of Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA), the bacterial cells were lysed in 200 µl of Solution II (0.2 M NaOH and 1% SDS) at 0°C for 5 mins. 150 µl of Solution III (3.0 M KOAc and 2.0 M acetic acid) was then added to precipitate denatured protein and bacterial chromosomes for 5 mins. After 5 mins micro-centrifugation, 400 µl supernatant was transfered to another tube containing 800 µl cold 95% ethanol to precipitate DNA. A DNA pellet was obtained by 5 mins micro-centrifugation and washed

once with 75% ethanol . The final dried DNA was redissolved in 30 µl of TEN buffer.

Screening for plasmid containing linkers

XhoI-SalI double digestion and KpnI-SalI double digestion were used for screening pXZF. XhoI-SalI digestion was carried out in 25 µl reaction mixture with XhoI and SalI enzyme together at 37°C for 1 hour. For KpnI-SalI double digestion , the KpnI reaction was performed first in a 20µl volume at 37°C 1 hour. 5 µl of a mixture of 2.5 µl of 5M NaCl, 2µl of 10 unit/µl SalI and 95.5 µl H_2O was added and incubation continued for another 1 hour. Reaction was stopped by adding stop reagent prior to gel electrophoresis.

XhoI-SalI and SmaI-SalI double digestions were used to screen for pXZG. XhoI-SalI digestion was done as described above. For SmaI-SalI digestion, the reaction was first carried out in SmaI for 1 hour in 20 µl. Then 5 µl of 2.4 µl 5M NaCl , 1.0µl 10unit/µl SalI, 96.6µl H_2O was added for SalI digestion.

Bacterial Cell Storage

0.85ml fresh bacterial culture and 0.15ml glycerol were mixed in a vial and placed at -70°C . To check that the bacteria were alive, the surface of the frozen material was scratched with a sterile needle and streaked onto the ampicillin plate(20µg/ml) for overnight growth.

Large Scale Plasmid Isolation

5ml of bacterial culture grown from a single colony was transferred to 250 ml nutrient broth containing 20µg/ml ampicillin and subjected to 16-20 hours vigrous shaking at 37°C.

Plasmids were isolated by an alkaline lysis method(Maniatis et al., 1982). Bacterial cells were harvested by centrifugation in a J-14 rotor (Beckman) at 8 Krpm for 10 mins and resuspended in 5 ml solution I. 10 ml solution II was added to lyse the cells and 7.5 ml solution III to precipitate proteins and bacterial chromosomes. Plasmids were separated from cell debris by centrifugation at 12 Krpm for 15 mins in a J-21 rotor (Beckman). To 18 ml plasmid containing liquid, 12ml isopropanol was added at room temperature and the mixture allowed to stand for 15 mins. Plasmid DNA was pelleted by 12 Krpm centrifugation for 10 min in a J-21 rotor (Beckman) and resuspended in 4.0 ml H_2O.

Plasmid Purification

Plasmid DNA obtained by the alkaline lysis method was further purified by cesium chloride (CsCl) gradient centrifugation. 4.4 g CsCl, 0.4ml ethidium bromide (10mg/ml) and 4.0ml plasmid containing solution was centrifuged at 65Krpm for overnight in a VTi65 rotor (Beckman). The plasmid DNA band (the lower of two bands) was collected with an 18-G sized needle attached to a syringe. A volume of water equal to twice the volume of the plasmid DNA containing CsCl solution and a volume of ethanol equal to twice the total diluted volume were added sequentially. The mixture was stored at -20°C overnight. After centrifugation at 10 Krpm for 15 mins in a J-14 rotor, DNA pellet was saved and resuspended in 200µl TEN buffer. Ethidium bromide was removed from the DNA by phenol extraction in semi-darkness. The DNA was then precipitated with ethanol overnight at -20°C and redissolved in 200µl TEN buffer .

DNA was diluted and the absorbance at 260 and 280nm read. DNA concentration in µg/ml equals A260 X dilution factor X 50. DNA purity was determined by A260/A280, which should be close to 2 for pure DNA.

CaMV DNA Inoculation of Plants

Turnips (*Brassica rapa* L. cv. Just Right) were used as hosts. Plants were grown in a growth chamber with 12 hour of light and 12 hours of dark. The temperature for these two periods was approximately 72ºF and 66ºF, respectively. The plants were watered daily and fertilized twice a week. Before inoculation, plasmid DNA was digested with SalI to release viral DNA from the vector. 20 µl of inocula containing 20mg/l of each plasmid, 2 X SSC (0.3 M NaCl and 30 mM Na_3citrate) and 2g/l celite were rubbed with a gloved finger onto three-and-a half week old turnip leaf, three leaves per plant.

CaMV Virion Isolation from Leaves.

The procedure was done by following Hull et al.'s method (1976) with some modifications. After 3-4 weeks of infection, plant leaves were homogenized in a Waring blender in 400ml virus isolation buffer (68.05 g monobasic potassium phosphate ca 750ml water, pH adjust by adding KOH pellet, q.s. 1000ml with water, and 7.5 g sodium sulfate). The homogenate was filtered through cheesecloth and the filtrate stirred overnight in the presence of urea(60mg / ml) and triton-X-100 (25µl/ml). The homogenate was centrifuged in a J-21 rotor (Beckman) at 5,000 rpm for 10 mins. The supernatant was collected and centrifuged at 18.5 Krpm for another 3 hours in a Ti45 rotor (Beckman). The pellet was resuspended in 1.0 ml glass-distilled water and centrifuged at 7,000rpm for 10 mins in a J-21 rotor.The

supernatant was layered onto a continuous sucrose gradient made from 10% and 40% sucrose and centrifuged in a SW 25.1 bucket rotor (Beckman) at 22.5 Krpm for 3 hours at 4°C. The viral band was recoved by puncturing the side of the tube with an 18 G syringe-needle. An equal volume of water was added to the viral suspension and the mixture centrifuged at 45 Krpm for 1 hour in a Ti75 rotor (Beckman). The viral particles were finally stored in 2.0 ml sterile water at -20°C.

50µl virus solution was diluted in 0.95ml H_2O and absorbance at 260 and 280nm read. The concentration (mg/ml) was calculated as (A260 X dilution factor)/7. The virus yield (mg/Kg) was determined by concentration X 2ml / weight of leaves (kg).

CaMV Virion Inoculation of Plants

20 µl of 2 µg /ml viral pericles in 1% K_2HPO_4 and 2g/l celite was rubbed onto three-week-old turnip leaves, three leaves per plant.

CaMV DNA Isolation from Leaves.

Viral DNA was prepared according to the method of Gardner and Shepherd (1980). 2 gm of leaf tissue from plants infected for 3 to 4 weeks was weighed , ground in liquid nitrogen and suspended in 10ml TEU solution (0.2 M Tris-HCl, 0.02 M EDTA and 1.5 M urea). The contents were poured into a 30ml Corex tube and the mortar was rinsed with 2ml 10% triton X-100. After centrifugation at 10 Krpm for 15 mins in a J-14 rotor (Beckman), the supernatant was filtered through Micracloth, layered onto a 2.0 ml TEU-sucrose layer (7.5 g sucrose, 10ml 10% triton X-100 to 50ml with TEU), and centrifuged 34 Krpm for 2.5 hours in a Ti75 or Ti65 rotor. The liquid was

removed by using a pasteur pipet attached to a vacuum aspirator. 0.2 ml viral resuspension buffer (0.1 M Tris-HCl, pH 7.5 and 2.5 mM $MgCl_2$) with 2ul of 1mg/ml DNase were added to the pellet and the mixture incubated at 37°C for 10 mins. 4 µl 0.5M EDTA, 50 µl 2.5mg/ml proteinase K, 12.5 µl 20% SDS were added and incubation was continued at 65°C for 10 mins. The reaction was stopped by phenol extraction by adding 30 µl 1M Tris-HCl, pH 8.0, 6 µl 5M NaCl, 300 µl phenol. DNA was precipitated with 0.75ml 95% ethanol at -20°C overnight and dissolved in 30 µl TEN buffer.

Alkaline Denaturing Gel

An alkaline agarose gel was used to examine single stranded DNA caused by gaps in CaMV DNA. 0.4 g of agarose was add to 40 ml water and heated until the agarose dissolved. When the contents cooled to about 50-60°C, 0.4 ml of 100 X alkaline electrophoresis buffer was added and the mixture poured into a gel mold. Before loading the samples, the gel was soaked in alkaline electrophoresis buffer (Table II) about 30 minutes. About 500ng DNA was loaded with alkaline loading buffer (0.2 N alkali(NaOH or KOH), 5% glycerol and 0.025% bromphenol blue) and electrophoresis was performed at 50 volts for the desired time.

Restriction Enzyme Digestion

XhoI -SalI, and Asp718 - EcoRI digestions; XhoI - SalI, and EcoRI digestions; Asp718 - EcoRI, and XhoI - EcoRI ; EcoRI, and Asp718 - EcoRI digestions were used to characterize progenies of CabbS and XZG, UM130 and XZG, CabbS and XZF,and UM130 and XZF coinoculation, respectively. 2 µl DNA preparation from leaves in 20µl reaction solution was used at 37°C for 1 to 2 hours. XhoI-SalI digestion was carried out in SalI buffer at the same

time, as mentioned. For Asp718 - EcoRI double digestion, Asp718 digestion was performed first and 10 µl of 25 µl 1M Tris-HCl, pH 7.0, 75 µl H_2O , 20 units of EcoRI were added for the second reaction. 1.5% agarose gels in TAE buffer or 1.0 % agarose gels in Loening buffer were used for electrophoresis.

In addition, EcoRI digestion was performed for the viral DNA isolated from plants coinoculated with SΔII and CabbS or SΔII and NY8153. ClaI and XbaI digestion were used for Ca-BB1 vs Ca-NB2 and NY8153 vs Ca-BB1 progeny DNA, respectively.

CHAPTER III

RESULTS

pXZF and pXZG

Construction and structure

XZF and XZG were designed to have 12bp insertions at unique sites of ORFII and III of CabbS, for maintaining their infectivity. When pCS101 was digested at its XhoI site, the sticky ends of 4bp filled and ligated with an 8bp KpnI linker, pXZF was formed (Fig. 4). The construction of pXZG was started from pUM41, which had 4bp added at KpnI site of pCS101 by cutting off the sticky ends of the KpnI site with T4 exonuclease-polymerase and ligating with an 8bp SmaI linker. Digestion at the SmaI site of pUM41 and directly ligating with an 8bp XhoI linker resulted in pXZG (Fig. 5).

To reduce self-circularization of the parent plasmid during the ligation raction, the linear plasmid was dephosphorylated. T4 DNA ligase will not ligate DNA fragments lacking a 5' phosphate group. Since our commercial linkers are also non-phosphorylated, the linkers had to be phosphorylated before their use. Phosphorylation of the linker with 32P-ATP also provided a way to test whether the linker was ligatable or not. When good ligated linker is exposed to ligase, a ladder pattern should be seen on autoradiographs of gel electrophoresis of the products. I found that not all commercial linkers are ligatable under standard conditions.

Figure 4. Construction of KZF.

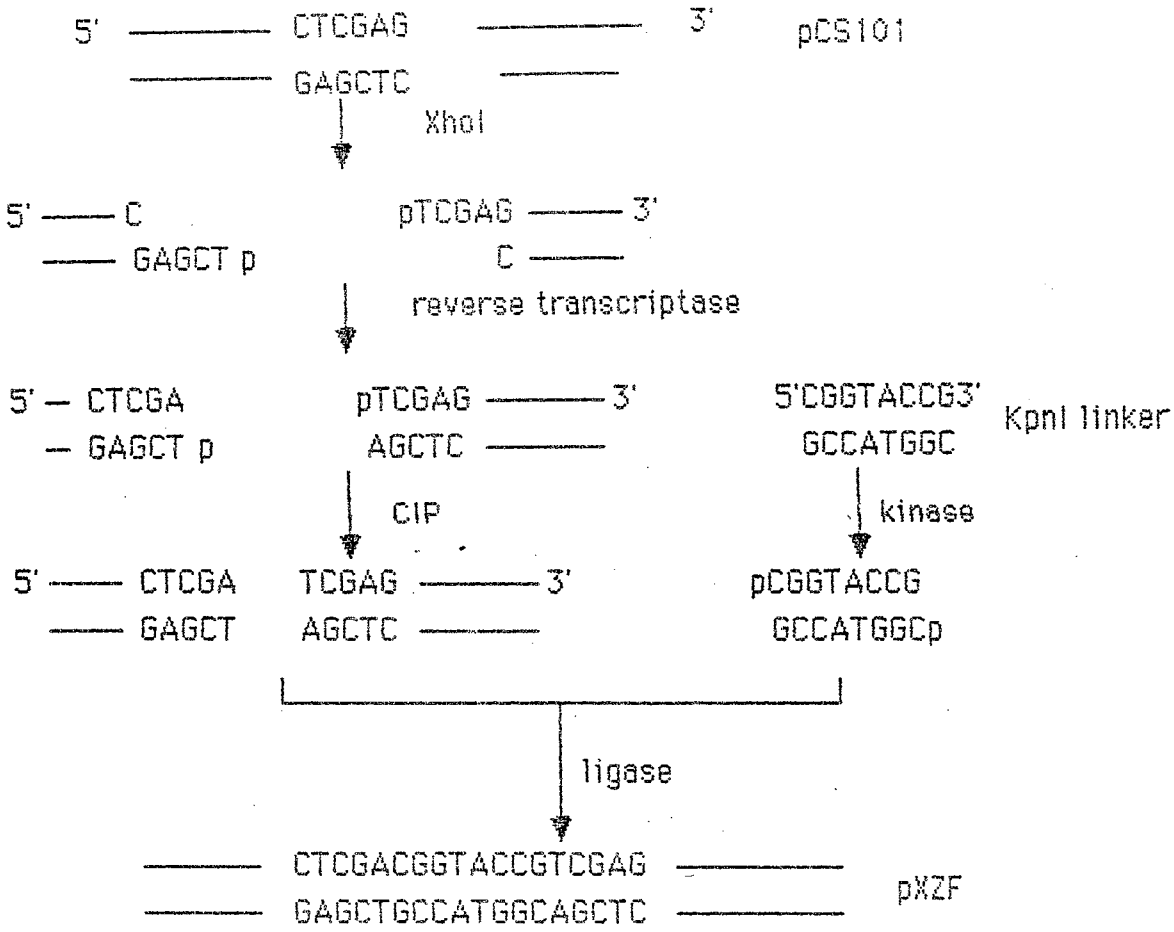
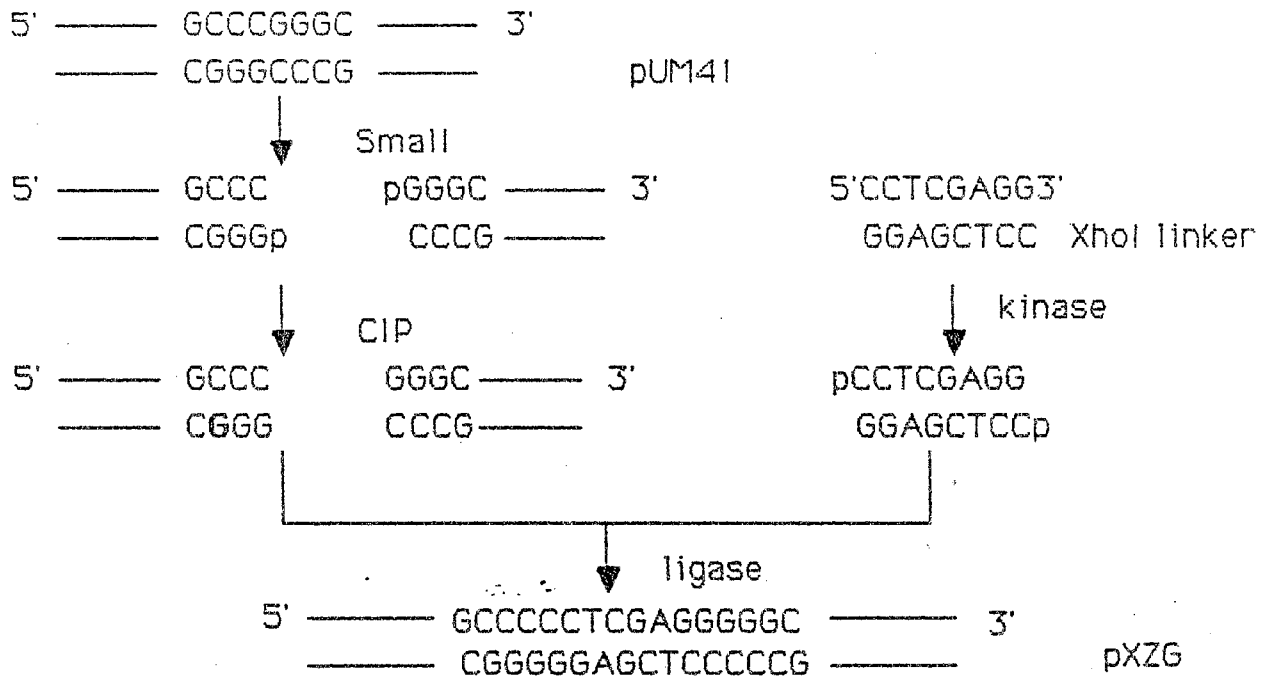

5' —— CTCGAG —— 3'
—— GAGCTC ——
pCS101
XhoI
5' —— C
—— GAGCT p
pTCGAG —— 3'
C ——
reverse transcriptase
5' — CTCGA
— GAGCT p
pTCGAG —— 3'
AGCTC ——
5'CGGTACCG3'
GCCATGGC
KpnI linker
CIP
kinase
5' —— CTCGA
—— GAGCT
TCGAG —— 3'
AGCTC ——
pCGGTACCG
GCCATGGCp
ligase
—— CTCGACGGTACCGTCGAG ——
—— GAGCTGCCATGGCAGCTC ——
pXZF

Figure 5. Construction of XZG


5' GCCCGGGC 3'
CGGGCCCG
pUM141
Small
5' GCCC pGGGC 3'
CGGGp CCCG
5'CCTCGAGG3'
GGAGCTCC XhoI linker
kinase
CIP
5' GCCC GGGC 3'
CGGG CCCG
pCCTCGAGG
GGAGCTCCp
ligase
5' GCCCCCTCGAGGGGGC 3'
CGGGGGAGCTCCCCCG
pXZG

Another way to increase linker insertion efficiency is to redigest the plasmid after ligation. Plasmids containing linkers will not be cleaved and will remain as relaxed circular forms, while self-ligated parental plasmids will be cleaved into linear molecules that do not transform bacteria in the next step. After redigestion a relaxed circle plasmid band and a linear plasmid band were seen on agarose gel electrophoresis. That was interpreted to mean that I had linker insertion in some plasmids.

After bacteria were transformed with the above ligation mixture, single colonies were picked and grown in broth. Plasmids were isolated from small bacterial cultures.

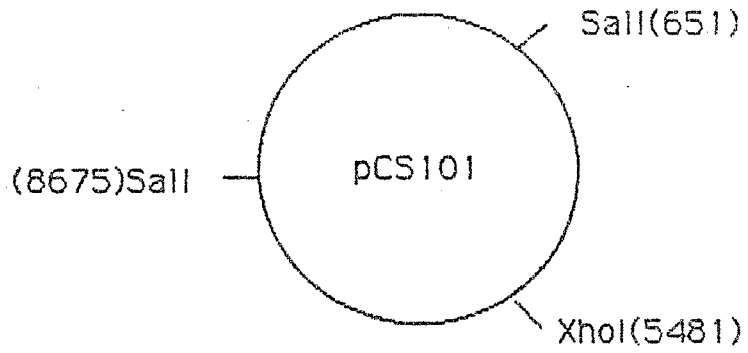
XhoI-SalI, and KpnI-SalI were used to screen for pXZF (fig.6). Since there is no XhoI site at 5481 in pXZF, XhoI and SalI digestion will give two fragments of 4363 and 8032bp , instead of three fragments of 3194, 4363 and 4838bp as in the parental plasmid pCS101. Eight out of 12 plasmids screened had the expected pXZF pattern of 4363 and 8032bp fragments (fig. 7). To ensure that a KpnI linker was inserted at 5481, KpnI and SalI digestion was performed. The additional KpnI linker at the 5481 site (fig.8) will give two shorter fragments of 5170 and 389bp in KpnI and SalI digestion, while for pCS101 it is 5228bp. The result is shown in figure 9.

XhoI-SalI digestion of pXZG should produce fragments of 4830, 4363, 2796 and 398bp , while 4830, 4363 and 3194 bp fragments are produced from parental pUM41 (fig.10). 3 out 10 plasmids screened had pXZG pattern (fig. 11).

Infectivity and symptoms

Both XZF and XZG were infectious. Plants inoculated with XZG showed symptoms as early as plants inoculated with CabbS (about 2 weeks post

Fig. 6. XhoI, SalI restriction map of pCS101 and pKZF


SalI(651)
(8675)SalI
pCS101
XhoI(5481)

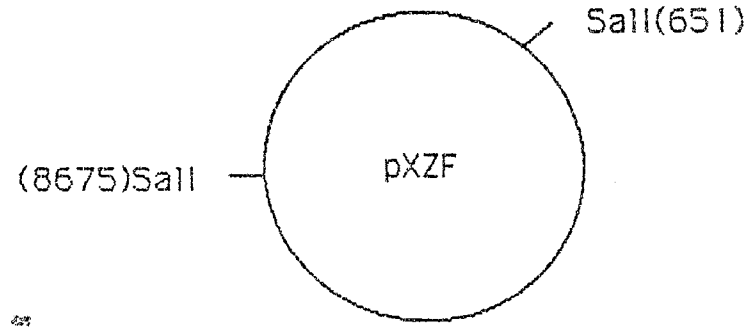

SalI(651)
(8675)SalI
pXZF

Figure 7. XhoI and SalI digestion: screening for pXZF. Lane 1 to 12, DNA from transformants. Lane 13, pCS101. The digests were electrophoresed on a 1.0% agarose gel.

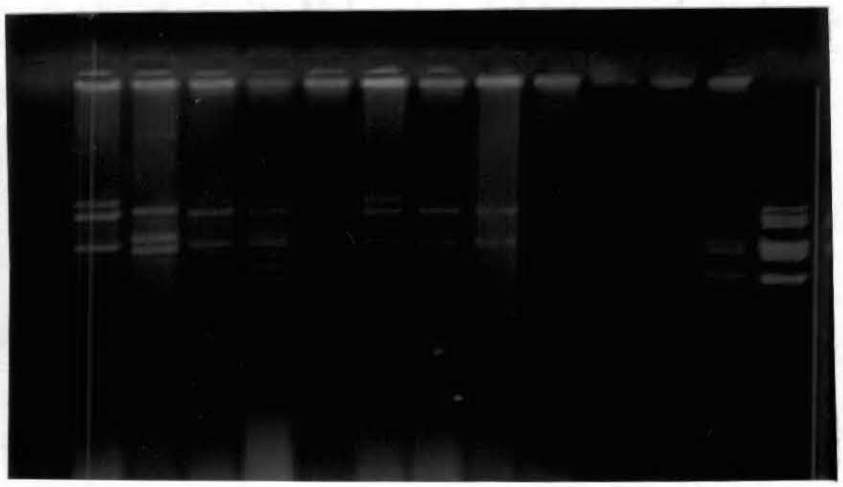
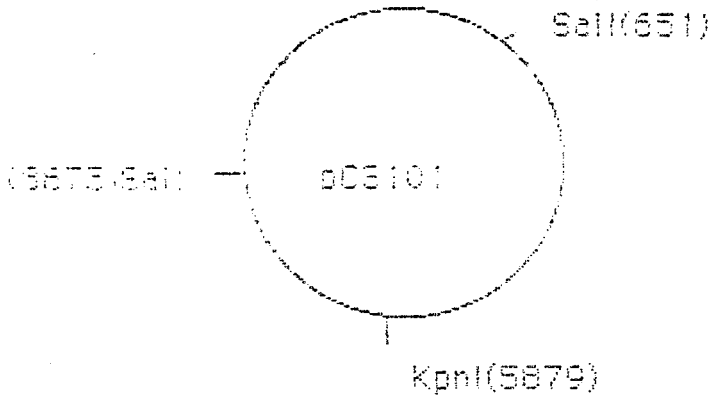

4363
3194
1 2 3 4 5 6 7 8 9 10 11 12 13

Fig. 8. KpnI, SalI restriction map of pCS101 and pXZF


SalI(651)
(5875)SalI
pCG101
KpnI(5879)

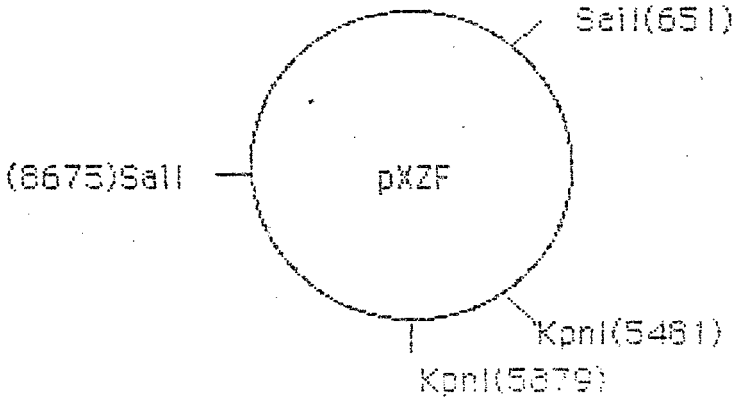
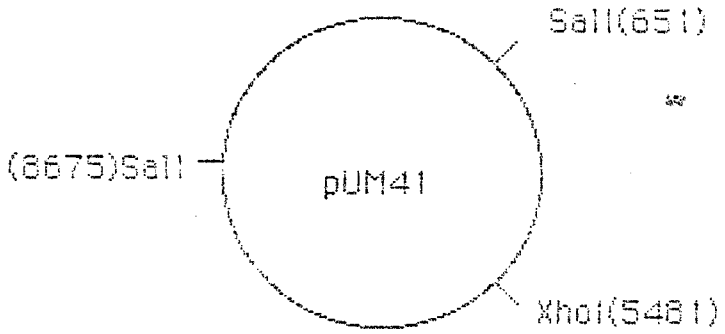

SalI(651)
(8675)SalI
pXZF
KpnI(5461)
KpnI(5879)

Figure 9. KpnI and SalI digestion of pCS101 and pKZF. Lane 1, pKZF. Lane 2, pCS101. Electrophoresis on a 1.5% agarose gel.


5170
5228
1
2


5170
5228
1
2


SalI(651)
(8675)SalI
pUM41
XhoI(5481)

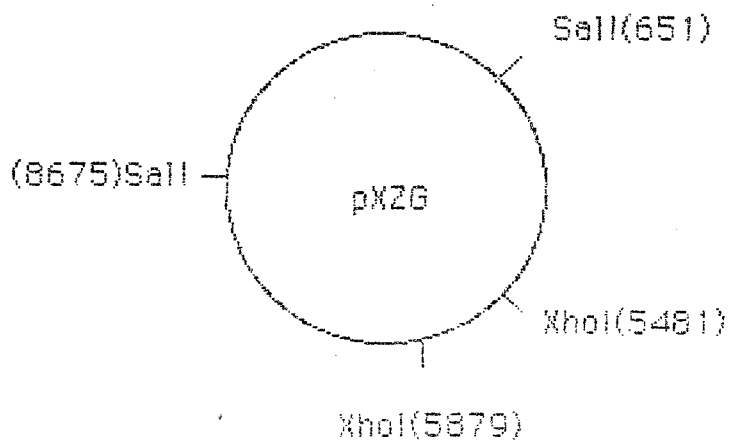
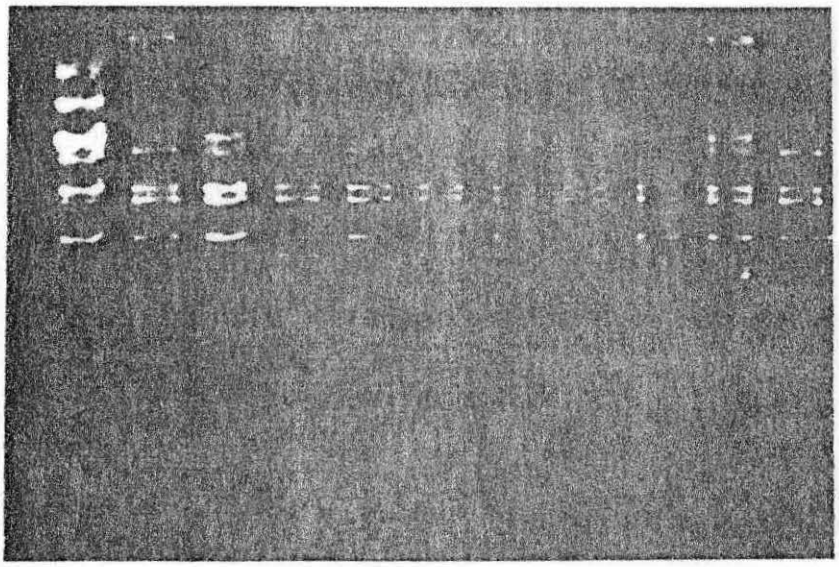

SalI(651)
(8675)SalI
pXZ6
XhoI(5481)
XhoI(5879)

Figure 11. XhoI and SalI digesion: screening for pXZG. lane 1, pUM41. Lane 2 to 11, DNA from transformants. Electrophoresis on a 1.5% agarose gel.


3194
2796
1 2 3 4 5 6 7 8 9 10 11

inoculation). The appearance of symptoms on plants inoculataed with pXZF DNA was delayed about 1 week. Only one to three days delay of apperance of symptoms was observed in XZF inoculated plants. However the infetious severity was not reduced as judged by the yields of virus from XZF and CabbS infected plants (3.68mg/Kg yields for XZF comparing 3.19mg/Kg for CabbS). On inoculated leaves , local lesion caused by XZF are sharp, defined yellow spots much smaller than those formed by CabbS, UM130 or XZG.

Stability of XZF and XZG DNA during growth in plants

KpnI-SalI, and XhoI-SalI digestions were used to check that the linkers were still present in XZF and XZG DNA recovered from plants. The results were positive.

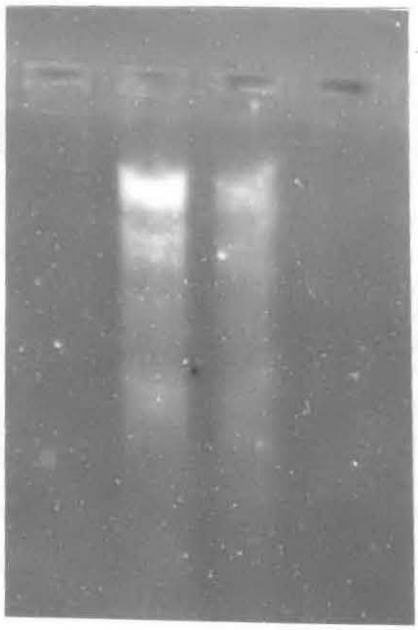
Alkaline denaturing electrophoresis was also performed to check whether three gaps were maintained in XZF DNA or not. The three gaps in the DNA of most isolateds of CaMV will produce three single-stranded DNAs under denaturation conditions. Figure 12 shows that XZF DNA has the same pattern as CabbS DNA.

Allele dominance

XZG vs CabbS

Allele dominance was tested by inoculating turnip plants with a mixture of purified virions, isolating virion DNA from the infected plants and restriction enzyme digestion of the recovered DNA. Viral DNA extracted from plants inoculated with CabbS and XZG was digested by XhoI and SalI to distinguish the progeny type. As mentioned above, 2796bp fragment is a marker for XZG DNA, while that of 3194bp is diagnostic for CabbS in this

Figure 12. Alkaline denaturing of CabbS and XZF DNA. Lane 1, Cabbs DNA. Lane 2, XZF DNA. Electrophoresis on a 1.0% agarose alkaline gel.



1 2

combination . A equal mixture of XZG and CabbS DNA existed in plants inoculated with equal amounts of XZG and CabbS inocula (Fig. 13). When inocula contained three times more CabbS than XZG, traces of XZG DNA were still found in the progeny as shown in the XhoI-SalI restriction digestion pattern.

XZG vs UM130

UM130 is derived from CabbS by insertion of an EcoRI linker at the SmaI site at 2040bp of pUM41. The EcoRI digestion pattern of UM130 differs from those of XZG or XZF in having a shorter fragment, 1632bp instead of 2009bp (fig.14). As shown in figure 15 , DNA resulting from an equal mixture of UM130 and XZG gave patterns after EcoRI digests of viral DNA showing fragments characteristic of both in 8 out of 8 plants. Since the XZG pattern could result from incomplete digestion at the 2040 site, XhoI-SalI digestion was used to distinquish whether XZG was really there or not. The presence of the XZG specific fragment 2796bp in the XhoI and SalI digest confirmed the presence of XZG result. Virion DNA from five other plants coinoculated in a separate experiment with equal amounts of UM130 and XZG gave a pattern after EcoRI digestion with major XZG fragment and only a of trace that for UM130 .

XZF vs CabbS

When viral DNAs were subjected to combined KpnI and EcoRI digestion, a 1234 bp fragment or 1632bp fragment was specific for XZF or CabbS, respectively(fig.16) .When the amount of XZF in the inoculum was three times that of CabbS, the fragments of progeny DNA digested with KpnI and EcoRI contained both1234bp and 1632bp fragments(fig.17). The presence of

Figure 13. XhoI and SalI digestion of DNA prepared from equal amounts XZG and CabbS coinoculated turnips. Lane 1. CabbS. Lane 2, pXZG. Lane 3 to 9, viral DNA from plants. Electrophoresis on a 1.0% agarose gel.

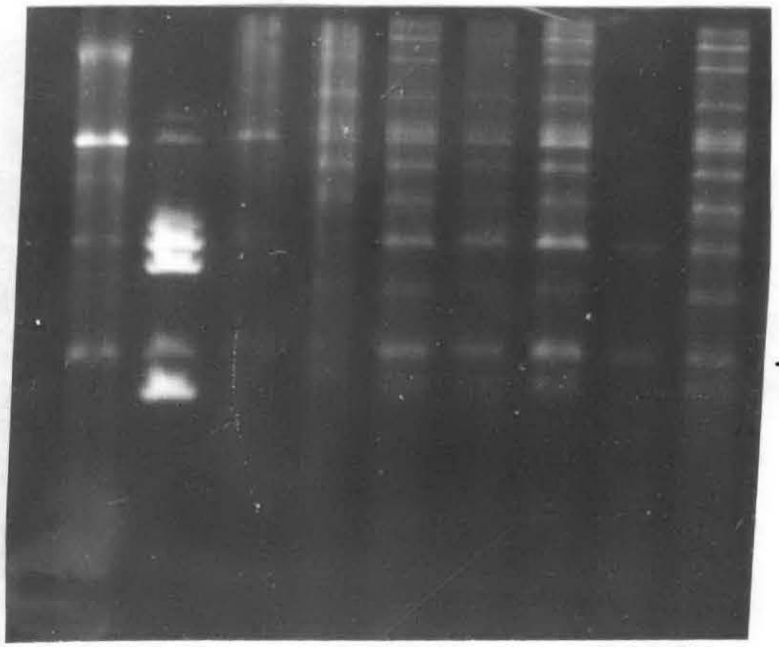
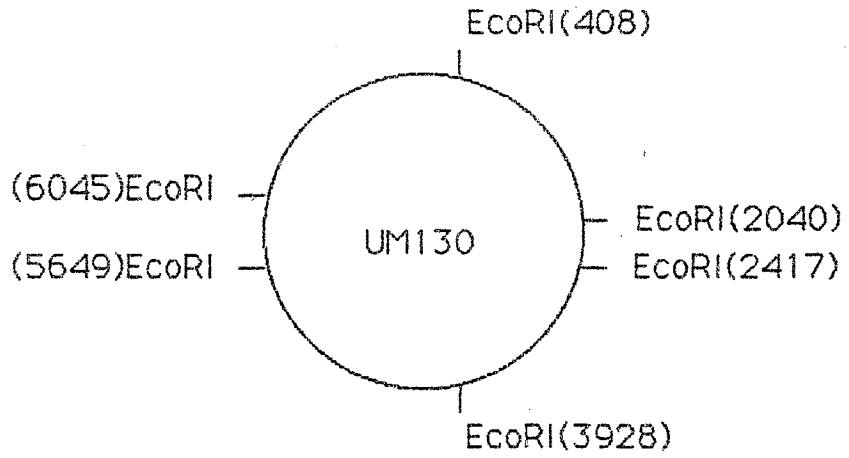

3194
2796
1 2 3 4 5 6 7 8 9

Fig. 14. EcoRI restriction map of UM130 and XZG or XZF


EcoRI(408)
(6045)EcoRI
UM130
EcoRI(2040)
(5649)EcoRI
EcoRI(2417)
EcoRI(3928)

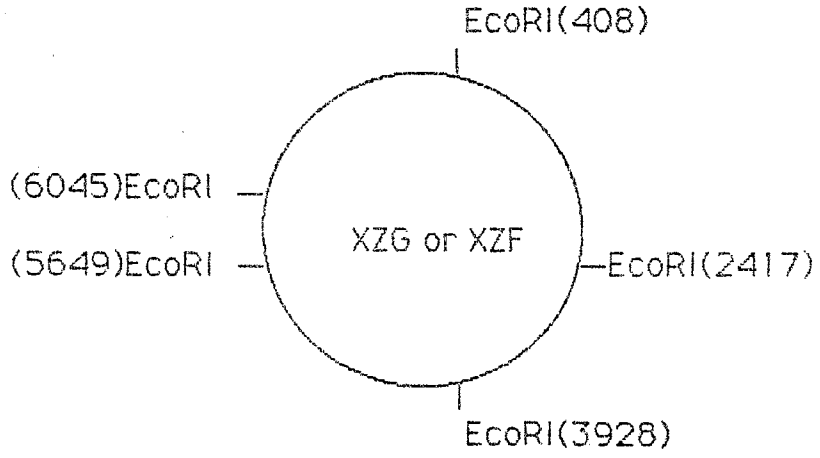

EcoRI(408)
(6045)EcoRI
XZG or XZF
(5649)EcoRI
EcoRI(2417)
EcoRI(3928)

Figure 15. EcoRI digestion of DNA prepared from equal amounts KZG and UM130 coinoculated turnips. Lane 1, pUM130. Lane 2, CabbS. Lane 3 to 10, viral DNA from plants. Electrophoresis on a 1.2% agarose gel.

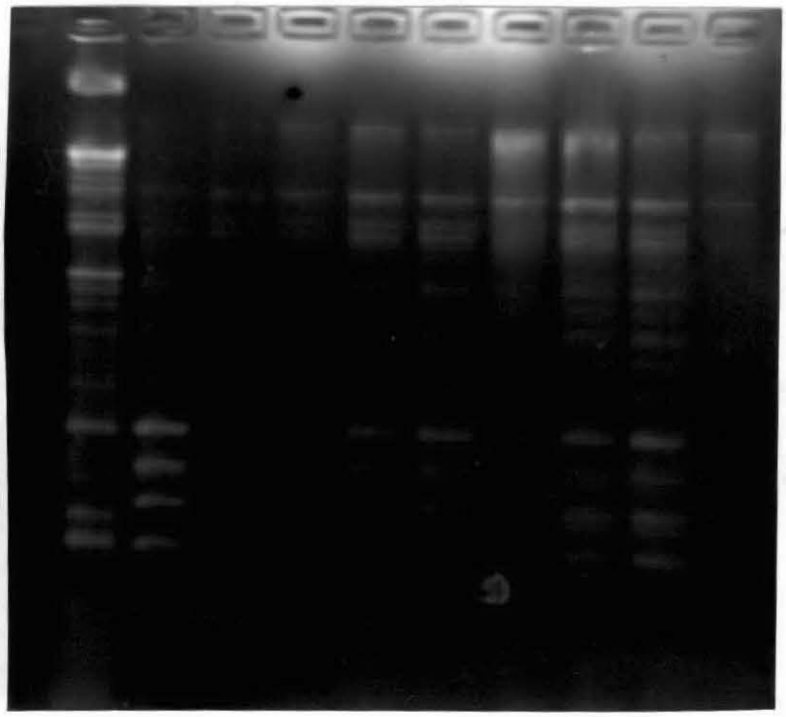
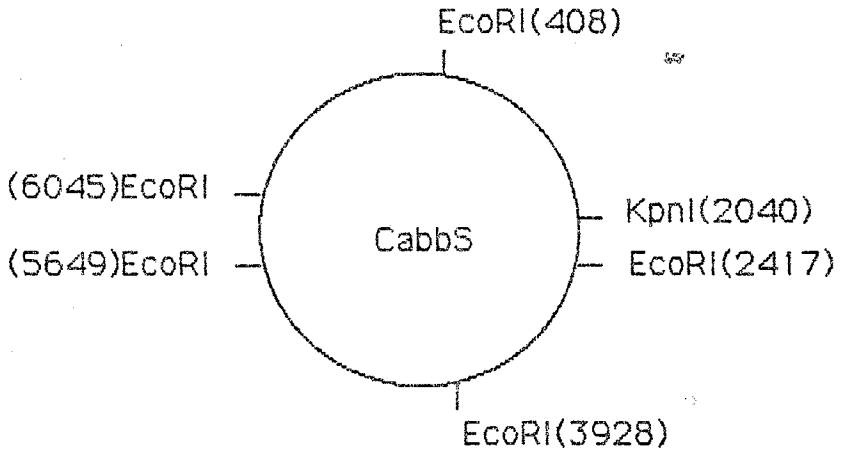

1632
2009
1 2 3 4 5 6 7 8 9 10

Figure 16. KpnI, EcoRI restriction map of CabbS and KZF.


EcoRI(408)
(6045)EcoRI
(5649)EcoRI
CabbS
KpnI(2040)
EcoRI(2417)
EcoRI(3928)

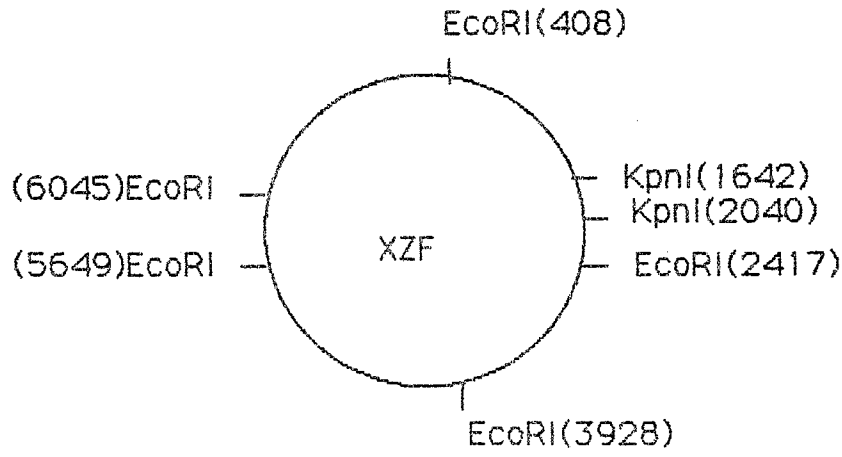
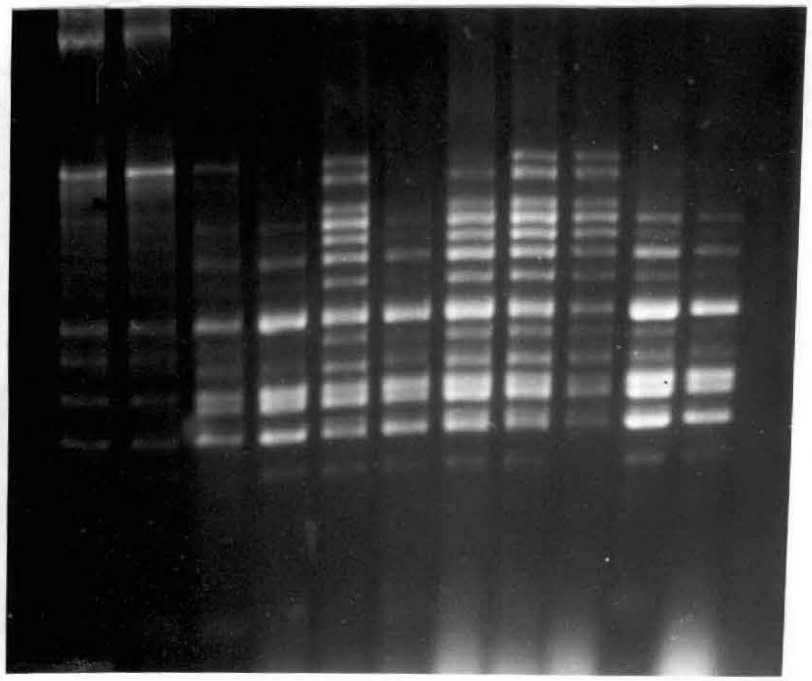

EcoRI(408)
(6045)EcoRI
(5649)EcoRI
XZF
KpnI(1642)
KpnI(2040)
EcoRI(2417)
EcoRI(3928)

Figure 17. KpnI and EcoRI digestion of viral DNA prepared from turnip plants that had been coinoculated with a concentration ratio of three XZF to one CabbS. Lane 1, EcoRI digestion of CabbS DNA. Lane 2, XhoI and EcoRI digestion of CabbS DNA. Lane 4 to 11, KpnI and EcoR digestion of viral DNA from plants. Electrophoresis on a 1.5% agarose gel.


1632
1234
1 2 3 4 5 6 7 8 9 10 11

CabbS DNA was also proved by SalI and XhoI analysis.When CabbS and XZF were present in equal amounts in the inoculum of plants, XZF progeny could not be detected any more by Asp718 and EcoRI digestion. Asp718 is an isoschizomer of KpnI. I found that KpnI is very ineffecient in cutting CaMV DNA, probably due to its substrate preference. 10 times more units of KpnI than Asp718 needed to be used in the reaction to obtain complete digestion.

XZF vs UM130

In plants inoculated with equal amounts of UM130 and XZF, viral DNA isolated showed major UM130 and minor XZF pattern in Asp718 and EcoRI digestion from 4/7 plants (fig.18). Two plants had UM130 and one plant had XZF. If the XZF content in the inocula was two time that of UM130, XZF was the only DNA in the progeny in 5/5 plants as shown by EcoRI digestion. A ratio of 1.5 or more UM130 to XZF in the inocula resulted in only the UM130 pattern from 6/6 plants by Asp718 and EcoRI digestion fig.18).

Table VI summarizes the above description.

SΔII vs CabbS

The deletion of 105 bp from 1537 to 1643 in SΔII reduces the size of the CabbS 2009bp fragment to 1894bp in EcoRI digestion. When the concentration of SΔII was 11.5 times greater CabbS in coinocula, still no 1894 bp fragment could be detected in the EcoRI digests of progeny DNA (fig. 19).

SΔII vs NY8153

Figure 18. Aspa718 and EcoRI digestion of viral DNA extracted from plants inoculated with UM130 and XZF. Lane 1, pUM130. Lane 2, pXZF. Lane3, XZF DNA. Lane 4 and 5, DNA from plants inoculated with equal amounts of UM130 and XZF. Lane 6 to 8, DNA from plants inoculated with a concentration ratio of 1.5 or more UM130 to XZF. Lane 9, UM130 DNA from plants. A XZF specific fragment 1234bp is indicated.

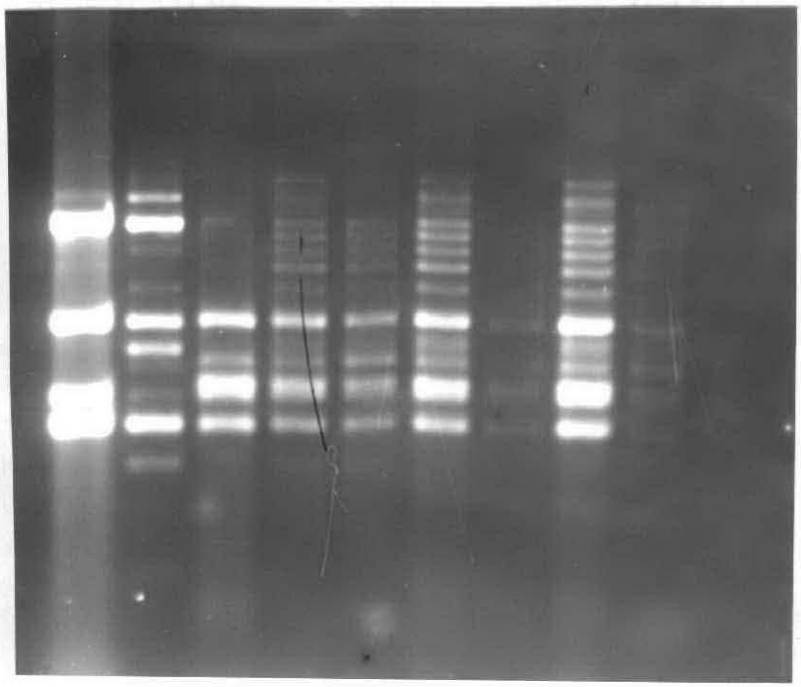

1234
1 2 3 4 5 6 7 8 9

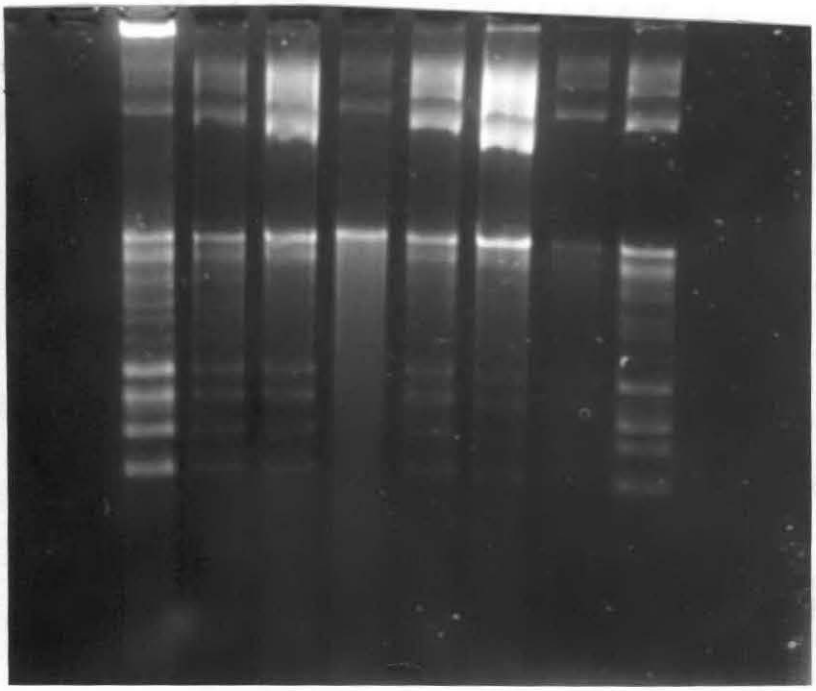
Table IV

ALLELE DOMINANCE OF CaMV BETWEEN XZG AND CabbS, XZG AND UM130, XZF AND CabbS, AND XZF AND UM130

ratio	CabbS vs XZG	UM130 vs XZG	CabbS vs XZF	UM130 vs XZF
1:1	8 m	8 m 5 G, trace 130	8 C	4 130, trace F 2 130 1F
1:2				5 F
1:3			8 m	
1.5:1				6 130
3:1	8 C, trace G			

C: CabbS
F: XZF
G: XZG
m: mixture of parents
130: UM130

Figure19. EcoRI digestion of viral DNA extracted from plants inoculated with SAll and CabbS. Lane 1, CabbS DNA from plants. Lane 2 to 7, DNA from plants inoculated with SAll and CabbS in a ratio ranged from 11.5:1 to 1:11.5. Lane 8, CabbS DNA from plants. Electrophoresis on a 1.5% agarose gel. Fragments 1894 and 2009bp are indicated.


2009
1894
1 2 3 4 5 6 7 8

Differences between the EcoRI digestion patterns of NY8153 (4340, 3172 and 458bp) and SAII (2325,1894,1721 and 1466bp) made it easy to test whether they coexisted or not in progeny when they were coinoculated. A mixed pattern was detected.

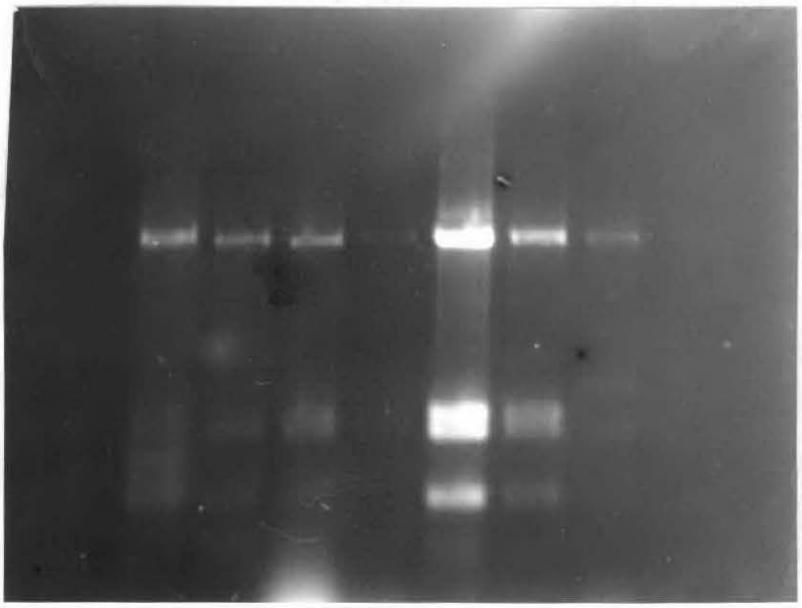
Ca-BB1 vs Ca-NB2

When Ca-BB1 and Ca-NB2 were subjected to ClaI digestion, the only difference between the fragments was a 1440bp fragment in Ca-BB1 and 1674bp fragment Ca-NB2. Only when Ca-NB2 was 11.5 times over Ca- BB1 in the coinocula, could Ca-NB2 DNA be detected in progeny (fig.20).

Ca-BB1 vs NY8153

XbaI digestion of NY8153 and Ca-BB1 will result in 3892, 2430, 864 and 844bp fragments for NY8153 and 3892,2430 and 1287bp fragments for Ca-BB1. A mixture of Ca-BB1 and NY8153 DNA patterns was seen in XbaI digestion of viral DNA isolated from plants coinoculated with Ca-BB1 and NY1853 at a concentration ratios from 1 : 10 through 10 : 1.

Figure 20. ClaI digestion of viral DNA from plants inoculated with Ca-BB1 and Ca-NB2. Lane 1, Ca-BB1 DNA from plants. Lane 2 to 6, DNA from plants inoculated with Ca-BB1 and Ca-NB2 in a ratio of 11.5:1 to1:11.5. Lane 7, Ca-NB2 from plants. Electrophoresis on a 1.2% agarose gel. Fragments 1440 and 1674bp are indicated.


1674
1440
1 2 3 4 5 6 7

CHAPTER IV

DISCUSSION

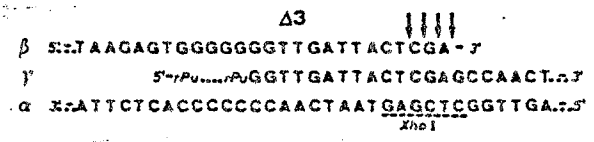
Daubert et al. (1983) have introduced a series of small insertions into the various translational reading frames of CaMV. They found that a 12bp in-phase insertion in ORF III destroyed infectivity. However my construct pXZG and pUM130, both have 12bp insertions at ORFIII , and are as infectious as the parents. The construction of Daubert et al.'s has 12bp inserted at nucleotide 2149 of CabbB strain, ours at 2040bp of CabbS. What makes the difference is interesting. The insertion amino acid sequneces pro.leu.glu.gly.ala. of XZG and pro.gly.phe.arg.ala. of UM130 probably didn't significantly change the structure of their protein products.

ORF II encodes an aphid transmission factor (Armour et al., 1983; Woolston et al., 1983.). It is not essential for infection, as it can be either deleted or expanded by small insertions (Howell et al., 1981; Gronenborn et al., 1981; Dixon et al., 1983). Both deletion and insertion at ORF II retard infectivity. XZF, with a 12bp insertion at ORF II causes disease with about one week delay of symptom appearance. In addition, XZF causes much smaller local lesions on inoculated leaves. It has been shown that the ORF II product influences the structure of the viral inclusion body or firmness with which virions are held within the viroplasm (Givord et al., 1984). I also observed Ca-NB2, which has an insertion of 234bp at ORFII of CM4-184 abolished or reduced the severity of vein clearing. The exact function of ORF II remains to be elucidated.

XZF has the 12 bp insertion very close to G3 (fig.21). It has been reported (Pietrzak and Hohn, 1985) that long polylinkers inserted beside G3 affected the stability of the insertion in progeny viral DNA in plants by acting as recombination targets. XZF retained its linker insertion during plant infection. DNA sequence around G3 is thought as the plus-strand primer-binding site for polymerase(Pfeiffer and Hohn, 1983; Hull and Covey, 1983; Hohn et al., 1985). That XZF viron DNA isolated from plant shows three single-stranded bands in alkaline denaturing gel, demonstrates the small insertion at the XhoI site does not disrupt the polymerase starting DNA synthesis at the G3 site. Known ORFII deletion mutant SA103 produced only genome-length single strands(Armour et al., 1983).

When XZG mixed with CabbS was used to inoculate plants, no allele dominace was observed in the DNA recovered from progeny virions. Even when the amount of CabbS was three fold more than that of XZG in the inocula, traces of XZG DNA were detected. In the case of XZG coinoculated with UM130, 8 plants had a mixture of parental DNA and five had mostly XZG and small amounts of UM130 in the progeny viral DNA. UM130, which has an extra 12bp inserted at nucleotide 2040 of CabbS, has been observed to convert completely to CabbS, even when the amount of UM130 was three times over that of CabbS in the inocula (Melcher and Essenberg, 1985). XZG has a 12bp linker inserted at the same site as UM130. The only difference between them is the 8 bp DNA sequence, GGAATTCC for UM130 and CCTCGAGG for XZG. We attribute allele dominance in the CabbS and UM130 combination to mismatch repair(excision loop structure) of a heteroduplex during recombination. In many systems, mismatch repair for many markers involves the preferential correction of one or the other strand . In *S. pneumoniae*, Roger (1977) found that opposite strands were not equally

Figure 21. Structure of the G3 discontinuity. XhoI linker insertion site is indicated.


Δ3
β 5'-TAAGAGTGGGGGGGTTGATTACTCGA-3'
γ 5'-rPu.....rPuGGTTGATTACTCGAGCCAACT-3'
α 3'-ATTCTCACCCCCCCAACTAATGAGCTCGGTTGA-5'
Xho I

effective in transformation. Also in S. *pneumoniae* a deletion of C in the sequence 5'-----TTCCCTA------3' was corrected with high efficiency, whereas a deletion of C in the sequence 5'-----GACCTT----- 3' had an intermediate efficiency of correction (Sicard et al., 1985). The efficiency of methyl-directed DNA mismatch-repair of *E.coli* acting on phage M13 was reported to be controlled by two parameters: the sequence environment of the mismatch and the structure of the mismatch itself (Kramer et al., 1984)

The equal mixture pattern (8 plants) or XZG predominance pattern (5 plants) resulting when XZG and UM130 were coinfected, could be due to no correction of mismatches. When XZG and UM130 recombination happens, mismatches will form in the heteroduplex instead of a loop structure as in XZG and CabbS combination.

------CGGGGGAATTCCCCCG------ UM130

------GCCCGGAGCTCCGGGC------- XZG

It appears that most single base mismatches can be repaired. A/C, C/T, A/A, C/T and G/C could be corrected very efficienly, T/T efficiently, and A/G and C/T not at a detectable frequency. This single base repair specificity is identical in *E.coli* and *S.pneumoniae* systems (Kramer et al., 1984; Dohet et al., 1985; Claverys et al., 1980, 1981 and 1983). But the correction of multiple mismatches could be complicated . In *S.pneumoniea*, the correction of a pair of mismatches could be changed by placing the second mismatch eight base pairs from the first, and two mismatches, normally not corrected singly, were recognized when both were present (Claverys et al., 1983). This again comes back to the possible effect of neighbouring sequences and structure of mismatches.

When XZF was coinoculated with CabbS in equal amounts, allele conversion to CabbS happened in 8/8 plants. But when XZF was three times great than CabbS in inocula, both parents were recovered. So probably I could say XZF is only weakly converted. As mentioned above SΔII and Ca-NB2 are much more strongly converted. Also as in the *E.coli* system, large deletions are better substrates for conversion than small deletions (Benz and Berger, 1973). The other posibility is that CabbS is competitivly advantage over XZF, since XZF has delayed appearace of symptome.

The interaction of XZF and UM130 is complicated to analyze, due to the presence of two heterologous regions.When XZF was inoculated with UM130, CabbS allele dominance over UM130 at ORFIII was hardly seen. It happened just when XZF (CabbS allele), was two fold more than UM130. In one to one mixture of XZF and UM130, 6 out of 7 plants had UM130 DNA , while 6 out of 6 plants had UM130 when UM130 was 1.5 times more than XZF in the coinoculation. XZF differs from CabbS in having an extra 12bp at the 1642bp site, that making a heterologous region 398bp away from the site of the converted allele in CabbS and UM130 interaction. When pUM24, which has a 40bp deletion from 6299 to 6338 , was mixed with pUM130, the heterologous sequence present didn't prevent UM130 allele conversion to CabbS in 8 of 10 plants (Melcher and Essenberg, 1985). Why should the 12bp heterology at 1642 depress conversion? One hypothesis is that near the 1642bp position is a specific recognition site for the initiation of conversion. Another explanation is that exact pairing between homologues, a prerequisite for normal recombination and gene conversion, is not possible in heterologous regions. That heterologies will prevent allele dominance has been observed when UM130 was mixed with CM4-184 or NY1853 (they have 5% sequence difference scattered throughout the whole viral genome).

I also observed several cases in ORFII. When SΔII , which is a deletion mutant within ORFII from 1537 to 1643 of CabbS DNA (Givord et al., 1984), was mixed with CabbS complete conversion to CabbS happened even when SΔII was three times high than CabbS in the inoculum. Allele dominance was abolished when SΔII was coinoculated with NY1853. The Ca-NB2 allele was lost when it was coinoculated with Ca-BB1. Ca-BB1 is derived from CM4-184 with the whole ORFII deleted and an the XhoI (9bp) linker inserted instead. Ca-NB2 has a dhfr gene 234bp inserted at XhoI site of Ca-BB1 (Brisson et al., 1984). Ca-BB1 coexisted with NY1853 at a wide range of concentration ratios from 1 :10 ratio or 10 : 1 ratio of Ca-BB1 to NY1853. Whether Ca-NB2 would be converted at the presence of NY1853 would be interesting to test.

The12bp extra in UM130 also depressed allele dominance that happened in ORF II. In a one to one mixture of XZF and UM130 , 3/7 plants kept XZF viral progeny, while no XZF were detected in plants when XZF was mixed with CabbS.

Various recombination models postulate heteroduplex structures between two parental DNAs on which mismatch correction is supposed to occur. A study of mismatch correction mechanism in CaMV is likely to be of general significance to the theory.

SUMMARY

pXZF and pXZG have been constructed by inserting 12bp into specific sites of pCS101 to study allele dominance in CaMV.

pXZF was obtained by XhoI digestion of pCS101, filling the ends with reverse transcriptase and ligation with a KpnI linker. pXZG was made by SmaI digestion of pUM41 and ligating with XhoI linker.

pXZF and pXZG were inoculated onto turnips to determine their infectivity. Both pXZF and pXZG were infectious. With pXZF about one week delay of symptom appearance occurred, XZF also differed from the parent by having smaller local lesions on inoculated leaves.

Allele dominance was investigated by inoculating turnip plants with mixtures of purified virus, extracting virion DNA from infected plants and analyzing with restriction enzymes.

No allele dominance was observed between XZG and CabbS DNA, in contrast to the allele dominance of CabbS over UM130, which has the 12bp insertion at the same site with XZG. Allele dominancae was also hardly ovserved in UM130 and XZG combination tested in 8 plants, while in 5 plants XZG seemed dominant over UM130.

There were mixtures of CabbS and XZF DNA in virion progeny when XZF was three times over XZF in inocula, but not when XZF and CabbS were in equal amounts.

The dominance of CabbS over UM130 at ORF II was non-existent in XZF and UM130 combination. 6/7 plant had UM130 DNA when infected with equal

amounts of UM130 and XZF. The extra 12bp at UM130 also interfered with allele converion of XZF at ORF II site.

Allele loss in ORF II was observed when other mutants SΔII and Ca-NB2 were used in mixed infection with their homlogous parents but not with heterologous strains.

The possible mechanism of allele dominance happened in CaMV was discussed.

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VITA[2]

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