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Turnip crinkle virus (TCV) and its sub-viral RNAs.

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TURNIP CRINKLE VIRUS (TCV) AND ITS SUB-VIRAL RNAs

A Dissertation Presented

by

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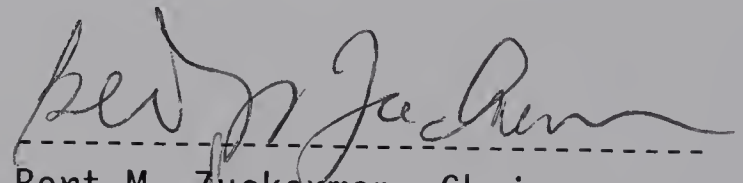

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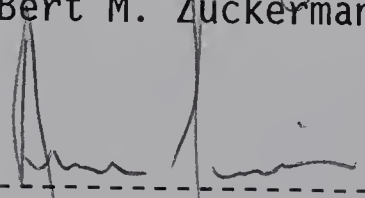

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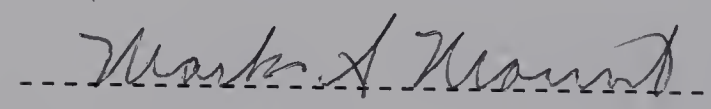

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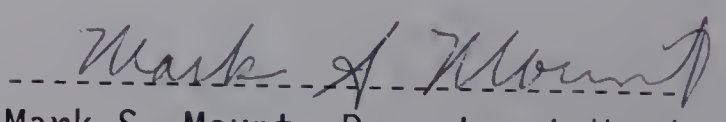

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ABSTRACT

TURNIP CRINKLE VIRUS (TCV) AND ITS SUB-VIRAL RNAs

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TCV isolate, TCV-M, supports a family of satellite (sat-) RNAs. The virulent sat-RNA C intensifies TCV symptoms on turnip cultivar Just Right. In this thesis, I report that sat-RNA C (356 b) exacerbates symptoms on all hosts where TCV produces visible symptoms including cultivars of *Brassica rapa* and *Arabidopsis thaliana*. This finding has led to studies of TCV-resistance using *A. thaliana*, a small plant with a well characterized genome, as a host. After screening over 6,000 M2 *A. thaliana* plants from EMS treated Columbia cultivar seeds and 22 ecotypes of *A. thaliana*, ecotype Dijon was found to be resistant to TCV.

A second isolate, TCV-B, supports an RNA species with a size similar to that of sat-RNA C. Northern hybridization and cDNA cloning and sequencing demonstrate that this RNA is actually a defective interfering (DI) RNA, denoted DI RNA G. Infection of turnip with virus derived from cloned transcripts of TCV-B resulted in *de novo* generation of a DI

RNA, DI1 RNA. Unlike DI RNAs associated with other plant viruses (or animal viruses), TCV DI RNAs intensify TCV symptoms. To understand sequences required for DI RNA infectivity, a series of mutation have been generated in a full length cDNA copy of DI RNA G. Stepwise deletions at base 98 of DI RNA G, at which an *Apa* I linker had been inserted, has shown that DI RNA GA (DI RNA G with a 8-base insertion at base 98) harboring deletions of bases 74-98 or less are infectious. However, deletion of bases 73-98 or more abolishes RNA infectivity. DI RNA GA with a deletion of bases 107-124 is infectious. However, DI RNA GA harboring a deletion of bases 107-138 is not infectious. I have found that infectivity of RNA harboring 31- or 32-base deletions can be restored by inserting foreign sequences into deletion sites. This implies that at least 71 bases (including the 8-base insertion) in DI RNA GA near the 5′ end are not specifically required for RNA infectivity. By combining deletions of infectious clones to generate larger deletions (40 or 43 bases deleted), I demonstrate that the infectivity of the RNA is abolished. This result suggests that size of the DI RNA is important in maintaining RNA infectivity.

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CHAPTER I

INTRODUCTION

Plant viruses have been known to be responsible for many destructive plant diseases for almost a century. However, little is understood about the molecular and cellular bases of disease induction. The complexity of plant response to viral infection makes studies of virus and plant interaction extremely difficult. Although numerous cytological and biochemical changes occur in infected plants, it is not clear whether these changes are the cause or the result of disease. The complexity of the virus itself also adds to the difficulty of these studies. In order to study viral disease induction and virus-host interaction in a more defined way, it is necessary to use a virus and host system as simple as possible.

In the research presented here, I have used turnip crinkle virus (TCV) and its sub-viral RNAs to study viral infection processes. I have also used *Arabidopsis thaliana* to study the response of a plant to TCV infection.

Turnip Crinkle Virus (TCV)

TCV is a small (30nm) isometric plant virus with a single stranded, positive sense RNA genome of 4 kb, recently classified in the Carmovirus group (Morris and Carrington, 1988). TCV is the cause of mottling and stunting of various *Brassica* plants in Scotland, England, and Yugoslavia (Broadbent and Heathcote, 1958; Hollings and Stone, 1972). It does not not naturally occur in the United States. However, at least two isolates, a Berkeley isolate (TCV-B) and a Massachusetts isolate (TCV-M) have been extensively studied in this country.

The genomic sequence and structure of TCV-B has been determined from cDNA clones (Carrington *et al.*, 1987). The 4051 base genomic RNA has 3 open reading frames (ORFs) from which 4 proteins are possibly produced (Carrington *et al.*, 1989). The first ORF encodes the putative replicase (28 KDa and 88 KDa -- a read-through product) and begins at base 63 near the 5'end, extending to base 2388. This ORF is punctuated by an amber termination codon at base 813. A second ORF specifies the coat protein (38 KDa) and is expressed from a 1.45 kb subgenomic RNA. Translation of ORF 2 begins at base 2743 and extends to base 3796. ORF 3, the smallest ORF, encodes an 8 KDa protein. This ORF begins at base 2357 and terminates at base 2573, overlapping the 3' end of ORF 1 by 34 bases. ORF 3 is also expressed through a subgenomic RNA. Recently, isolate TCV-M has also been

cloned and sequenced (Carpenter, unpublished results). The genomes of the two isolates share approximately 99% similarity with base changes scattered throughout the genome.

Because TCV can be dissociated and reassembled readily in solution, it also provides a useful model for studying the assembly of icosahedral RNA viruses. Using an RNase protection technique, Wei and others (Wei *et al.*, 1990) have found two sequence clusters in the TCV-B genomic RNA that are protected after dissociation of the virus by remaining tightly bound to coat protein subunits. These two sequence clusters are located around base 800 and base 3500, within the putative replicase ORF and the coat protein ORF, respectively. These two sites are, therefore, thought to be involved in coat protein binding for possible expression regulation and/or assembly initiation.

Both isolates are known to be associated with several low molecular weight sub-viral RNAs (Altenbach and Howell, 1981; Hillman, *et al.*, 1987). Their small size, dispensability for viral replication and involvement in viral pathogenicity, make the TCV sub-viral RNA system extremely attractive for studying viral replication and disease processes at the molecular level (Simon and Howell, 1986; 1987; Simon, *et al.*, 1988; Carpenter, *et al.*, 1991a, manuscript submitted; 1991b, manuscript submitted).

Satellite (Sat-) RNAs

By definition, sat-RNAs are small sub-viral RNAs that generally do not share sequence homology with their helper virus (Murant and Mayo, 1982) and are encapsidated in viral particles. Sat-RNAs do not in general encode any proteins, unlike satellite viruses which encode their own coat protein. Sat-RNAs depend entirely on helper virus for replication and infectivity, yet sat-RNAs are not required for helper virus replication and infection (Fritsch *et al.*, 1978; Gould *et al.*, 1978).

One of the unique characteristics of plant viruses is their frequent association with satellites including satellite viruses and sat-RNAs. Sat-RNAs have been identified associated with a number of plant viruses in at least 6 different virus groups, including nepoviruses (eg., tobacco ringspot virus (TobRV), tomato black ring virus (TBRV) and arabis mosaic virus (ArMV)); cucumoviruses (eg., cucumber mosaic virus (CMV) and peanut stunt virus (PSV)); sobemoviruses (eg., lucerne transient virus (LTV), velvet tobacco mottle virus (VTMV) and subterranean clover mottle virus (SCMV)); tombusviruses (eg. cymbidium ringspot virus (SyRV)); and TCV in the carmovirus group; etc. (for reviews, see Francki, 1985; Simon, 1988). Sat-RNAs can be either linear or circular. A sat-RNA associated with TobRV (sTobRV) is present in both circular or linear forms in infected tissues. However, only the linear form is

encapsidated in virions. Sat-RNAs associated with sobeviruses are circular and are commmonly referred to as virusoids.

Sat-RNAs usually interfere with helper virus replication and modulate the symptoms induced by the helper virus. Symptoms can become more severe or can be attenuated, depending on different strains of satellites, helper viruses or hosts involved (Palukaitis, 1988).

At least 13 similar sat-RNAs are known associated with CMV which have different effects on CMV symptoms. Some CMV sat-RNAs intensify symptoms resulting in necrosis (Kaper and Waterworth, 1977) or chlorosis (Garcia-Arenal *et al.*, 1987; Gonsalves *et al.*, 1982; Palukaitis, 1988), some attenuate viral symptoms (Mossop and Francki, 1978; Garcia-Arenal *et al.*, 1987; Palukaitis, 1988), while others have no effects on symptom production (Palukitis, 1988). CMV sat-RNA can cause a lethal necrosis in tomato but attenuation of symptoms in other species (Waterworth *et al.*, 1979).

The mechanisms involved in symptom modulation by sat-RNAs is not understood. Since there is no sequence homology between the plus strands of CMV sat-RNAs and CMV genomic RNAs, the effects on symptoms may not be a consequence of direct sequence interaction between the infectious strands of the sat-RNAs and their helper viruses. By making chimeric molecules between an ameliorative sat-RNA and a necrogenic sat-RNA of CMV,

Kurath and Palukaitis (1989) have determined that a 150 nucleotides segment in the 3′ region of the necrogenic sat-RNA is responsible for inducing necrotic symptoms. Within this region, sequence between nucleotide 286 and 310 is completely conserved among all necrogenic sat-RNAs and is different among all ameliorative sat-RNAs (Kaper *et al.*, 1988). Sleat and Palukaitis (1990) were able to alter a CMV sat-RNA from nonnecrogenic to necrogenic by only a single nucleotide change with this region. It is suggested that this highly conserved region in necrotic sat-RNAs is involved in induction of necrotic symptoms due to some unknown function(s) residing within the region (Sleat and Palukaitis, 1990). Attenuation of CMV symptoms by some sat-RNA strains, on the other hand, is suggested to be due to competition between sat-RNAs and their helper CMV for limited viral or cellular factors necessary for viral and sat-RNA replication (Kaper and Torsignant, 1984). However, the symptom attenuation is not always correlated with repression of helper virus synthesis. Transgenic tobacco plants expressing biologically active sat-RNAs show much less CMV symptoms, while accumulating normal levels of the helper virus (Baulcombe *et al.*, 1988).

Sat-RNA associated with TobRV (sTobRV, 359 b) is another example of a sat-RNA that attenuates viral symptoms induced by the helper virus (Gerlach *et al.*, 1987). In this

case, there is a close correlation of symptom attenuation and viral yield reduction by the sat-RNA (Buzayan *et al.*, 1986).

Sat-RNAs do not always need to replicate *in planta* to have an effect on virus accumulation and symptom expression. Ponz and coworkers (Ponz *et al.*, 1987) found that replication of sTobRV is not supported by cherry leafroll virus (CLRV), another nepovirus; however, coinoculation of CLRV with sTobRV strongly decreases the yield of CLRV and symptoms induced by CLRV in inoculated cowpea leaves, not in uninoculated leaves. The interference was also observed in studies involving *in vitro* translation of CLRV. They conclude that replication is not a prerequisite for a sat-RNA to interfere with viral synthesis and symptom expression.

One important implication of attenuation of viral symptoms by sat-RNAs concerns using attenuative sat-RNAs as a means of protecting plants against viral pathogenic effects. However, application of sat-RNA to plant protection against viral disease has been cautioned by different authors. As mentioned earlier, Sleat and Palukaitis (1990) were able to convert the sat-RNA phenotype on tomato from amelioration to necrogenesis by only a single nucleotide change in the sat-RNA, an event which could occur frequently in plant tissues.

TCV isolate TCV-M naturally supports a family of sat-RNAs, denoted as sat-RNA F (230 b), sat-RNA D (194 b), and sat-RNA C (356 b) (Simon and Howell, 1986). Sat-RNA F is almost identical to sat-RNA D with the exception of a 36-base insert near its 3′ end that is absent in sat-RNA D. Sat-RNA F and sat-RNA D fulfill the definition of a sat-RNA in all aspects. They share no sequence similarity with TCV (with the exception of seven nucleotides located at the 3′ end of TCV and all three sat-RNAs) and require the helper virus for accumulation in plants. Sat-RNA C is an unusual hybrid molecule consisting of a 5′ domain very similar to full length sat-RNAs D and F, and a 3′ domain composed of two regions at the 3′ end of TCV genomic RNA (Fig. 1.1, Simon and Howell, 1986). A second hybrid sat-RNA between sat-RNA D and TCV has recently been identified. Sat-RNA CX (420 b) has sat-RNA D sequence at the 5′ portion, and a single 230 nucleotide sequence at the 3′ portion from the TCV 3′ end (Cascone, *et al.*, in preparation).

Among the TCV sat-RNAs, sat-RNA C is the only one that modulates symptoms caused by TCV (Altenbach and Howell, 1981). Sat-RNA D and sat-RNA F have no apparent influence on symptom expression (Altenbach and Howell, 1981; Simon *et al.* 1988). In the absence of sat-RNA C, TCV-m (TCV-M helper virus alone) or TCV-m plus sat-RNA D with or without sat-RNA F causes slight crinkling and stunting. In the presence of sat-RNA C, infected plants become much more crinkled, stunted and darkly pigmented. This symptom

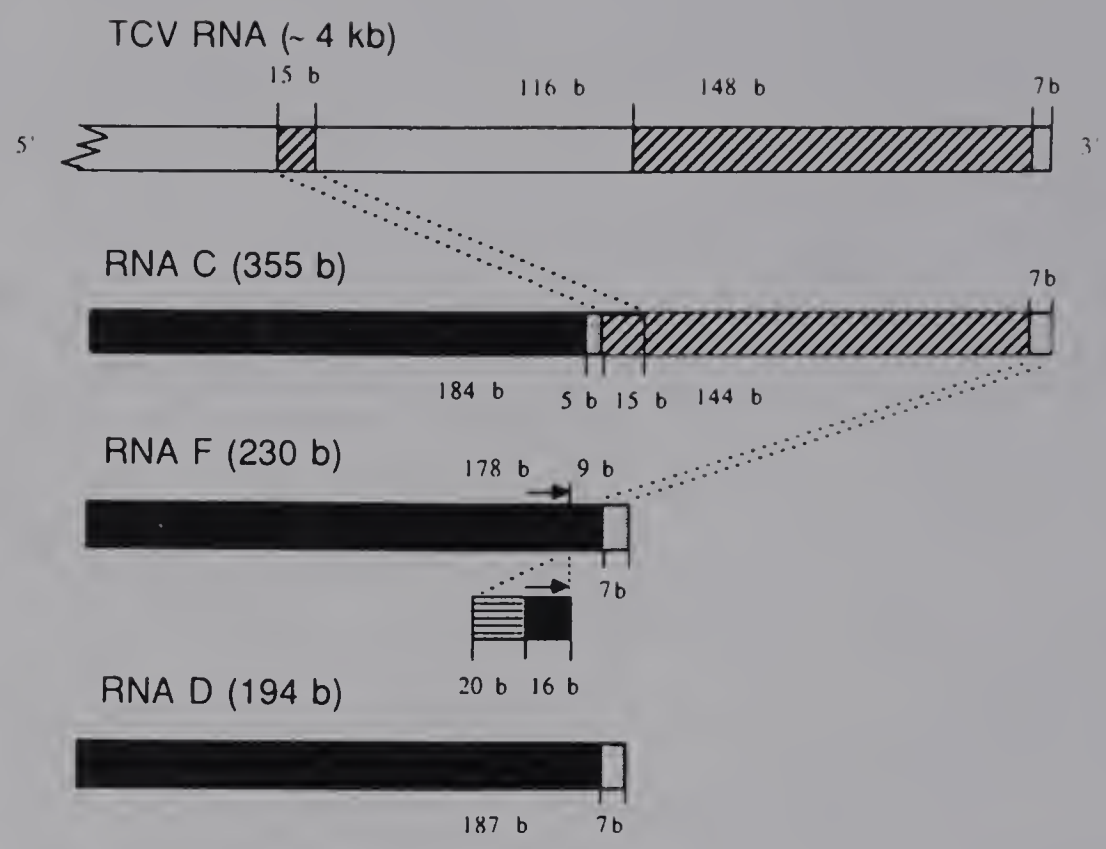


Fig. 1.1 Schematic representation of the homology among the satellites and the 3′ end of the helper virus genome (Simon and Howell, 1986).

intensification caused by the virulent sat-RNA C reveals an interesting phenomenon of host-virus-satellite interaction in disease development and expression in the TCV system.

Since sat-RNA C does not encode any proteins (Altenbach and Howell, 1984), the nature of the intensification of viral symptoms by the sat-RNA is not understood. However, recent advances in *in vitro* synthesis of infectious RNA copies of TCV sat-RNAs (Simon and Howell, 1987) has made it possible to study the effect of modulating specific domains of sat-RNA C. Studies with chimeric satellites containing the 5′ 155 bases of sat-RNA F ligated to the 3′ 200 bases of sat-RNA C reveal that the 3′ TCV-similar domain of sat-RNA C is responsible for symptom intensification by the sat-RNA (Simon and Howell, 1988). This finding led to the hypothesis that the 3′ domain of RNA C and perhaps, the very similar 3′ untranslated region of TCV are responsible for symptom production by interacting or interfering with some unidentified component present in plant cells. The intensification of the symptoms produced by TCV by sat-RNA C may therefore be related to an increased dosage of this domain in infected plants (Simon and Howell, 1986).

The mechanism of sat-RNA replication is not yet understood. It was suggested that sat-RNA of tobacco ringspot virus (sTobRV, 359 bases) replicates through a rolling circle mechanism (Kiefer *et al.*, 1982) based on the findings that infected plant tissue contains linear,

circular and multimeric forms of both polarities of the sTobRV RNA (Sogo and Schneider, 1982; Kiefer *et al.*, 1982; Linthorst and Kaper, 1984). In this model, a circular form of the positive sat-RNA acts as a template during replication, giving rise to multimeric forms of the negative strand sat-RNA intermediate (Kiefer *et al.*, 1982) which then either serves directly as template to produce multimeric positive strands, or, is self-cleaved into monomeric minus strands which then can serve as template for the formation of positive stands (Haseloff *and Gerlach,* 1989). The positive multimeric strand then undergoes a self-catalyzed cleavage (Buzayan *et al.*, 1986; Haseloff and Gerlach, 1989; Prody *et al.*, 1986) to produce unit-length forms. The resulting 5′ hydroxyl and 2′,3′-cyclic phosphate termini (Prody *et al.*, 1986; Buzayan *et al*, 1986; Bruening *et al.*, 1988) are required for a spontaneous non-enzymatic ligation reaction which gives rise to circular forms of the sat-RNA (Buzayan *et al.*, 1986). Since only high molecular weight forms of (-) strands of some sobevirus virusoids have been found in infected tissues, it was suggested that the multimeric positive strands are, more likely, directly synthesized from multimeric (-) strands which do not undergo self-processing (Davies *et al.*, 1990). However, in most virus systems, including TCV, no circular forms of linear sat-RNAs have been found although multimeric forms of these sat-RNAs also can be readily found *in vivo*. It is,

therefore, unlikely that replication of these sat-RNAs follows the same mechanism as postulated for TobRV sat-RNAs and virusoids.

All TCV sat-RNAs are present in both monomeric or multimeric forms in infected plant tissue (Simon *et al.*, 1988, Carpenter *et al.*, 1991a, manuscript submitted). Formation of multimeric forms of these sat-RNAs is thought to involve a replicase driven copy choice mechanism (Carpenter *et al.*, 1991a, manuscript submitted), the mechanism proposed previously for the formation of TCV chimeric sat-RNA(s) and sat-RNA recombinants (Cascone *et al.*, 1990). Recombinant sat-RNAs occur when specific deletions are made in the 5′ portion of sat-RNA C. The sat-RNA sequence is "corrected" by recombination between sat-RNA D and sat-RNA C when both are present in the TCV inoculum. Recombinant molecules generated have sat-RNA D sequence at the 5′ end and sat-RNA C sequence at the 3′ end (Cascone *et al.*, 1990), or with a short stretch of TCV genomic sequence in between (Cascone *et al.*, in preparation). Based on observations that the right side junction sequences found in the sat-RNA recombinants and chimeric sat-RNA C are similar to either the 5′ end of TCV genomic RNA (motif I) or the 5′ends of the sat-RNAs (motif II) (Fig. 1.2, Cascone *et al.*, 1990), a replicase driven copy choice model was proposed to explain formation of recombinants (Fig. 1.3 A, Cascone *et al.*, 1990) and sat-RNA C (Fig. 1.3 B, Cascone *et al.*, 1990). In this model, the

Fig. 1.2 Comparison of sequences between TCV genomic RNA and the junction points of discontinuous RNAs associated with TCV. Comparison of sequences between the 5′ end of TCV and the junction points in the recombinant (D/C) sat-RNAs (**A**), the sequences between the 5′ end of sat-RNA F (Simon *et al.*, 1988), the junctions of discontinuous sequences in sat-RNA C and the sequence upstream of one of the TCV subgenomic RNAs (ORF 3) (**B**), and the junction of sat-RNA CX, the sequence upstream of one of the TCV subgenomic RNA (ORF 2) and a double recombinant of sat-RNA D, TCV and sat-RNA C (**C**) (Cascone *et al.*, 1990; Cascone *et al.*, in preparation).

A

TCV 5' end 5'- NGUAAUCUGCAAAUCCCUGCACCCGCCUA ...

D/C Recombinant ... AUCCCACUCGAAAGAGUCGAAUCCC . AGACCCUCCAG ...

Motif I

B

Sat-RNA F 5' end 5'- GGGAUAAAAAUAAGGGAUUCAUAC ...

Sat-RNA C (2) ... CCAGCCAAAGGGUAAAUGGGACC AAAAACGGCGGCAGCACC ...

Sat-RNA C (1) ... AAAGAAUCCCAGACCCUCCAGCC AAAG . . GGUAAAUGGGAC ...

TCV sub-genomic (3) . . . GGGAUAUAAAGAAGAA . CUUAGTG ...

Motif II

C

Motif III

SAT-RNA CX ... GAGUCCAAGACCCUGUCUUAGUAGUUAGCUCUCUCU ...

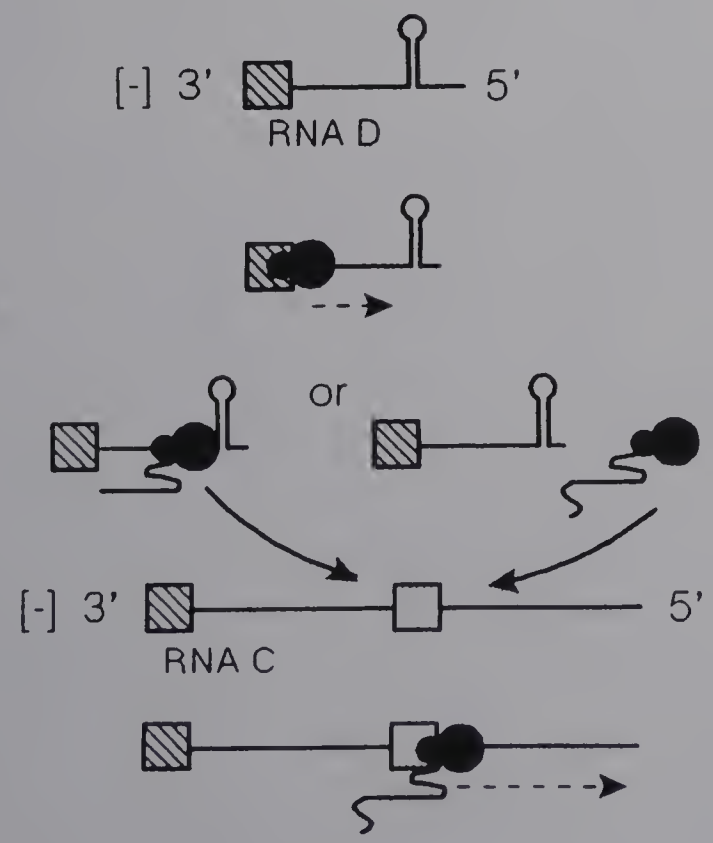
TCV sub-genomic (2) . . . G . CUUUCUA . . CA ACUCUCUCU ...

Double recomb. ... CCAAGACCCUGCCUCUUAGUAGUUCUCAAAAGAA .....

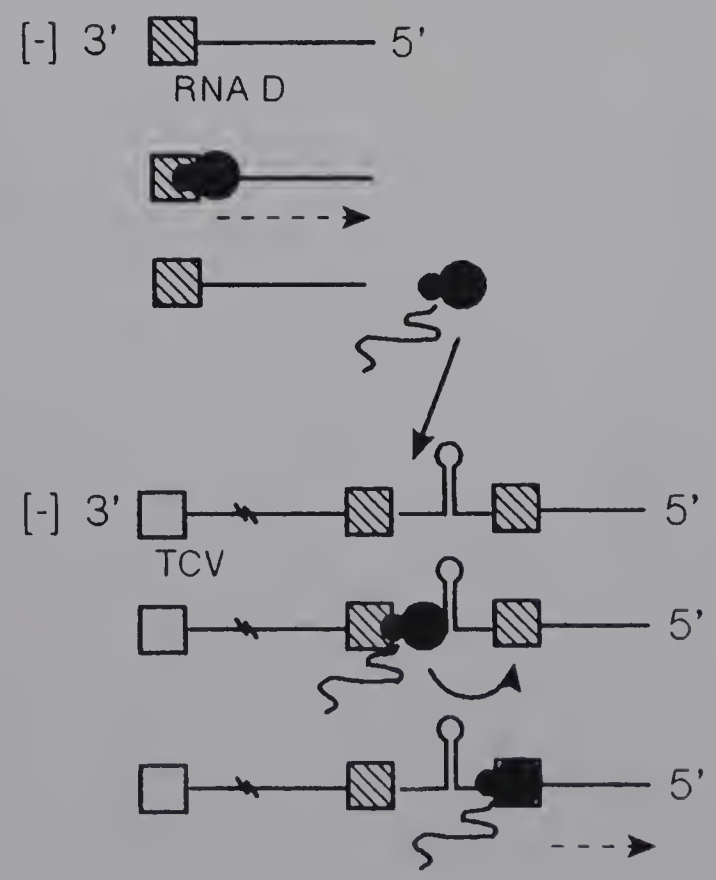
Motif I.....

Fig. 1.3 Model for the generation of (**A**) recombinant sat-RNAs, (**B**) sat-RNA C and (**C**) sat-RNA dimers. The model suggests that during replication of (-) strand templates, the replicase (represented by two filled-in circles) dissociates from the template due to a yet undetermined constraint (represented by a hairpin structure) or after completion of the daughter strand, and then reinitiates synthesis at one of two recognition signal (motifs 1 and 2, represented by shaded boxes) without first releasing the nascent (+) strand. The two recognition signals are shaded differently (Cascone *et al.*, 1990; Carpenter *et al.*, 1991a, manuscript submitted).

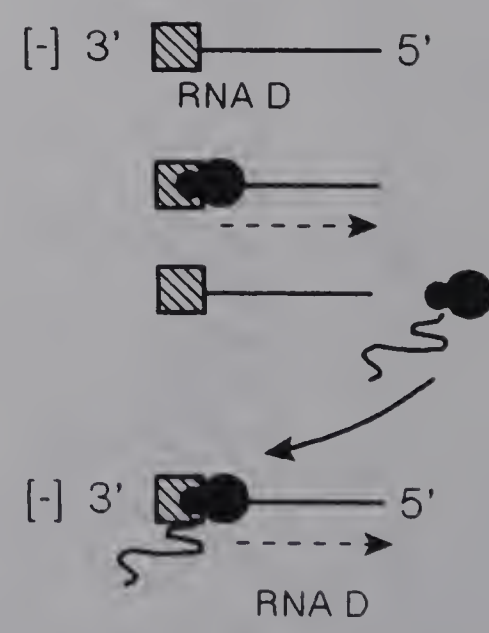
A: Recombinant Satellite



B: Sat-RNA C



C: Dimers



replicase is thought to dissociate from the template along with the nascent (+) strand, while copying viral or subviral (-) strands, and reinitiate synthesis at internal replicase recognition sequences (the motifs) on different templates thus generating recombinant sat-RNAs as well as chimeric sat-RNAs, such as sat-RNA C. The evidence supporting the copy choice model for formation of multimeric forms of sat-RNAs comes from the following observations: 1) As with like sat-RNA recombinants, approximately 30% of sat-RNA multimers have non-template nucleotides at junctions between the monomer units, and 2) deletions found at the 3′ end of sat-RNA D monomeric units in multimers are also found at the 3′ end of sat-RNA D in recombinants. These similarities between junction sequences of multimeric sat-RNAs and sat-RNA recombinants indicate that these molecules are possibly formed in the same way (Fig. 1.3 C, Carpenter *et al.*, 1991a, manuscript submitted).

Defective Interfering RNAs (DI RNAs)

DI RNAs are a second type of sub-viral RNA associated with animal and plant viruses which can modulate virus disease symptoms. As delineated by Huang and Baltimore (1970), DI RNAs are defective versions of viral RNAs that have lost essential coding sequences required for viral movement, replication, maturation, or packaging. A helper

virus is required to restore the deleted functions (Holland, 1985; Schlesinger, 1988). DI RNAs, in general, interfere with replication of homologous infectious helper virus (Huang and Baltimore, 1970). Most DI-RNAs are encapsidated separately form their helper virus genomes in their own particles (DI particle, or DIP). However, unlike satellite viruses in which the small RNA encode its own capsid proteins, DIP coat proteins are still encoded by the helper virus genome since DI-RNAs have no coding capacity (Huang and Baltimore, 1977; Barret and Dimmock, 1986).

DIs have been found associated with nearly all groups of animal viruses, including DNA and RNA viruses (Huang and Baltimore, 1970), and have also been found associated with a few plant RNA viruses, such as wound tumor virus (WTV), a plant virus member of the Reoviridae (Anzola *et al.*, 1987); some members of the Tombusviruses, such as tomato bushy stunt virus (TBSV) (Hillman *et al.*, 1985; 1987; Morris and Hillman, 1989), and cymbidium ringspot virus (CyRSV) (Burgyan *et al.*, 1989); and, plant Rhabdoviruses, such as sonchus yellow net virus (SYNV) (Ismail and Milner, 1988). The only DI RNAs associated with plant viruses that have been characterized at the molecular level, so far, are the DI RNAs associated with TBSV (Morris and Hillman, 1989), CyRSV (Rubino *et al.*, 1990) and TCV (this thesis). TBSV DI RNAs range from 350 to 500 bases in size. One of these small DI RNAs is a mosaic molecule derived completely from

the viral genome and attenuates symptom. The sequence of the DI RNA is derived from the 3′ and 5′ ends, as well as internal sequences of the TBSV genome (Hillman *et al.*, 1987; Morris and Hillman, 1989). Similarly, a DI RNA associated with CyRSV is 499 nucleotides long, containing six stretches of sequence derived from CyRSV genomic RNA. The 5′ and 3′ ends of this DI RNA are identical to those of the genomic RNA.

In animal virus systems, DI RNAs often compete with the non-defective virus for limited replication components, resulting in a decrease in the accumulation of viral genomic RNA. This interference with viral replication results in protection against viral-induced cytopathic effects in cell culture and in some cases has been implicated in virus disease modulation in whole animals (Huang, 1988). Analogous phenomena have also been observed for plant DI RNA infections. For example, *Nicotiana clevelandii* plants coinoculated with TBSV and DI RNA accumulate substantially lower amounts of virus and produce milder symptoms than plants inoculated with DI-free TBSV (Hillman *et al.*, 1985; Hillman *et al.*, 1987). Similarly, reduction in viral RNA has been detected in tobacco plants infected with DIs of CyRSV (Burgyan *et al.*, 1989). This suppression of viral genomic RNA accumulation is also observed in tobacco protoplasts after inoculated with TBSV and its DIs (Jones *et al.*, 1990). In this study, protoplasts were inoculated with increasing ratios of

DI:genomic RNA; the results suggest that DI RNA-mediated suppression of genomic RNA synthesis results from competition for factors essential for viral replication in plants. Together, these results suggest that DIs may be important determinants in the course of acute, self-limiting viral infection (Huang and Baltimore, 1970). For this reason, using DI RNAs as tools to study molecular basis of disease symptom expression and induction, and to explore approaches for possible anti-viral agents against plant viruses (Kaper and Collmer, 1988; Morris and Hillman, 1989), as well as animal viruses (Huang, 1988), has been suggested.

Little is known about the generation and replication of DIs (Lazzarini *et al.*, 1981; McClure *et al.*, 1980). As suggested by Huang and Baltimore (1970), production of DIs is a two step process. The first step is DI generation from the inoculum containing helper virus alone. Formation of one DI molecule is sufficient to meet the requirement in the first step. One possible model for generation of DI RNAs and other discontinuous RNAs such as RNA recombinants involves a "copy choice" mechanism (Lazzarini *et al.*, 1981; Holland, 1985; Kirkegaard and Baltimore, 1986) similar to that described above for TCV recombinants. In this model, viral RNA polymerase is thought to switch, along with the nascent strand, to either a different, random sequence on the same template to produce DI-RNAs or a different template to produce RNA recombinants. The "copy choice"

model is also thought to be the mechanism for generation of TBSV DI RNAs since no sequence conservation has been observed at the deletion junctions or in the deleted blocks (Hillman *et al.*, 1987), as might be expected for a possible splicing mechanism (Mount, 1982).

The second step in DI production is amplification at the expense of helper virus and ultimate domination of the virus yield. Studying negative-strand RNA animal viruses, Huang and Baltimore (1970) proposed a model to explain amplification of DI-RNAs at the expense of their helper viruses. They speculate that if the rate of RNA polymerization is the same on all virus-specific templates, a small RNA molecule would be completed more quickly than a larger one. This is analogous to a phenomenon which occurs during the *in vitro* synthesis of bacteriophage *QB* RNA. Small segments of *QB* RNAs compete successfully with large *QB* RNA for replicase and ultimately dominate the reaction (Mills *et al.*, 1967).

The dispensability of DIs to their helper virus and their relatively small sizes suggest that DIs share minimum essential characteristics with helper viruses and provide only cis-acting recognition signals for replication and encapsidation, while all trans-acting elements are provided by the helper virus (Lazzarini *et al.*, 1981). In addition, in order to be replicated and amplified in cells, DI RNAs must be of sufficient size to be recognized by the helper virus replicase and encapsidated by viral proteins. By

performing competition experiments between different sendai virus DI RNA species of different sizes, Re and Kingsbury (1988) found that DI RNAs smaller than 1600 bases were gradually eliminated from virions in mixed infections with larger DI RNAs. To identify minimum sequences required for the replication and encapsidation of DI RNAs of Sindbis virus, Levis *et al*. (1986) determined the effects of a series of deletions on the biological activity of the DI genome. They demonstrated that more than 90% of the sequences in the DI RNA have no essential role in the replication or packaging of the molecule. Only 162 nucleotides at the 5′ terminus and 19 nucleotides at the 3′ terminus are specifically required for replication and packaging. However, a DI transcripts containing only these 19 and 162 bases was not detected in their amplification assay, indicating that other sequences probably are important in keeping the DI a certain size to fulfill the packaging constraints.

To gain insight into the mechanism of DI generation, Kimiko and Nomoto (1989) recently constructed a poliovirus DI particle *in vitro*. The infectious DI RNA was synthesized from a cloned poliovirus cDNA by using bacteriophage T7 RNA polymerase. This cloned cDNA carried a deletion in the genome region corresponding to nucleotide positions 1663-2478 which encodes viral capsid proteins. Insertion of 4 nucleotides at position 2978 (which disrupts an open reading frame) abolished the infectivity, while insertion

of 12 nucleotides (leading to addition of three amino acids) had no effect on infectivity. From this observation, they suggested that a nonstructural protein(s) may function as a cis-acting protein(s) for DI replication.

Lastly, the generation and interfering capacity of DI RNAs may vary enormously in different cell types (Choppin, 1969; Huang and Baltimore, 1970; Perrault and Holland, 1972; Holland *et al.*, 1976). In the case of the positive-strand animal viruses, even the final size and structure of the DI genome is strictly controlled by the cell type which generates the DIs (Stark and Kennedy, 1978), indicating host factor(s) involvement in DI-RNA formation.

Plant Resistance to Viral Infection

Plant resistance to viral infection can take many forms. As a general term, plant disease resistance represents situations in which plants either do not support viral replication (immunity); support viral replication while failing to develop visible symptoms (tolerance); support viral replication only in virus invasion sites and fail to systemicaly spread virus, resulting a localized infection (subliminal infection). Sometimes cells at the center of these local areas become necrotic in which case the reaction is termed hpersensitive. Hypersensitive reactions are a type of resistance response of a plant to

pathogen infection and involves the quick death of cells surrounding the infection site to form localized necrotic lesions. However, a hypersensitive reaction is not a prerequisite for localization of viral infection (Konate *et al.*, 1983; Zaitlin, 1987). The mechanisms that lead to necrosis may be quite different from that which lead to localization.

Plant resistance can be conferred at different stages of disease development and by different mechanisms. As suggested by Fraser (1987), at least 5 possible biochemical mechanisms are involved in plant resistance to viral infections. Resistance could be at the stage of viral transmission, that is, a plant resistant to a virus vector could lead to its resistance to a virus carried by the vector (Gibson and Pickett, 1983). Resistance could be at the stage of establishment of infection; or, resistance could be due to restriction of viral spread in plants (Holmes, 1938). More over, plants can protect themselves by inhibiting viral accumulation at the level of transcription, translation, or assembly (Wyatt and Kuhn, 1979; Maule *et al.*, 1980; Fraser and Gerwitz, 1980) or by inhibiting viral symptom formation (tolerance) (Kooistra, 1968).

The best studied plant virus resistance loci are the tobacco N and N' genes, which confer resistance to tobacco mosaic virus (TMV), and the Tm genes in tomato, conferring resistance to the tomato strain of TMV. The N gene, which

is naturally found in *Nicotiana glutinosa* (Holmes, 1929), is a single dominant gene which results in the hypersensitive response to all strains of TMV. All natural varieties of tobacco are susceptible (systemic infection) to TMV common strains (*Vulgare*, U1, OM) (Holmes, 1960). However, tobacco species, varieties, or cultivars that possess the dominant N gene are resistant to all strains of TMV (hypersensitive response). The N gene-controlled hypersensitive reaction has been mapped to the Hg chromosome in *N. glutinosa* and has been incorporated into different tobacco cultivars by crossing (Holmes, 1938; Valleau, 1952). The resulting cultivars all became hypersensitive to TMV infection. However, the function of the N gene product has not been identified. In the past 20 years, it has been suggested that the N gene is involved in the activation of an anti-viral factor (AVF) that can eliminate viral replication analogous to the interferon system in mammals (Antignus *et al.*, 1977; Sela *et al.*, 1978, 1981; Gera *et al.*, 1983). More recently, the involvement of the N gene in interactions with the viral movement protein and therefore inhibition of systemic viral movement has also been postulated (Moser *et al.*, 1988).

The N′ gene, originally found in *Nicotiana sylvestris* (Melchers *et al.*, 1966), is a second dominant gene conferring hypersensitivity to TMV. Unlike the N gene, the N′ gene localizes many but not all strains of TMV (Valleau, 1952; Fraser, 1983). By comparison of abilities to localize

different TMV strains in N and N′ gene plants, Fraser (1983) found that the size of lesions on N′ plants are more variable than on N plants and the N′ gene allows greater amount of virus multiplication per lesion than the N gene. Also, the ability of different TMV strains to multiply and cause lesions of different sizes on N′ plants, but not N plants, are strongly correlated with differences in the thermal stabilities of their isolated coat protein subunits (Fraser, 1983). This result indicates a possible involvement of viral coat protein in the N′ gene-conferred hypersensitive response in *N. sylvestris*. Recently, by making recombinant viruses between systemic and local lesion strains of TMV, Saito *et al* (1987) found that the production of systemic or local lesion symptoms by *N. sylverstris* is exactly correlated with the origin of the virus coat protein. More recently, by making a single point mutation in the TMV coat protein gene, Knorr and Dawson (1988) were able to localize a systemic strain of TMV on *N. sylvestris*. To determine the action of viral coat protein in eliciting the hypersensitive response in *N. sylvestris*, Culver and Dawson (1989) used *in vitro* synthesized TMV RNA transcripts which were missing the translational starting site of the coat protein gene. These coat protein free-RNA mutants move poorly out of inoculated leaves yet produce a systemic mosaic symptom 9 to 12 weeks after inoculation. All these results strongly suggest that TMV coat protein is

involved in eliciting hypersensitive reactions in N' plants although coat protein is not involved in development of systemic symptoms.

In tomato, two genes, Tm-1, Tm-2 and its allele, $Tm\text{-}2^2$, have been recognized as conferring resistance to some strains (Tm-1 and Tm-2) or all natural strains of TMV ($Tm\text{-}2^2$) tested (Pelham, 1966; Hall, 1980; Watanabe *et al.*, 1987). Tm-1 was originally found in progeny of crosses between cultivated tomatoes and some wild species of tomatoes (Pelham, 1966, 1972). Since TMV symptoms are suppressed in both Tm-1 homozygous tomatoes (Tm-1/Tm-1) and Tm-1 heterozygous tomato (Tm-1/+) plants, Tm-1 is assumed to be a dominant gene (Pelham, 1972). To understand the mode of resistance conferred by these genes, Watanabe *et al* (1987) measured the syntheses of all viral-coded proteins and TMV-specific RNAs in cell suspensions of different tomato lines (Tm-1/Tm-1, Tm-1/+ and +/+). After inoculation with wild type tomato strain (strain L) of TMV, the production of viral proteins and RNAs were markedly reduced in Tm-1 protoplasts but not in the protoplasts without the Tm-1 gene. The reduction of viral proteins and RNAs was not found in Tm-1 protoplasts inoculated with a resistance-breaking strain, Lta1, suggesting that Tm-1 confers resistance at the level of virus replication.

The resistance conferred by the Tm-2 or $Tm\text{-}2^2$ gene differs from the Tm-1 gene in that expression is only in whole plants or in leaf disks but not in isolated

protoplasts (Motoyoshi and Oshima, 1975, 1977; Stobbs and MacNeill, 1980). Further more, Meshi *et al*. (1989) found that a stain of TMV, Ltb1, could multiply in tomatoes with the Tm-2 gene. The Ltb1 phenotype was mapped to the 30-kD protein, a putative movement protein of TMV (Watanabe *et al*., 1987). Ltb1 virus has 2 amino acid changes in the 30-kD protein compared with the wild type L strain. *In vitro* generated viral RNAs with these two changes multiplied in tomatoes with the Tm-2 gene and induced essentially the same symptoms as those caused by Lta1. All these results suggest that the Tm-2 gene is probably closely related to the function of the 30-kD protein, presumably in virus cell-to-cell movement (Meshi *et al*., 1989).

It should be clear from the work described above that the resistance of plants to virus infection has been extensively studied yet the physical and biochemical mechanisms of resistance is still poorly understood. So far, no plant resistance gene has been isolated and no function has been defined for any plant resistance loci. The complexity of the plant response to pathogen infection constitutes a major difficulty for detailed study of the molecular events involved. Traditionally used plants, such as tobacco and tomato, have relatively long life cycles and large, complicated genomes. Such characteristics make these plants difficult systems to use for the study of plant resistance at the molecular level.

Thesis Plan

In this thesis, I describe my studies on pathogenicity and infectivity of TCV sub-viral RNAs and plant resistance to TCV infection. I have analyzed the effect of virulent sat-RNA C on disease symptoms for over 20 plant varieties in 5 different species of cruciferous plants. By testing large numbers of *A. thaliana* plants, I have found a TCV-resistance ecotype, Dijon. Working on a second TCV isolate, TCV-B, I have identified DI RNAs (DI RNA G and DI1) associated with this virus. Detailed studies of DI RNA pathogenicity and infectivity have been pursued. In Chapter I, I give an introduction on TCV virus, sat-RNAs, DI RNAs and plant resistance to viral infection. In Chapter II, I describe symptom intensification by the virulent sat-RNA C of TCV-M on different cruciferous hosts. Based on the finding that sat-RNA C causes lethal effects on *A. thaliana* plants, studies of the plant response to TCV infection and TCV-resistance have been initiated. In Chapter III, I present my research in determining protein and RNA accumulation in *A. thaliana* upon TCV-M infection by two-dimensional gel electrophoresis. Also, in this chapter, I describe how a TCV resistant ecotype of *A. thaliana*, Dijon, was found via screening thousands of *Arabidopsis* plants. In Chapter IV, I report my work on the identification and characterization of DI RNAs associated with TCV-B by Northern hybridization and cDNA cloning and

the determination that TCV symptoms are intensified by DI RNAs. Finally, in Chapter V, I present my work on analysing sequences required for DI-RNA infectivity by mutagenesis of DI RNA G, as well as attempts to synthesize artificial DIs from a full length genomic cDNA clone of TCV-B.

CHAPTER II

SYMPTOM INTENSIFICATION ON CRUCIFEROUS HOSTS BY THE VIRULENT SATELLITE RNA OF TURNIP CRINKLE VIRUS

Introduction

A naturally occurred TCV wild type isolate, TCV-M, is associated with a family of small sat-RNAs (sat-RNA D and sat-RNA F) and chimeric sat-RNAs (sat-RNA C and sat-RNA CX) (Altenbach and Howell, 1981; Murant *et al.*, 1988; Cascone *et al.*, in preparation). Sat-RNA C (356 b) is the only virulent satellite and has been shown to intensify TCV-M helper virus (TCV-m) symptoms on *Brassica rapa* spp. *rapifera* (turnip) cv. 'Just Right' (Altenbach and Howell, 1981; Simon, 1988; Simon and Howell; 1986). Turnip plants infected with TCV-m, lacking sat-RNA C, exhibit symptoms consisting of mild stunting, slight leaf crinkling and vein-clearing. The addition of sat-RNA C to the inoculum results in dark green plants which are severely stunted and have tightly crinkled leaves.

Many satellites have been identified which modulate the symptoms of their helper viruses (for recent reviews, see Kaper and Collmer, 1988; Simon, 1988). Satellites

associated with tobacco ringspot virus (Gerlach, *et al.*, 1987) and tomato bushy stunt virus (Gallitelli and Hull, 1985) are implicated in the attenuation of disease symptoms produced by their helper viruses. Satellites associated with cucumber mosaic (Kurath and Palukaitis, 1989), arabis mosaic (Davies and Clarke, 1983) and groundnut rosette viruses (Murant *et al.*, 1988) intensify symptoms on specific hosts in an undetermined manner. To date, symptom intensification by sat-RNA C has only been demonstrated for turnip cv. Just Right (Altenbach and Howell, 1981; Simon and Howell; 1987). In this chapter, I will describe my work on symptom modulation by sat-RNA C on other TCV hosts. Furthermore, I will describe my determination of host range and systemic viral spread of TCV-M on different cultivars within five species of crucifers. The majority of this work has been published in Phytopathology (80:238-242, 1990).

Materials and Methods

Inocula and Inoculations

The wild-type strain of TCV-M containing sat-RNAs C, D, and F was originally obtained from Roger Hull, John Innes Institute, England. TCV containing only sat-RNA D (TCV-m + D) was prepared by inoculating turnip with gel purified TCV. As previously reported (Simon and Howell, 1987), it has not been possible to maintain a stock of TCV-m without sat-RNA D.

For each plant, with the exception of *Arabidopsis*, the inoculum contained 10 *ug* total RNAs extracted from infected turnip leaves with either TCV-m plus sat-RNAs C, D and F (TCV-M) or TCV-m plus sat-RNA D (TCV-m + D). The RNAs were diluted in 70 *ul* RNA infection buffer (50 mM glycine/30 mM K_2HPO_4/0.02% bentonite, 0.5% Celite, pH 9.2) to meet a final RNA concentration of 0.14 *ug/ul*. Each *Arabidopsis* seedling took about one fourth the amount of inoculum as turnip, cabbage or mustard. Inoculations were carried out by rubbing the first two (for all the plants except *Arabidopsis*) or four true leaves (for *Arabidopsis*) with RNA inocula by fingers or by a glass rod at 2-leaf stage (for turnip, cabbage or mustard) or 4-leaf stage (for *Arabidopsis*).

Plant Materials and Growth Conditions

Plants used in this study were obtained as seeds from the following sources: *B. rapa* spp. *chinensis* 'Pak Choi Joi Choi' (Park Seed), 'Hon Tsai Tai' (Sakata Seed), 'Sha Ho Tsai' (Sakata Seed), 'Tsai Shim' (Sakata Seed); *B. rapa pekinensis* 'Michihili Jade Pagoda' (Park Seed), 'Green Rocket' (Park Seed); *B. r. perviridis* 'Tendergreen' (Park Seed), 'Savanna' (Park Seed); *B. r. rapifera* 'Just Right' (Burpee), 'Tokyo Cross' (Park Seed); *B. juncea* 'India' (Park Seed); *B. napus American* 'Purple' (Park Seed); *Arabidopsis thaliana* ecotypes La-0, Col-0, Ag-0, Bus-0, No-0 (generous gift of F. Ausebel, Harvard Medical

School); wild type ecotype Columbia and its ethylene-insensitive mutant Eth-1 (Chris Sommerville's lab, Michigan State University); ethylene-insensitive mutants Eth^r 1-2-3, 1-6A-1, 1-6B-1, 1-8A-1, 1-8B-1, 2-1-1 and their parental line Columbia (Howard Goodman's lab, Harvard Medical School); *Raphanus sativus* 'April Cross' (Park Seed), 'Inca' (Park Seed), 'Champion' (Park Seed), 'Cherry Belle' (Park Seed), 'French Breakfast' (Park Seed), and 'Icicle' (Park Seed).

Plants were grown in growth chambers under a 16 hr day. Temperature was 19°C day and 17°C night. Symptoms were generally first visible after 10 days. Scoring of symptoms was based on the analyses of at least six plants.

RNA Extraction and Analysis

RNA was extracted from all leaves of approximately the same stage of maturity about two weeks post-inoculation as previously described (Simon and Howell, 1986). Leaves were ground in liquid nitrogen. About 0.5 ml volume of leaf powder was quickly mixed in RNA extraction buffer containing 0.2 M tris (pH 9), 0.4 M LiCl, 25 mM EDTA and 1% (w/v) SDS immediately before phenol extraction. Total RNA was extracted 2 times with water saturated phenol, followed by one chloroform extraction to remove proteins. After ethanol precipitation, the RNA pellet was resuspended in 2 M LiCl solution and pelleted by a 2 min spin in a microfuge at 4°C to selectively precipitate RNAs from DNA and other

impurities. The RNA pellet was dissolved in 0.4 ml ddH_2O and ethanol precipitated. Total RNA extractions were subjected to electrophoresis on either 50% Urea, 5% polyacrylamide gels in 0.5x TBE (1x is 45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA) for sat-RNA detection or 1% nondenaturing agarose gels in 0.5x TBE for genomic RNA detection. The concentration of total RNA in each sample was determined spectrophotometrically. Gels were stained with ethidium bromide for direct visualization. Photographic negatives were scanned with a soft laser scanning densitometer (Biomed Instruments Inc., Fullerton, CA) and levels of TCV-m genomic RNA and sat-RNA C were normalized to internal rRNA controls in each lane.

Results

Previous studies using turnip cv. Just Right as a host indicated that sat-RNAs D and F were avirulent when inoculated separately or together on seedlings along with gel-purified genomic TCV (Altenbach and Howell, 1981; Simon and Howell, 1987). Sat-RNA C however, was virulent, intensifying the symptoms of TCV. In these experiments, it was not possible to maintain TCV-m free of sat-RNA D for more than a single passage, at most. Therefore, all TCV inocula used in this study contained sat-RNA D to assure uniform results.

Effect of Sat-RNA C on Different Cruciferous Plants

To determine the effect of sat-RNA C on various hosts other than turnip cv. Just Right, potential TCV hosts including 23 cultivars in 5 different species were inoculated with buffer (mock) or buffer along with TCV preparations which included sat-RNA C, sat-RNA D and sat-RNA F (TCV-M) or TCV which had been previously freed of sat-RNA C and sat-RNA F (TCV-m + D). Two to three weeks post-inoculation, plants were visually scored for the presence (or absence) of symptoms (Fig. 2.1). On all hosts where symptoms were detected following inoculation with TCV-m + D, sat-RNA C was found to intensify their symptoms. Plants infected with TCV-m + D (with the exception of *A. thaliana*) exhibited mild symptoms consisting of slight stunting and leaf crinkling. Plants infected with TCV-M exhibited greater stunting and crinkling as well as dark green leaves. Especially severe symptoms were associated with infection of all strains of *A. thaliana* (Fig. 2.1 A, 2-2). *A. thaliana* infected with TCV-m + D were more severely affected than other TCV-infected hosts, exhibiting symptoms including leaf browning, stunting and inhibition of bolting. *A. thaliana* infected with TCV-M became increasingly necrotic and were dead by 18 days post inoculation (Fig. 2.1 A, 2.2). The results of these analyses are summarized in Table 2.1. As expected, not all crucifers were hosts for TCV. Although several cultivars of *R. sativus* (radish) were reported to be severely

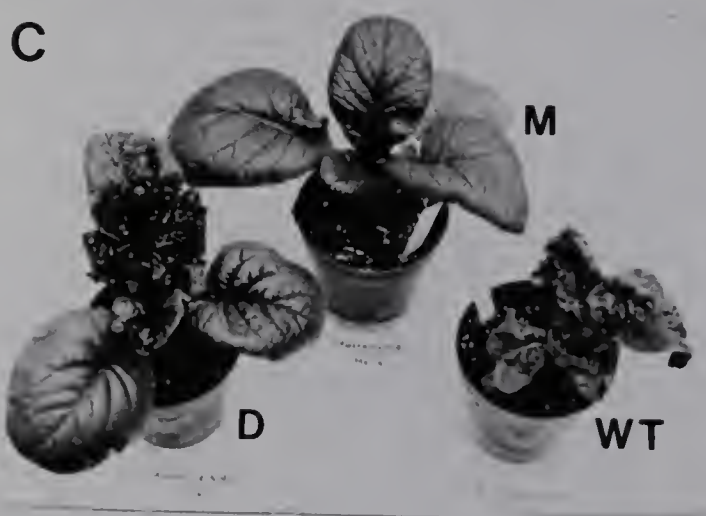
Fig. 2.1 Symptoms of selected crucifers infected with turnip crinkle virus plus either sat-RNAs C, D and F (TCV-M) or sat-RNA D alone (TCV-m + D). Plants were photographed two weeks after being inoculated with buffer (M), TCV-m + D (D) or TCV-M (W). **A**, *Arabidopsis thaliana* No-0; **B**, *Brassica. rapa* spp. *perviridis* 'Savanna'; **C**, *B. r. perviridis* 'Tendergreen'; **D**, *B. r. rapifera* 'Tokyo Cross'; **E**, *B. r. chinensis* 'Pak Choi Joi Choi'; **F**, *B. r. chinensis* 'Hon Tsai Tai'; **G**, *B. r. chinensis* 'Sha Ho Tsai'; **H**, *B. r. pekinensis* 'Tsai Shim'. There was no discernible difference in symptoms among different *A.thaliana* ecotypes. 'Sha Ho Tsai' and 'Tsai Shim' cultivars were tolerant to TCV infection and exhibited no discernible symptoms whether or not sat-RNA C was included in the inoculum.


A
M
D
WT


E
M
D
WT


B
M
D
WT


F
M
D
WT


C
M
D
WT


G
M
D
WT


D
M
D
WT

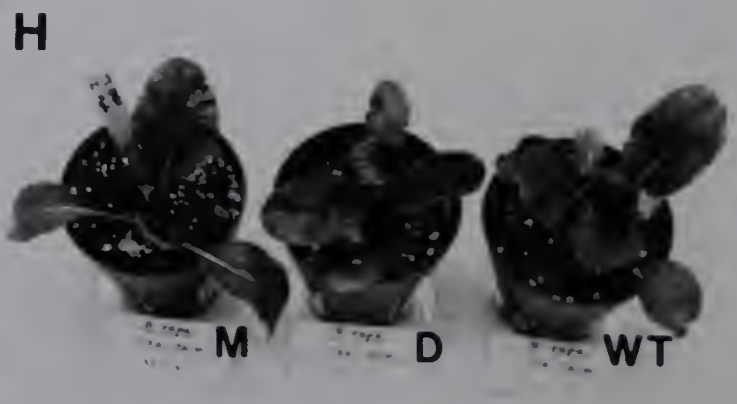

H
M
D
WT

Fig. 2.2 Symptoms of some ecotypes of *Arabidopsis thaliana* plants infected with TCV-m + D and TCV-M. Plants were photographed two weeks after being inoculated with buffer (Mock), TCV-m + D or TCV-M. **A**, Col-0; **B**, Bus-0; **C**, Ag-0; **D**, Cvi-0, **E**, La-0. As with No-0 plants shown in Fig. 1.1 A, all these *A. thaliana* ecotypes were severely affected by TCV-m + sat-RNA C infection, and all plants eventually died.

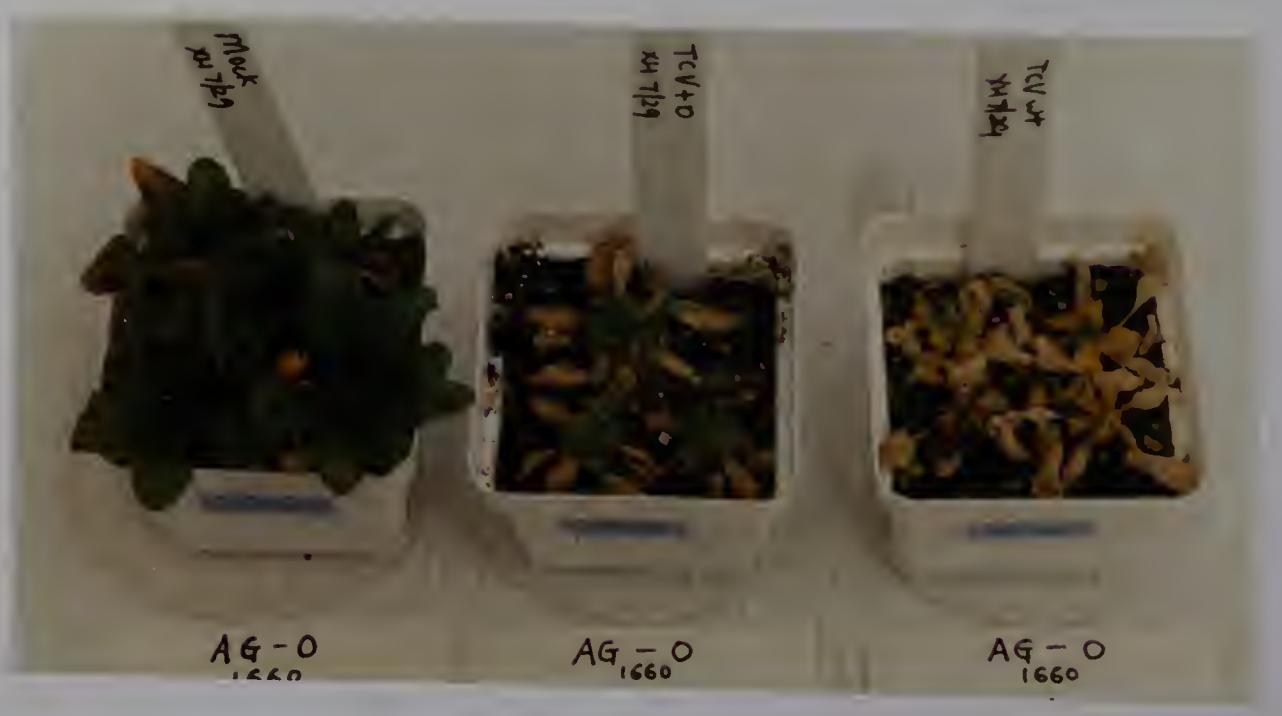
A


Mock
TCV-m +D
TCV-M
CoL-0
Mock
COL-0
TCV+D
CoL-0
TCV wt

B


BUS-0 1820
BUS-0 1820
BUS-0 1820

C


AG-0
AG-0
AG-0

Mock TCV-m + D TCV-M

D

E

Table 2.1 Response of some cruciferous plants to inoculation with turnip crinkle virus (TCV) plus one or several of its sat-RNAs

Potential plant host	Presence of systemic viral infection	Expression or non-expression of symptoms after inoculation with	
		TCV + D[a]	TCV WT[b]
Brassica rapa			
spp. *chinensis*			
Pak Choi hybrid	yes	+[c]	++
Hon Tsai Tai	yes	+	++
Sha Ho Tsai	yes	-	-
Tsai Shim	yes	-	-
spp. *pekinensis*			
Michihili Jade Pagoda	yes	-	-
Green Rocket Hybrid	yes	-	-
spp. *perviridis*			
Tendergreen	yes	+	++
Savanna	yes	+	++
spp. *rapifera*			
Tokyo Cross	yes	+	++
Just Right	yes	+	++
B. juncea			
India	no	-	-
B. napus			
American Purple	no	-	-
Arabidopsis thaliana			
La-0	yes	++	+++
Col-0	yes	++	+++
AG-0	yes	++	+++
Bus-0	yes	++	+++
No-0	yes	++	+++
Raphanus sativus			
April Cross	no	-	-
Inca	no	-	-
Champion	no	-	-
Cherry Belle	no	-	-
French Breakfast	no	-	-
Icicle	no	-	-

[a] Inoculum contains TCV plus the sat-RNA D.
[b] Inoculum contains TCV plus the sat-RNAs D, F and C.
[c] + = slight stunting and/or crinkled leaves, ++ = severe stunting and/or dark green crinkled leaves, +++ = severe necrosis and plant death, - = no detectable difference from mock-inoculated plants.

affected by infection of TCV (Broadbent and Heathcote, 1958), none of the cultivars of radish I inoculated exhibited symptoms, nor was the presence of TCV genomic RNA detected by acrylamide or agarose gel electrophoresis (data not shown).

All cultivars of *B. rapa* tested were hosts for TCV. However, not all cultivars exhibited symptoms in response to viral infection. Two of four cultivars of *B. r. chinensis* as well as both cultivars of *B. r. pekinensis* were tolerant to infection by TCV-m + D or TCV-M, exhibiting no visible symptoms over mock inoculated plants (Fig. 2.1 G, H).

All ethylene-insensitive mutants of *A thaliana* columbia showed the same response to TCV-m + D and TCV-M as their respective wild type Columbia (Fig. 2.3). The reason for using two Columbia backgrounds was that different strains propagated in different labs might be slightly different. As observed for all other *A. thaliana* wild ecotypes tested, these plants were dead 2 to 3 weeks post-inoculation in the presence of sat-RNA C.

Symptoms Induced by TCV on *A.Thaliana* Are Affected by Age of the Plant at Inoculation

In some early experiments, *A. thaliana* plants were inoculated at late stages when plants had at least 4 fully expanded leaves. Some of these plants, especially La-0, although showing intense symptoms after inoculation with

Fig. 2.3 Symptoms of selected plants of *Arabidopsis thaliana* ecotype Columbia and their respective ethylene-insensitive mutant lines inoculated with buffer (Mock), TCV-m + D and TCV-M. Plants were photographed 2 weeks post-inoculation. **A**, Plants of Columbia and its mutant line Eth-1; **B**, Plants of Columbia and its mutant line Eth^r 1-6B-1. There were no discernible differences in symptoms among different mutant lines and their respective Columbia background.

Mock TCV-m + D TCV-M

A

B

TCV-M, were still alive at least until 20 days after inoculation. However, plants inoculated at early stages were all killed by sat-RNA C. These observations suggested that these plants responded to TCV infection differently at different development stages. To confirm the effects of plant ages at inoculation on susceptibility, two sets of *A. thaliana* ecotypes La-0, Cvi-0, Col-0 and No-0 were inoculated with TCV-M and TCV-m + D at two different ages, 4 days apart. The younger plants (23 days old) were inoculated when plants had 4 leaves just as 1/4 to half size as fully expanded leaves. The older plants (27 days old) were inoculated when plants had 4 fully expanded leaves with another 4 leaves emerged. Severity of symptoms was scored and compared between the plants inoculated at different ages. All Cvi-0, Col-0 and No-0 plants had similar symptom severity when inoculated 4 days apart (data not shown). However, older (even just 4 days older) La-0 plants appeared less susceptible to both TCV-M and TCV-m + D (Fig. 2.4). In another independent experiment, La-0 and Col-0 were inoculated 8 days apart. At the time of inoculation, the younger plants (20 days old) were about the same size as younger plants described in above experiment. Again, Col-0 plants were killed 20 days after inoculation regardless of inoculation ages. Younger La-0 plants appeared much more susceptible to the virus infection than older ones (data not shown). From these experiments, I conclude that *A. thaliana* La-0 plants

Fig. 2.4 Symptoms of *Arabidopsis thaliana* La-0 plants inoculated at different ages with buffer (Mock), TCV-m + D and TCV-M. Plants were photographed 18 days post-inoculation. **A**, La-0 plants inoculated at younger age (23 days after planted). **B**, La-0 plants inoculated 4 days later (27 days after planted). Diagrammed plants indicating the size at inoculation time are shown at left side of photographs. Older La-0 plants appeared less susceptible to TCV infection than younger plants.

Mock TCV-m + D TCV-M

A

B

respond to TCV infection differently at different biological age. The decrease of sensitivity to TCV infection as plants aging were also observed in other *A. thaliana* such as No-0 and Bus-0. However, the insensitivity in these plants appeared only when the plants were inoculated at a much older age, for example, a 8-full leaf stage (data not shown). I should point out that, in these experiments, I did not only use "day" to infer the age of plants because in different experiments, slightly different conditions, such as watering schedule, etc., would make difference in germinating and growing rates of plants, especially for *Arabidopsis*. The ages of plants were, therefore, judged as sizes, as well as number of true leaves emerged at the time of inoculation.

TCV-m Genomic RNA and Sat-RNA Levels Are Not Reduced in TCV Tolerant Plants

In order to determine if the levels of viral and/or satellite accumulation were reduced in tolerant plants, total leaf RNA was prepared from plants two weeks post-inoculation and subjected to electrophoresis on 1% non-denaturing agarose gels for visualization of genomic TCV-m RNA or denaturing 5% polyacrylamide gels, for sat-RNA detection (Fig. 2.5). Gels were stained with ethidium bromide and photographic negatives scanned by densitometer to quantitate the relative levels of genomic TCV and sat-RNA C present in each plant. The results, presented in

Fig. 2.5 Accumulation of TCV genomic RNA and sat-RNA C in infected cultivars of *Brassica rapa*. Five micrograms of total RNA isolated from mock (M), TCV-m + D (D) or TCV-M (W) infected plants was subjected to electrophoresis on **A**, 1% non-denaturing agarose gels or **B**, 5% acrylamide/50% UREA gels followed by staining with EtBr. These gels and similar ones were scanned by densitometer to estimate the relative TCV genomic and sat-RNA C levels that are presented in Table 2.2. Only *B. rapa* 'Pak Choi' and 'Hon Tsai Tai' exhibited disease symptoms upon infection with TCV-m + D or TCV-M.

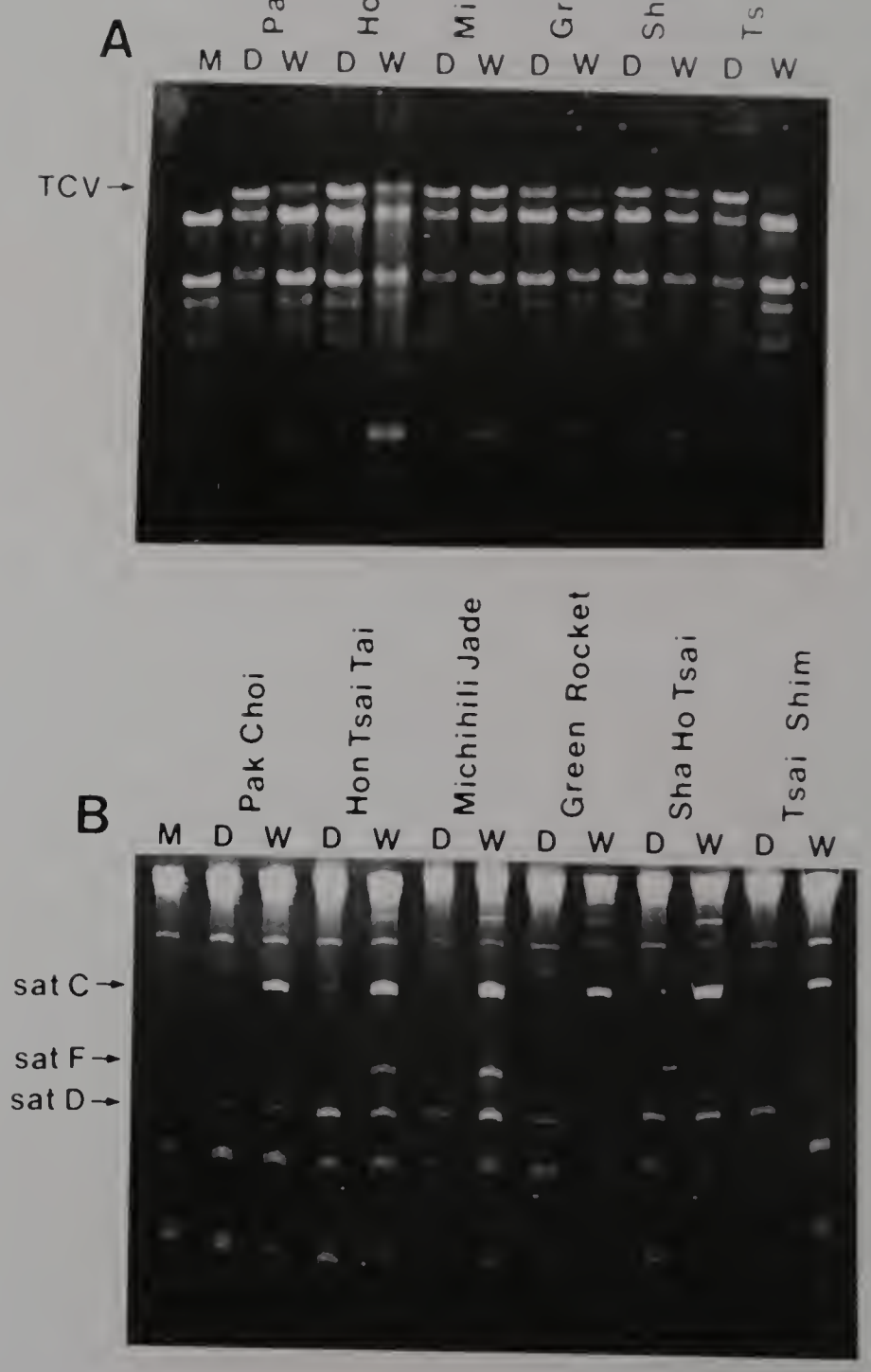

A
M D W D W D W D W D W D W
Pak Choi
Hon Tsai Tai
Michihili Jade
Green Rocket
Sha Ho Tsai
Tsai Shim
TCV
B
M D W D W D W D W D W D W
Pak Choi
Hon Tsai Tai
Michihili Jade
Green Rocket
Sha Ho Tsai
Tsai Shim
sat C
sat F
sat D

Table 2.2, indicate that there was no statistical significance at the $P < 0.05$ level in the accumulation of TCV-m or sat-RNA C in tolerant and susceptible *B. rapa* cultivars using the Mann-Whitney U test (Table 2.2). Therefore, we conclude that approximately equal levels of genomic TCV-m and sat-RNA C were present in all *B. rapa* cultivars whether or not symptoms were produced.

Sat-RNA F Is Not Responsible for Symptom Intensification

Because TCV M differs from TCV-m + D by the presence of sat-RNA F as well as sat-RNA C, it is necessary to demonstrate that sat-RNA F is not also involved in symptom production. *B. rapa* and *A. thaliana* plants which exhibit intensified symptoms when infected with TCV-M (Table 2.1 and Fig. 2.1), were inoculated with TCV-m + D along with *in vitro* synthesized sat-RNA C transcripts. There were no discernible differences in visible symptoms between plants which did or did not contain sat-RNA F (Fig. 2.6). I therefore conclude that intensified symptoms in plants infected by TCV M versus TCV-m + D were due to the presence of sat-RNA C.

Discussion

One characteristic which distinguishes plant viruses from most animal viruses is their common association with sat-RNAs. TCV is unique among plant viruses in its ability

Table 2.2 Relative levels of TCV genomic RNA and sat-RNA C accumulating in infected *B. rapa* tolerant and susceptible cultivars

B. rapa cultivar	Inoculum	Reletive accumulation[a] TCV	Sat-RNA C
Pak Choi	TCV + D	79 ± 38 (4)	
	TCV M	57 ± 22 (5)	290 ± 43 (5)
Hon Tsai Tai	TCV + D	92 (1)	
	TCV M	45 ± 22 (5)	429 ± 116 (5)
Michihili Jade Pagoda	TCV + D	57 ± 16 (3)	
	TCV M	75 ± 38 (3)	267 ± 160 (3)
Green Rocket	TCV + D	92 ± 1 (2)	
	TCV M	68 ± 34 (2)	306 ± 88 (2)
Sha Ho Tsai	TCV + D	71 ± 21 (3)	
	TCV M	49 ± 32 (4)	390 ± 247 (4)
Tsai Shim	TCV + D	103 ± 50 (4)	
	TCV M	72 ± 25 (5)	263 ± 96 (6)

[a] Values represent area under peaks produced by densitometer scanning, normalized to internal rRNA controls within each lane.

[b] Numbers in parentheses are the number of plants analyzed. Standard deviations are given

Fig. 2.6 Symptoms of selected plants of different *Arabidopsis thaliana* ecotypes and *Brassica rapa* spp. *chinensis* 'Pak Choi Joi Choi' inoculated with buffer (Mock), TCV-m + D, TCV-m + D + RNA C *in vitro* transcripts and TCV-M. Plants were photographed 3 weeks post-inoculation. **A**, *A. thaliana* No-0; **B**, *A. thaliana* Col-0; **C**, *A. thaliana* La-0; **D**, *B. r. chinensis* 'Pak Choi Joi Choi'. There was no discernible difference in symptoms between the plants infected with TCV-m + D + C and TCV-M, indicating that sat-RNA F which was present in TCV-M inoculum did not contribute to symptom intensification. Therefore, it is sat-RNA C that is responsible for symptom intensification on cruciferous plants.

A


Mock
TCV-m + D
TCV-m + D
+
C
TCV-M
NO-0 1833
Mock
NO-0 1833
TCV+D
NO-0 1833
TCV+C
NO-0 1833
TCV wt

B


CoL-0
Mock
COL-0
TCV+D
CoL-0
TCV+C
CoL-0
TCV wt

C

Mock | TCV-m + D | TCV-m + D + C | TCV-M

D

Mock | TCV-m + D | TCV-m + D + C | TCV-M

to support the simultaneous replication of a group of different sat-RNAs. Sat-RNA F, which differs from sat-RNA D mainly by the insertion of a 36 base sequence (Simon and Howell, 1986) and sat-RNA D were previously found to be avirulent on turnip cv. Just Right whereas sat-RNA C was a virulent satellite, exacerbating the normally mild symptoms produced by TCV alone (Altenbach and Howell, 1981; Simon *et al.*, 1988; Simon and Howell, 1987). Since virus sat-RNAs can have different effects on different hosts (reviewed in Kaper and Collmer, 1988), I was interested in determining what effects sat-RNA C might have on different hosts of TCV. For this report, I have tested 23 different cultivars from five species of crucifers and some ethylene-insensitive Columbia mutant lines for symptoms produced by sat-RNA C. My results indicate that all hosts of TCV which exhibit visible symptoms such as stunting and leaf crinkling upon viral infection produce more intense symptoms if sat-RNA C is included in the inoculum. I have also identified hosts which are symptomless when infected with TCV with or without sat-RNA C. Of the ten cultivars of *B. rapa* tested, four were tolerant to viral infection regardless of whether or not sat-RNA C was included in the inoculum. Lack of symptoms was not associated with a decrease in viral or sat-RNA accumulation.

Some *Arabidopsis* ecotypes, especially La-0, although highly susceptible to TCV infection, were less sensitive to TCV when inoculated at a late developmental stage. This

result correlates with other viral infections in which older leaves or plants produce less symptoms (Law *et al.*, 1989).

Ethylene, as one of the plant hormones, can influence a number of plant developmental processes. Ethylene is also known to accumulate in response to plant stresses including pathogen attack and wounding (Yang and Hoffman, 1984) and has been suggested to play a role in signal transduction in activating plant defense mechanisms against pathogen attack (Ecker and Davis, 1987). Several *Arabidopsis* Columbia mutant lines have been isolated which have lost the ability to respond to ethylene (Bleecker *et al.*, 1988). I have tested some of these mutants to determine whether or not these ethylene-insensitive plants also have altered response to TCV infection. My results show that these ethylene-insensitive mutants did not change their symptoms upon TCV infection. However, it would be interesting to ascertain if a TCV resistant line responses to ethylene differently from TCV sensitive lines if such a resistant line is available. Obviously, none of the *Arabidopsis* lines I tested here are resistant to TCV infection, although one of them showed a differential degree of symptom induction when inoculated late.

Little is known about how plant viruses and some sat-RNAs interact with their hosts to produce disease symptoms. Recent studies using tobacco mosaic virus engineered to contain deletions in the coat protein gene,

indicated a possible involvement of the coat protein in symptom production (Dawson *et al.*, 1988). Because of their small size, symptom modification domains are currently being mapped for several virulent satellite RNAs. Baulcombe and co-workers (Baulcombe *et al.*, 1988) and Kurath and Palukaitus (1989) independently determined that the determinants for yellow chlorosis and necrosis are located in distinct and separate regions of CMV sat-RNAs. Previously, studies with chimeric satellites containing the 5′ 155 base of sat-RNA F ligated to the 3′ 200 bases of sat-RNA C revealed that the 3′ of sat-RNA C is responsible for symptom intensification by satellites (Simon *et al.*, 1988). From this evidence, plus the striking similarity in the 3′ domains of TCV and sat-RNA C, we hypothesized that the virus and satellite might produce symptoms by interaction of their 3′sequences with a host component(s) (Simon *et al.*, 1988; Simon and Howell, 1986). Further intensification of symptoms by sat-RNA C could be due to a dosage effect brought about by the presence in infected cells of approximately ten fold more satellite molecules than genomic virus (Altenbach and Howell, 1981). If this hypothesis is correct, then sat-RNA C might have no effect on host morphology if infection by TCV alone produced no symptoms. The results reported here support this hypothesis.

CHAPTER III

PROTEIN AND RNA ACCUMULATION IN *ARABIDOPSIS THALIANA* UPON TCV INFECTION AND TCV-RESISTANCE IN *A. THALIANA*

Introduction

The interaction between plants and viruses can result in a diversity of host responses, depending on individual viral and host genomes. In a susceptible host, characteristic symptoms and systemic virus spread and accumulation occur (compatible reaction). In many instances, the genotype of different plants may express itself in a resistance response (incompatible reaction). It is the interaction between plant and virus that determines whether a compatible or incompatible reaction will occur.

Plant resistance can be either non-host, genetically-controlled or induced (acquired resistance), depending on concepts of underlying mechanisms (Fraser, 1987). Unlike non-host resistance, genetically-controlled resistance is conferred by individual resistant plants within a host species that harbor a resistance gene or genes against a particular virus normally attacking and

causing disease in that species. Genetically-controlled resistance is heritable and can be used for plant resistance breeding and manipulation.

During the course of virus invasion, a diversity of changes can be detected in host nucleic acid and protein metabolism. In early infection, inhibition of chloroplast proteins can occur (Doke and Hirai, 1970; Saunders *et al.*, 1989). For many host and virus combinations, large increases in peroxides activity has been found in hypersensitive infections (Sheen and Diachun, 1978; Wagih and Coutts, 1982a; 1982b), especially in cells surrounding necrotic local lesions. Peroxidase and polyphenoloxidase are known to catalyze the oxidation of phenolic compounds to quinines, which then polymerize to form the brown pigments associated with lesions, and which may be important in necrogenesis (Van Loon, 1983a). Change in RNase activity was also observed in different viral infections (Wyen *et al.*, 1972; Wagih and Coutts, 1982a, 1982b). Large induction of enzymes involved in the phenopropanoid pathway following viral infection has been suggested to be involved in defense against viruses via lignification (Legrand *et al.*, 1976; Collendavelloo *et al.*, 1983). Induction of so called pathogenesis related (PR) proteins upon viral infection probably have been most extensively studied (for reviews, see Bol *et al.*, 1990; Van Loon, 1985; Fraser, 1987). PR proteins usually are low molecular weight acidic proteins which accumulate to high

levels surrounding necrotic lesions in hypersensitive plants. It has been suggested that PR proteins may have a non-specific activity against microbial pathogens and insect pests (Cornelissen *et al.*, 1986; Richardson *et al.*, 1987), or may be involved in acquired resistance to viral infection (Sela, 1981; Van Loon and Antoniw, 1982; Richardson *et al.*, 1987). Since PR proteins can also be triggered by non-viral factors (Van Loon, 1983b, 1985), the specificity of these protein in virus infection is not clear.

The examples above demonstrate how complicated plant response to virus infection can be. Replication processes of a virus may interact with host metabolism at many different levels. As suggested by Fraser (Fraser, 1987), "certain host proteins might be directly involved in the biochemical process of viral replication that could be related to the production of symptoms. Other host proteins may be involved in recognition events during the development of pathogenesis or expression of resistance mechanism events". Because virus infection usually releases a cascade of events resulting in the disease syndrome, it is difficult to determine whether such changes are a cause or a result of disease. Studies are especially difficult when the host plant has a large, complex genome.

Plant resistance to viruses has attracted tremendous attention in plant virology studies; using plant resistance provides the most promising means of protecting plants

against viral infection. Several resistance loci have been identified in different plants. However, because of the large and complicated genomes of plants traditionally used for resistance breeding and studies, molecular analysis and characterization of resistance genes in these hosts are difficult. So far, no plant resistance gene has ever been cloned from these or any other plants.

Arabidopsis thaliana, a small cruciferous plant, possesses the characteristics of an ideal plant system for the study of viral disease resistance and plant-virus interactions. *A. thaliana* has a small haploid genome that is only 5 times yeast and 15 times E. coli, consisting of only 5 well characterized chromosomes (Pruitt and Meyerowitz, 1986; Meyerowitz, 1987; Meyerowitz, 1989) and a complete genome linkage map is available (Chang *et al.*, 1988; Koornneef *et al.*, 1983). *A. thaliana* also has a very short life cycle of approximately 6 weeks (Meyerowitz, 1987), and can be readily transformed by *Agrobacterium tumerfaceiens* (Valvekens *et al.*, 1988). *A. thaliana* is a host for several plant viruses including turnip yellow mosaic virus (TYMV) (Martinez *et al.*, 1989, meeting report) and cauliflower mosaic virus (CaMV) (Melcher, 1989). Most importantly, *A. thaliana* is also a host of TCV (see Chapter II)! All these characteristics have make it possible to study the molecular basis of plant resistance and plant response to viral infection using the TCV system.

Information gained from this simple system may provide a basic understanding of the mechanisms of plant resistance and molecular interactions between plants and viruses.

In this chapter, I report on my work screening for TCV resistance in *A. thaliana* plants, as part of an ambitious project on TCV resistance ongoing in the lab which include: identification of a possible resistance gene in *A. thaliana*, mapping the resistance gene(s) and eventually cloning and characterizing the gene(s). I also report on my work involving the study of susceptibility of *Arabidopsis* to TCV infection as a counterpart to the *A. thaliana* resistance studies. So far, most work on the molecular responses of plants to virus infection have focused on restriction of viral spreading or multiplication. Understanding molecular changes that occur during systemic virus infection and, especially, events that are involved in triggering plant susceptible responses at early infection stages, might suggest possible targets for engineering plant resistance by genetic and molecular manipulation.

Materials and Methods

Plant Materials and Inoculations

Arabidopsis thaliana wild type Columbia seeds were obtained from F. Ausubel, Harvard Medical School. These wild type Columbia plants were mainly used for analysis of

protein and RNA accumulation upon TCV infection. *A thaliana* M2 seeds derived from EMS treated Columbia seeds were also obtained from F. Ausubel. All the *A. thaliana* ecotypes screened for possible resistance to TCV were obtained from S. Sommerville, Michigan State University. These ecotypes are: Bur-0, Columbia, Di-0 (Dijon), Chi-1, Co-1, Columbia-glabrous, Etr, Rid, Estland, La-0, Montcalin, Bla-4, Nd-0, Pr-0, Bch-1, Msu12, Msu14, Msu15, Msu16, Msu24, Msu25 and Msu30.

All *Arabidopsis* seeds were planted onto damp Pro-Mix (Premier Brands Inc.) soil and vernilized at 4°C for 5 days to facilitate seed germination. After this cold treatment, plants were moved to room temperature and kept covered with plastic-wrap to keep moist for 5 days until germination. Plants were then moved to a growth chamber with a 16 h lightening/19°C day and dark/16°C night.

Plants were inoculated with either RNA infection buffer (mock) or total plant RNA extract containing either TCV-m + D or TCV-M, as described in Chapter II. Level of symptoms was scored 10 to 20 days post-inoculation.

Total Protein Extraction

Arabidopsis leaves at various times post-inoculation were ground to fine powder in liquid nitrogen. Proteins were extracted from about 500 *u*l volume of leaf powder in 200 *u*l urea-saturated protein-grinding buffer containing 9 M urea, 75 mM imidazole, pH7.5, 10% (v/v)

B-mercaptoethanol, 3% (w/v) CHAPs in a 1.5 eppendorf tube containing 66 mg powdered urea. While grinding, an additional 66 mg urea powder was added, and grinding continued until all urea was dissolved. Proteins were collected as supernatant after 10 min centrifugation at full speed (10,000 rpm) in a microcentrifuge.

Total RNA Extraction and *In Vitro* Translation

Total plant RNAs were extracted as described in Chapter II. RNA was extracted from plants previously inoculated at different time points and translated *in vitro* in a rabbit reticulocyte lysate (Stratagene *in vitro* translation kit) using conditions suggested by the manufacturer. After incubating 1 *u*g of RNA extract in 3 *u*l volume at 68°C for 30 seconds, 2 *u*l of ^{35}S-methionine (11.52 mCi/ml, ICN Biomedicals, Inc.) was added to each RNA extract for labeling the polypeptide products. Twenty microliter of quickly thawed and mixed lysate (stored at -80°C) was added to, and mixed well with, the RNA-label mixtures. The reactions were incubated at 30°C for 60 minutes for translation of proteins.

To check the specific activity of the labeled polypeptides, pieces of whatman paper were presoaked in 1 *u*g/*u*l unlabeled methionine, followed by dropping 2 *u*l of each *in vitro* translation sample onto each piece of the paper. After air drying, one drop of 10% (w/v) trichloacetic acid (TCA) was add to the pieces of paper to

precipitate the proteins. The paper was washed with 2% (w/v) TCA in a beaker 4 times. After drying, the paper was soaked in scientillation fluid, and the counts measured in a scintillation counter. Paper piece treated with 2*u*l free ^{35}S-methionine and 2 *u*l H_2O was used as a background control.

Two-dimensional (2-D) SDS-polyacrylamide Gel Electrophoresis

Two-dimensional gel electrophoresis was used to analyze proteins of both leaf extracts and *in vitro* products. The method used was recommended by Bio-Rad (Bio-Rad Bulletin #1144) with modifications by L. Schwartz (personal communication). The first dimension is isoelectrofocusing (IEF) during which proteins are separated according to their isoelectric points. The second dimension is SDS-polyacrylamide gel electrophoresis (SDS-PAGE) during which proteins are separated by molecular (subunit) size.

The 1-D gels were prepared in clean gel tubes with a diameter of 2.5 mm and a height of 14.8 cm filled with isoelectric focusing polyacrylamide solution containing a mixture of 8-5 and 10-3 amphyllines (2-D Pharmalyte, Pharmacia) at a ratio of 10:1 as described (Bio Rad Bulletin 1144) to a height of 13 to 13.5 cm. Twenty-five microliters of protein extract or 23 *u*l reaction solution of *in vitro* translation products were mixed with 5 *u*l IEF

sample concentrate (40% amphyllines with 2-D Pharmalyte 8-5 and 10-3 at the ratio of 10:1/20% *B*-mercaptoethanol/40% Triton X-100) and 4 *u*l glycerol added just before loading. The samples were loaded at the basic end of the focusing gels and overlaid with gel overlay solution containing 5 M urea, 5% (v/v) amphyllines of 8-5 and 10-3 at a ratio of 10:1, and 2% (v/v) Triton X-100. The upper (cathode) buffer was 0.4% (w/v) NaOH. The lower buffer (anode) was 0.05% (v/v) H_3PO_4. Electric focusing was conducted for 12 to 15 h at 400 V then 2 h at 800 V. Following the IEF, gels were carefully removed from the IEF tubes and marked with India Blue ink at one end. The pH gradient was measured from a control gel in which 25 *u*l of protein grinding buffer (without CHAPs) was loaded to replace 25 *u*l protein sample. This gel was cut into 5-10 mm pieces and soaked in 1 ml of degassed distilled water. After soaking for several hours, the pH was determined with a pH meter. Gels containing protein samples were soaked in 5 ml of SDS-reducing buffer containing 62.5 mM Tris, pH 6.8, 5.5% (v/v) *B*-mercaptoethanol, and 0.3% (w/v) SDS. After equilibration for 10 to 15 min, these gels were either immediately run on a second dimension gel or stored frozen (-20°C) in a glass tube sealed with parafilm.

The second dimension SDS gels were 1.5 mm thick and consisted of a 15 X 14 cm 12% acrylamide separation gel overlaid with a 15 X 2 cm 4% acrylamide stacking gel. Before loading, the IEF frozen gels were taken out from the

frozen tubes and equilibrated at room temperature for about 20 min in a petri dish. The focusing gel was sealed to the SDS gel with 1% agarose dissoloved in H_2O. The gel was run in 1X SDS-gel running buffer containing 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS. Electrophoresis was at 30 mA/gel, constant current, until the bromophenol blue dye ran to the bottom of the gel, about 5 to 6 hours. A 1 *u*l, 1:20 dilution, of low molecular protein standards (Bio Rad) were run along with the protein sample.

The second dimension gels were fixed in 50% methanol and 10% glacial acetic acid for one hour to overnight, followed by fixing in 2.5% gluteraldehyde for 30 min. Gels were silver stained according to Dick Rayne (personal communication). Gels were washed in 500 ml ddH_2O with 4 changes for 15 min each, followed by washing in 10 *u*g/ml fresh DTT solution for 30 min. Gels were then placed in 0.1% (w/v) $AgNO_3$ (1 mg/ml) for 30 min and rinsed in ddH_2O for 1 min. For development, gels were placed in 250 ml Na_2CO_3/formaldehyde solution (7.5 g Na_2CO_3, 250 ml H_2O, 125 *u*l 37% formaldehyde) for 2 to 10 min. The staining was stopped in 500 ml ddH_2O with 5 ml (1 capful) fixer (Kodak) for about 4 min. After rinsing with H_2O, the gel was photographed with a Fotodyne camera at a speed/aperture setting of 125/22.

Results

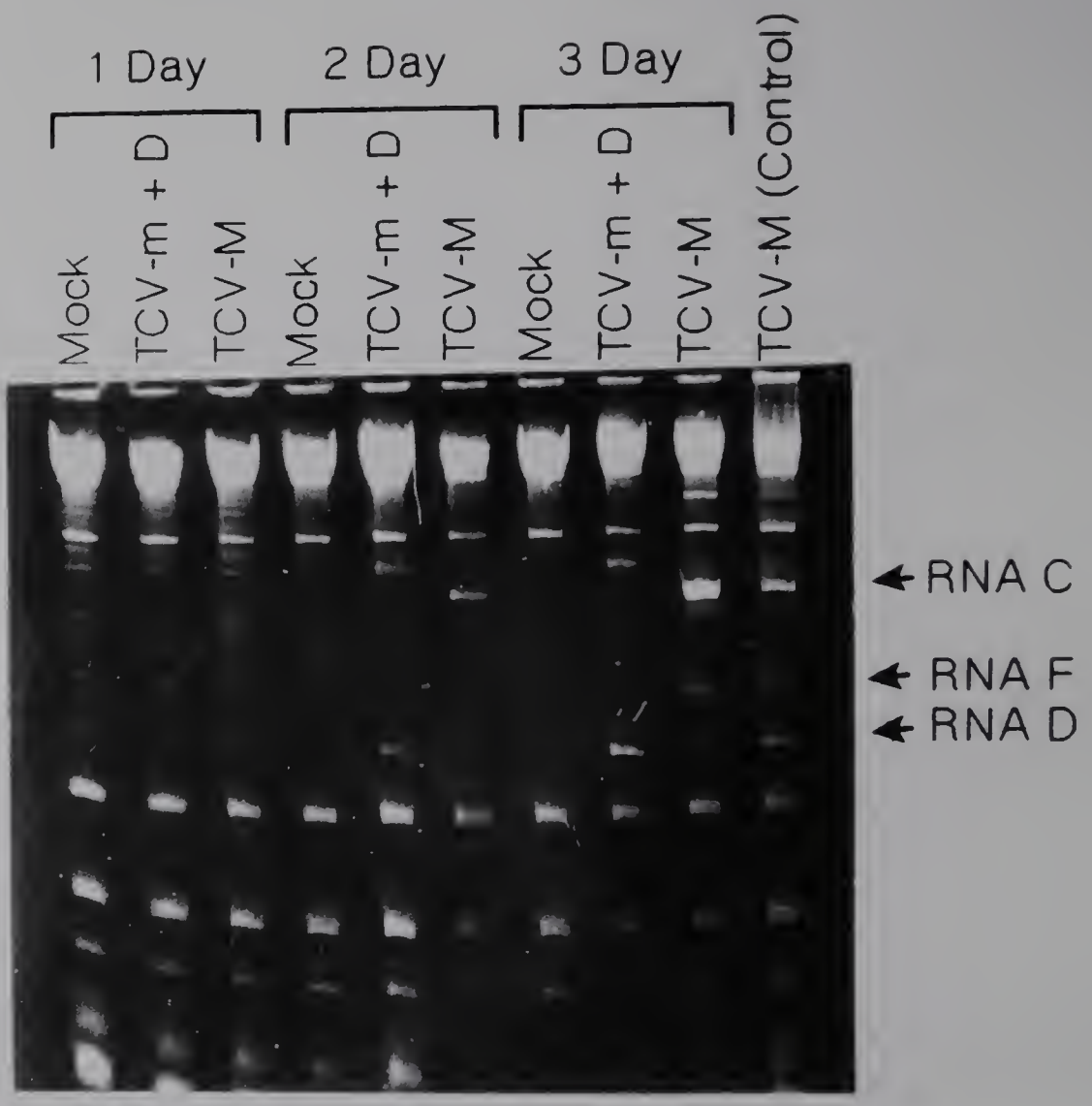
Early Accumulation of TCV Viral RNAs in Infected Susceptible *A. Thaliana* Plants

To determine the extent of sat-RNA accumulation over time in Columbia plants, total RNA was extracted from Columbia leaves that had been inoculated with either RNA infection buffer (mock), TCV-m + D, or TCV-M for 1 day, 3 days, or 6 days. Sat-RNA accumulation was detected followed electrophoresis on a 5 % denaturing polyacrylamide gel stained with ethidium bromide. Abundant levels of sat-RNAs C and D could be detected after staining from respective samples after 3 days of infection (Fig. 3.1). No visible sat-RNAs could be detected from 1 day infection samples. Since the first symptoms could be detected in infected Columbia plants 6 to 8 days after infection, this result demonstrates that TCV sat-RNAs accumulated at a high level at least three days before plants showed any discernible symptoms, indicating that TCV viral RNA replication is not the direct cause of symptom development in Columbia plants.

Differential Expression of Cellular Proteins in Mock and TCV Infected Susceptible *A. Thaliana* Plants

To determine whether there is differential protein accumulation in susceptible *A. thaliana* plants in mock versus TCV inoculated plants, total proteins were extracted from Columbia either 1 day, 3 days or 6 days

Fig. 3.1 Accumulation of TCV-M sat-RNAs in infected *A. thaliana* Columbia plants. Total RNA was extracted from infected plants that had been inoculated 1 day, 3 days or 6 days previously with buffer (mock), TCV-m + D or TCV-M. Four micrograms of total RNA was subjected to electrophoresis on 5% polyacrylamide/50% urea gels followed by staining with EtBr. RNA inoculum for each plant sample is indicated above each lane.


1 Day
2 Day
3 Day
Mock
TCV-m + D
TCV-M
Mock
TCV-m + D
TCV-M
Mock
TCV-m + D
TCV-M
TCV-M (Control)
RNA C
RNA F
RNA D

post-inoculation with either buffer (Mock), TCV-m + D or TCV-M. Extracted proteins were separated by two dimensional gel electrophoresis. The time points were chosen based on the observation that sat-RNAs appeared as early as 3 days after inoculation. Presumably, early events might be more important in determining plant susceptibility than changes appearing at later infection stages.

As presented in Fig. 3.2, polypeptides accumulating differentially following TCV infection fell into three categories: (1) proteins that only accumulated in infected plants, while absent or at low level in mock inoculated plants (Fig. 3.2 arrows e and 1; Fig. 3.3 arrows i, j, k, m, n); (2) proteins that were present in mock inoculated plants, while not discerable or decreased to low levels following virus infection (Fig. 3.2 arrows c, d, f, g, 2, 3); (3) proteins that were expressed at different level in TCV-m +D and TCV-M infected plants (Fig. 3.2 arrows a and b). Levels of polypeptide 'a' were not significantly reduced until 6 days after inoculation with TCV-M. The decrease of protein 'a' was not observed in mock and TCV-m + D inoculated plants. On the other hand, polypeptide 'b' was very low in TCV-m + D plants at 1 day and 3 day stages, reaching a high level in plants 6 days post-inoculation (Fig. 3.2). A cluster of proteins around 25 to 28 KDa was dramatically changed at the 6 day time point (Fig. 3.2 G, H, I open arrow X). Close ups of this area are shown in Fig. 3.3. Protein 'i', 'j', 'k' had similar molecular

Fig. 3.2 Two dimensional gel electrophoresis of proteins from infected *A. thaliana* Columbia plants. Total proteins were extracted from plants 1 day, 3 days or 6 days after inoculation with buffer, TCV-m + D or TCV-M. The first dimension isoelectric focusing gel contained a gradient of pH 6.8-4.8. The second dimension SDS polyacrylamide gel contained 12% acrylamide. The gels were stained for protein using silver nitrate. Solid arrows denote proteins which differed among the various inocula. Open arrows (X) indicate an area where several proteins accumulated differently among the different inoculations. Proteins in gels A, B and C were extracted from plants 1 day post-inoculation with buffer (**A**), TCV-m + D (**B**) or TCV-M (**C**), respectively. Proteins in gels D, E and F were extracted from plants 3 days post-inoculation with buffer (**D**), TCV-m + D (**E**) or TCV-M (**F**). Proteins in gels G, H and I were extracted from the plants 6 days post-inoculation with buffer (**G**), TCV-m + D (**H**) or TCV-M (**I**).

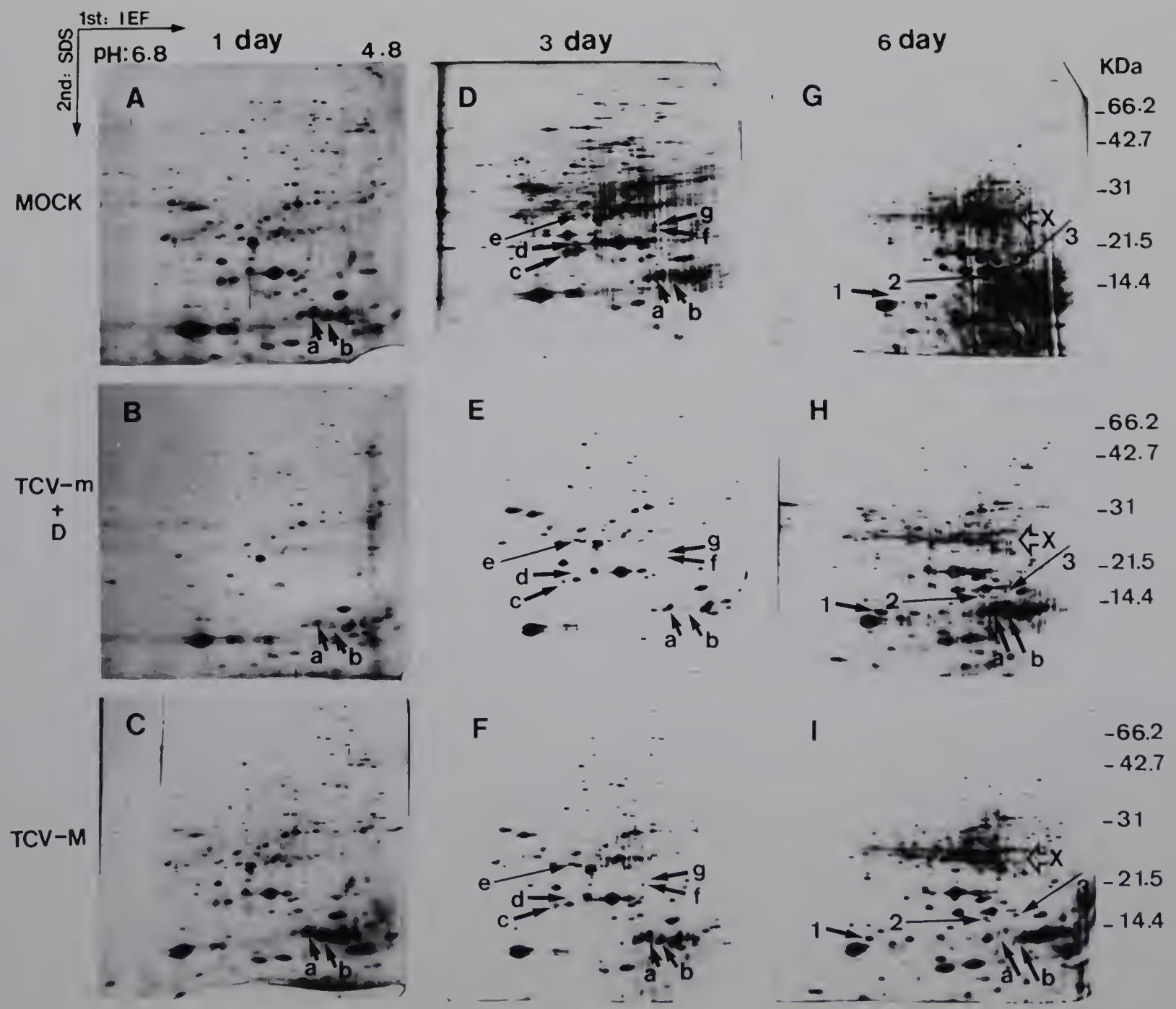
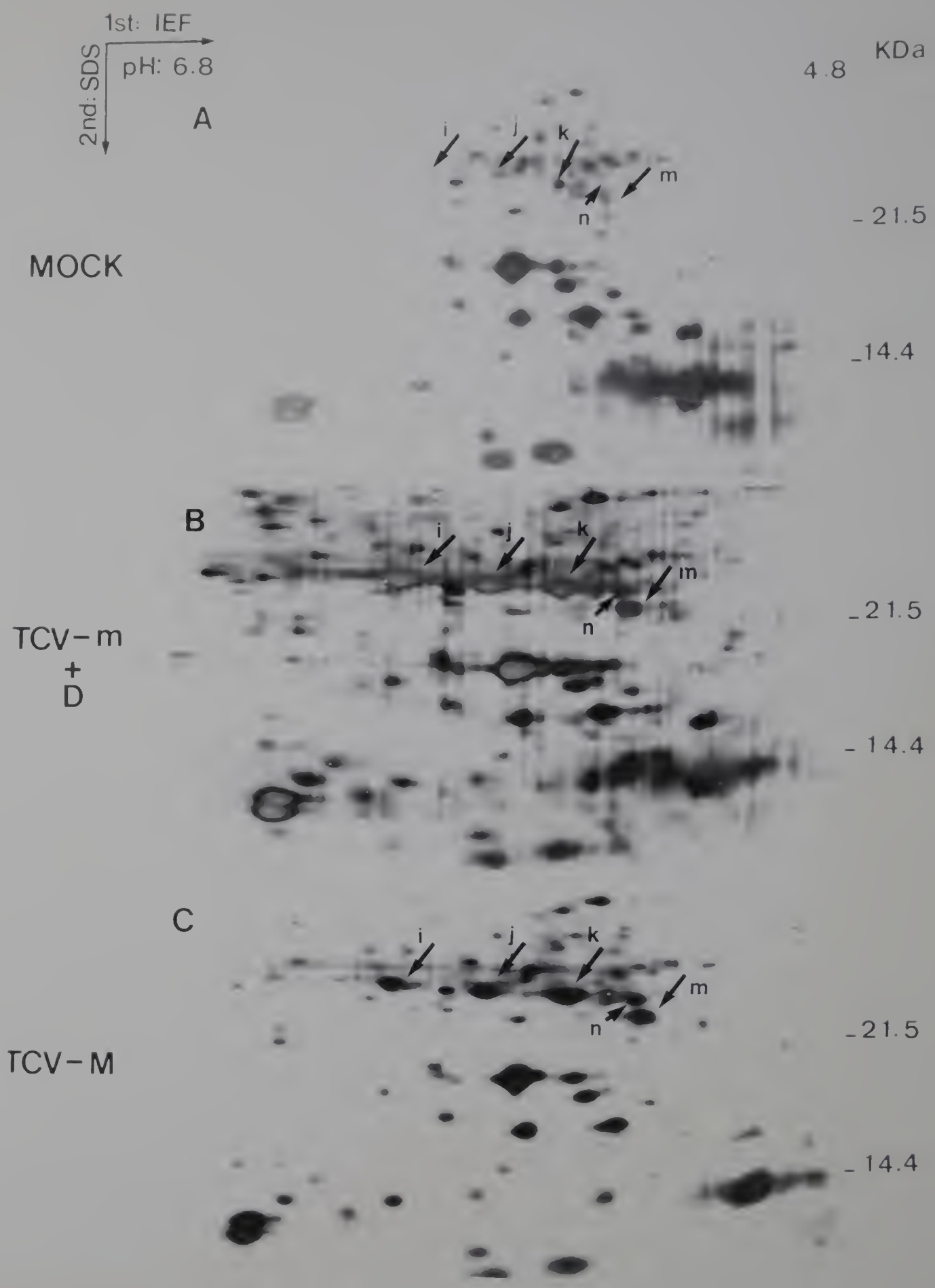

1st: IEF
2nd: SDS
pH:6.8
4.8
1 day
3 day
6 day
KDa
-66.2
-42.7
-31
-21.5
-14.4
MOCK
TCV-m + D
TCV-M
A
B
C
D
E
F
G
H
I
a
b
c
d
e
f
g
X
1
2
3

Fig. 3.3 Magnified 2-D gels of proteins from infected *A. thaliana* Columbia plants. Pictures of the gels representing plant protein extracted from 6 days post-inoculated plants as in Fig. 3.2 (G, H and I) were magnified for a better view of the area indicated by an open arrow (X) in Fig. 3.2 G, H and I. **A**, Proteins extracted from mock infected plants 6 days post-inoculation. **B**, Proteins extracted from TCV-m + D infected plants 6 days post-inoculation. **C**, Proteins extracted from TCV-M infected plants 6 days post-inoculation. The solid arrows indicate proteins that vary among different inoculations.


1st: IEF
2nd: SDS
pH: 6.8
A
KDa
4.8
i
j
k
m
n
- 21.5
MOCK
_14.4
B
i
j
k
m
n
_21.5
TCV-m
+
D
- 14.4
C
i
j
k
m
n
_21.5
TCV-M
- 14.4

weight and were separated by different isoelectric points. Polypeptides 'i', 'j', 'k' co-migrate with a putative TCV encoded replicase (28KDa) (Carrington *et al.*, 1989). However, because virus encoded replicases usually are present in very low levels (Mouches *et al.*, 1984), it is unlikely that proteins 'i', 'j', and 'k' could be the TCV encoded 28 KDa protein. Beside these proteins, there was no indication that any other deferentially expressed proteins were possible TCV encoded proteins from their molecular weight and isoelectric points. Therefore, it can be concluded that TCV infection indeed changes Columbia cellular protein expression at early stages of infection. TCV-M and TCV-m + D also appear to have different influences on expression or accumulation of cellular proteins.

Differential Accumulation of Cellular RNA in Mock and TCV-M Infected Susceptible *A. Thaliana* Plants

To determine if TCV-infection also changes the accumulation of cellular mRNAs in Columbia plants, total RNA was extracted from Columbia inoculated with either buffer or TCV-M for different periods of time and translated *in vitro* using a cell free rabbit reticulocyte lysate. Protein products were subjected to 2-D gel analysis. Because of the poorly developed gel for the 1 day TCV-M infection sample, it is difficult to interprete compare results for this time point. However, there are a

few differences discernable in Fig. 3.4 A and B (arrows indicate differently translated proteins between mock and TCV-M samples). At the 3 day stage (Fig. 3.4 C and D), several proteins translated using only the mock RNA sample or the TCV-M RNA sample (arrows). I conclude from this experiment that TCV infection influences RNA accumulation or translatability in plant cells as well.

Screening of TCV-resistance in *A. Thaliana* Plants

Over 6,000 Colombia plants from EMS treated seeds were inoculated with TCV-M in an attempt to screen for possible TCV-resistance in *A. thaliana* plants. Most of these plants died around 20 days post-inoculation. Fourteen plants, however, showed either reduced or no symptoms. To test if these 14 plants were truly resistant to TCV, progeny from these plants were rescreening. All the progeny plants died around 20 days after inoculations. These 14 plants were hence thought to have escaped from the initial inoculations. For further screening, plants of 22 different *A. thaliana* ecotypes were tested (see Materials and Methods for a listing of the ecotype tested). Most of them showed identical symptom production as susceptible Columbia control plants following TCV-M inoculation. However, ecotype Dijon plants inoculated with TCV-m + D or TCV-M appeared healthy until at least 22 days after inoculation (Fig. 3.5).

Fig. 3.4 Two dimensional gel electrophoresis of *in vitro* translated proteins from infected *A. thaliana* Columbia plant RNAs. Total RNA was extracted from plants 1 or 3 days post-inoculation with buffer (mock) or TCV-M. Gels A and B contain proteins translated from RNA of plants that had been inoculated with buffer (**A**) or TCV-M (**B**) for 1 day. Gels C and D contain proteins translated from RNA of plants that had been inoculated with buffer (**C**) or TCV-M (**D**) for 3 days. Arrows indicate proteins that only appeared in either 'mock' or 'TCV-M' samples.

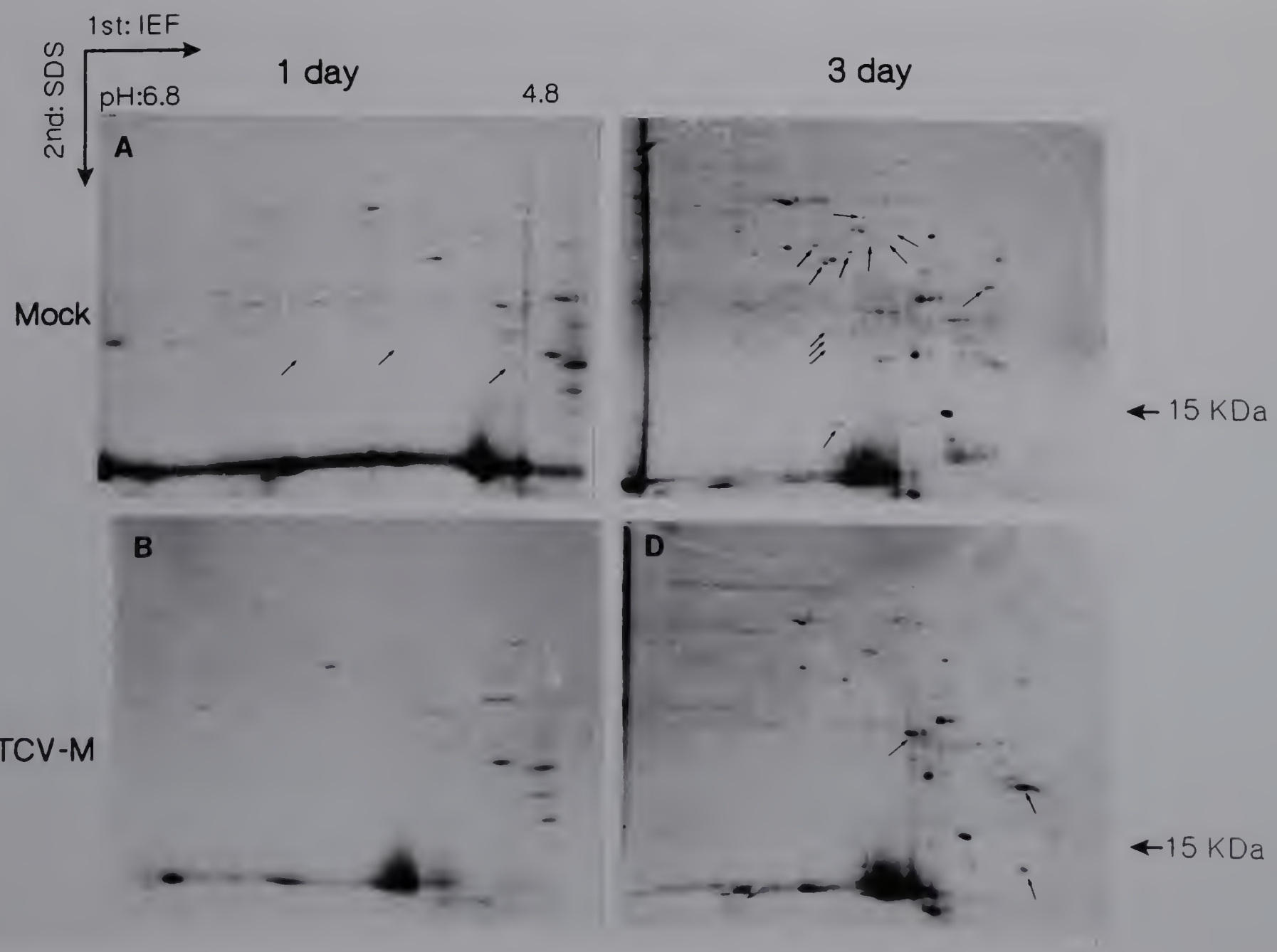

1st: IEF
2nd: SDS
pH:6.8
4.8
1 day
3 day
A
Mock
B
TCV-M
D
15 KDa
15 KDa

Fig. 3.5 Comparison of Columbia and Dijon inoculated with TCV. Dijon (1) and Columbia (2) were inoculated with either buffer (Mock), TCV-m + D or TCV-M at 21 days post-germination. (**A**) 8 days post-inoculation. (**B**) 10 days post-inoculation. (**C**) 12 days post-inoculation. (**D**) 14 days post-inoculation. (**E**) 18 days post-inoculation. (**F**) 22 days post-inoculation. Columbia plants begin to show symptoms 6 to 8 days after inoculation and died 22 days after inoculation. No visible symptoms were detected during this time for any Dijon plant.

Mock TCV-m + D TCV-M

A

B

Mock TCV-m + D TCV-M

C

D

E

F

To determine whether or not inoculations at different ages could lead to susceptibility in Dijon as previously found in La-0 plants (Chapter II), Dijon was inoculated with buffer, TCV-m + D or TCV-M at 4 different times, day 17 to day 23 after planting with a 2 day interval. At least 6 to 10 plants were inoculated for each time point. Columbia was also inoculated in exactly same way as a control. Twenty days after inoculation, all Columbia plants were dead, while all Dijon plants were healthy, regardless of inoculation age (Fig. 3.6). This suggests that, unlike La-0 plants, Dijon is resistant to TCV at all inoculation times tested.

These results suggest that the Dijon ecotype is resistant to TCV. TCV-resistance was possibly found in several other ecotypes as well, such as Msu 24 (Fig. 3.7 A) and Msu 30 (Fig 3-7 B). However, due to time limitation, no further tests were done to confirm resistance.

Discussion

In this chapter, I have reported my preliminary work on protein and mRNA accumulation in the susceptible *A. thaliana* ecotype Columbia after TCV infection. I have also reported my discovery of an *A. thaliana* ecotype which is resistant to TCV infection.

Fig. 3.6 Symptoms of Dijon inoculated at different ages. Dijon and Columbia were inoculated at 17 to 23 days after germination. Twenty days after inoculation, all Columbia plants were dead. Dijon showed the same degree of resistance independent of inoculation age. The ages of the plants at inoculation are indicated.

AGE OF INOCULATION

Fig. 3.7 Symptom expression of *A. thaliana* Msu-24 and Msu-30 plants following TCV inoculation. Msu-24 (A) or Msu-30 (B) plants were screened for TCV-resistance. Plants were inoculated with either buffer (Mock) or TCV-M. Unlike Columbia plants previously tested, Msu-24 and Msu-30 plants inoculated with TCV-M were still alive two to three weeks after inoculation.

A

B

By comparing cellular proteins accumulating in TCV inoculated vs. mock inoculated Columbia plants via 2-D gel analysis, several proteins were found to accumulate only in TCV-inoculated plants. These proteins were especially evident in plants 6 days after inoculation, the same time that plants begin to show symptoms. These proteins were not likely to be TCV encoded proteins based on their isoelectric focusing properties and molecular weights, as well as their abundance. Apparently, the differential accumulation of proteins is due to the virus infection.

Decreases in the accumultion of certain proteins in infected Columbia plants versus mock inoculated plants was also observed as early as 3 days post-inoculation before detectable symptoms, but after plants had accumulated appreciable levels of viral RNAs (Fig. 3.1). Therefore, symptom production was not a direct result of viral RNA replication. Another interesting finding was that a few proteins seem to be expressed differently in TCV-m + D versus TCV-M infected plants (Fig. 3.2, arrows a and b).

Possible explanations of differential protein expression early in infection are as follows: (i) TCV induces *de novo* expression of mRNAs coding for cellular proteins; (ii) TCV induces modifications which affects the stabilization/destabilization of pre-existing cellular proteins. Viroids, the smallest autonomously replicating pathogen consisting of only single strand RNA, is known to be able to modify host encoded proteins following infection

(Hiddinga *et al* 1988); (iii) TCV induces factors which suppress or induce further protein accumulation; (iv) Viral encoded proteins or viral RNAs bind directly to pre-existing cellular proteins, or vice versa, and influence their isoelectric points in 2-D gel as well as their stability. Evidence to support the last speculation came from the finding that the coat protein and 126 KDa protein of tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV) were associated with host chromatin, resulting in different symptoms (Van Telgen, 1985a, 1985b). Furthermore, cellular ubiquitin, a protein present in all eukaryotic organisms, has been known to conjugate with proteins encoded by number of plant viruses and a satellite (Hazelwood and Zaitlin, 1990).

Proteins that were expressed differently in TCV-m + D and TCV-M infected plants may be involved in the symptom intensification triggered by the virulent sat-RNA C. The disappearance of one protein (Fig. 3.2, arrow a) from 6 days TCV-M post-inoculated plants correlates with the appearance of symptoms. Another difference between TCV-M and TCV-m + D infections is the change in protein 'b' (Fig 3.2, arrow b). This protein apparently disappeared in 1 day and 3 day TCV-m + D infection and reappeared in 6 day infection. Since this protein was present at approximately the same level in both mock and TCV-M infected plants at

all stages and was abundantly present in TCV-m + D infection 6 days post-inoculation, these results are more difficult to interprete.

Although, the roles of the differentially expressed proteins is not known from these experiments, the relatively early responses and correlation with viral RNA accumulation and symptom expression suggests their importance in determining TCV pathogenicity or plant susceptibility. Understanding the precise role of differentially expressed proteins will necessitate further characterization. It is also not known whether changes observed early in Columbia infections are primary or secondary. However, relatively few changes in plant RNAs observed in *in vitro* translation experiment (Fig. 3.4) and the minor differences in protein accumulation in 1 day-infected plants may imply that these changes occur before bulk changes in plant metabolism. Furthermore, the observation that most early changes in plants 3 days after infection were the reduction of cellular proteins might suggest that at the beginning of infection, susceptibility was established by terminating the expression of plant defense proteins. Proteins involved in symptom production might be expressed later based on observations that production of new proteins contributed major changes in differential protein accumulation in plants 6 days after infection.

I have also identified a possible resistance gene in *A. thaliana* ecotype Dijon to TCV (Fig. 3.5, 3.6). Other ecotypes, Mus 25 and Mus 30 (Fig. 3.7), may also be resistant to TCV. Dijon inoculated at several different times post-germination did not express any symptoms, indicating that Dijon resistance is not dependent on age of inoculation as found for other viral infections (Law *et al.*, 1989).

Although no symptoms could be detected in Dijon, plants inoculated with TCV-M accumulated about 1/10 the level of viral RNA as Columbia plants although viral accumulation does not occur until 9-11 days post-inoculation (C. Carpenter, personal communication). Methods for detecting TCV in uninoculated leaves, as well as studies using protoplasts may help to determine whether this decreased viral RNA level is due to inhibition of viral movement or replication. Inhibition of symptom production in Dijon may be a direct consequence of the decrease in viral RNA accumulation. Analysis of proteins expressed in early Dijon infection may well complement studies involving early response of susceptible Columbia plants to TCV infection. Understanding fully the resistance of Dijon to TCV will rely on mapping, cloning and characterization of the resistant gene(s) involved. This work is now being actively pursued in the lab.

CHAPTER IV

TURNIP CRINKLE VIRUS DEFECTIVE INTERFERING RNAs INTENSIFY VIRAL SYMPTOMS AND ARE GENERATED *DE NOVO*

Introduction

Defective interfering (DI) RNAs have been found associated with a wide variety of animal viruses. First delineated by Huang and Baltimore (Huang and Baltimore, 1970), DI RNAs are defective versions of viral RNAs that have lost essential coding sequences required for independent replication, maturation, or packaging. DI RNAs are not infectious without the presence of a helper virus to restore the deleted functions (Holland, 1985). DI RNAs often compete with the nondefective virus for limited replication components, resulting in a decrease in the accumulation of helper virus. This interference with viral replication results in protection against viral-induced cytopathic affects in cell culture and in some cases has been implicated in virus disease modulation in whole animals (Huang, 1988). DI RNAs of animal viruses have also proved to be valuable subjects of study for many other important virological phenomena including the

identification of cis-acting sequences important in replication and encapsidation, fundamental studies on RNA replication and recombination, and more recently as transient expression vectors in animal cells (Schlesinger, 1988).

Although DI RNAs are generally considered as ubiquitous components of animal virus infections, they have not been common in plant virus infections (Anzola *et al.*, 1987; Hillman *et al.*, 1987; Morris and Hillman, 1989; Burgyan *et al.*, 1989). The only DI RNAs associated with an RNA plant virus which have been characterized at the molecular level are the small, symptom attenuating RNAs associated with the cherry strain of tomato bushy stunt virus (TBSV) (Hillman *et al.*, 1987) and CyRSV (Rubino *et al.*, 1990). The 396 b TBSV DI RNA, which is packaged along with the helper virus and requires the virus for infectivity, is a mosaic molecule derived from the 5′ and 3′ ends of the virus and internal viral sequences. Like many animal virus DIs, TBSV DI competes with the helper virus resulting in a reduced level of virus accumulation and a marked attenuation of viral symptoms in infected plants (Hillman *et al.*, 1987). Evidence supporting the origin of these DI RNAs from the TBSV genome has been demonstrated by high multiplicity passage (M.O.I) of a DI-free isolate (Morris and Hillman, 1989; Knorr and Morris, unpublished). These experiments are consistent with the origin of animal virus DI RNAs studied by high M.O.I.

passage in animal cell culture. Definitive proof of DI origin from a parental RNA virus genome, however, has not been demonstrated.

In this chapter, I report the identification of DI RNAs in association with an isolate of TCV which does not contain the previously described sat-RNA C. The availability of inocula derived from infectious transcripts of a complete clone of the viral genome (Heaton *et al.*, 1989) has permitted definitive demonstration of *de novo* generation of plant DI RNAs for the first time. Curiously, the DI RNAs also have the unusual ability to intensify symptoms caused by the helper virus.

Most of the work I describe in this chapter has been published in Proceeding of National Academic Science USA (86:9173-9177, 1989).

Materials and Methods

Virus Strains and Plant Inoculations

Two well-characterized isolates of TCV used in this study have been maintained as distinct laboratory isolates. One of the isolates, designated TCV-B, was originally acquired from Dr. R.J. Shepherd and has been propagated at U.C. Berkeley for many years in turnip. The complete sequence of this isolate has been recently determined (Carrington *et al.*, 1989) and infectious transcripts have been produced from complete cDNA clones of

the genome (Heaton *et al.*, 1989). Additional isolates which were derived from infectious transcripts of the clones pTCV-P1 (lambda promoter) and pTCV-T1d1 (T7 promoter) have been designated TCV-B-P1 and TCV-B-T1d1 respectively. The second laboratory isolate (TCV-M) has been used predominantly in the study of the TCV sat-RNAs (Altenbach and Howell, 1981; Simon and Howell, 1986; Simon *et al.*, 1988; Simon and Howell, 1988; Chapter II). It was originally obtained from Dr. R. Hull and normally contains three satellites (C, D, F). Isolates which have been modified by the curing of sat-RNAs from the inocula are indicated by the lower case initial of the original isolate as well as by designating the remaining sat-RNA(s) (for example TCV-m + D is the M isolate with only sat-RNA D), the same terminology as used in previous chapters. The viral genomic RNA from TCV-M has also been recently cloned and sequenced (Carpenter, unpublished result); the two isolates share over 99% sequence homology.

Inoculations were carried out using total RNA or virions isolated from previously infected plants. Five *ug* of total RNA or 70 ng of virions were diluted in 70 *ul* of RNA infection buffer (0.05 M glycine, 0.03 M K_2HPO_4, 0.02% bentonite, pH 9.2) or virion infection buffer (0.05 M Na_2HPO_4, pH 7.0), then mechanically inoculated onto leaves of 14 day old turnip cv. Just Right or Chinese cabbage cv.

Pak Choi Joi Choi seedlings which had been dusted with celite. Inoculated plants were grown in a growth chamber with a 16 h day, 70°F/55°F cycle.

Northern Hybridizations

Northern hybridizations were performed as previously described (Simon and Howell, 1986) except that 0.1-*um* pore, charged-modified nylon (Nytran; Scheicher and Shuell) replaced nitrocellulose. An oligonucleotide probe, complementary to the 3′ 20 terminal nucleotides of TCV-B genomic RNA (oligo 8), was labeled by incubation with 100 mCi γ-^{32}P-ATP (7000 Ci/mmole; NEN) and 10 units of polynucleotide kinase (Bethesda Research Labs) for 30 min at 37°C using buffer conditions suggested by the manufacturer. Full-length sat-RNA F probe was labeled using α-^{32}P-dATP (3000 Ci/mmole; ICN) and *E. coli* DNA polymerase I (Bethesda Research Labs) in a nick translation reaction.

Cloning and Sequencing of DI-RNA G and DI1 RNA

One *ug* of oligo 8 (20 b) was hybridized to approximately 0.5 *ug* of gel purified DI RNA G or DI1 RNA in 10 *ul* of hybridization buffer (0.4 M NaCl, 10 mM Pipes pH 6.4) at 80°C for 1 min then 75°C for 10 min followed by slow cooling to 60°C. First-strand cDNA was synthesized using 200 units of MMLV reverse transcriptase (BRL) in 50 *ul* of 50 mM Tris (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM

$MgCl_2$, 0.5 mM of each of dXTPs, 100 *u*g/ml BSA according to the manufacturer's instructions. After 1 h at 37°C, the first-strand cDNA and RNA were phenol/chloroform extracted in the presence of 1 *u*l of 10% SDS, followed by ethanol precipitation. Second-strand cDNA was synthesized using 20 units *E. coli* DNA polymerase I (BRL) in 100 *u*l of 25 mM Tris (pH 8.3), 100 mM KCl, 5 mM DTT, 5 mM $MgCl_2$, 0.5 mM of each of the dXTPs and 2 units of RNase H (BRL). The reaction was terminated after 2 h at 16°C by the addition of 1 *u*l of 10% SDS, followed by phenol/chloroform extraction and ethanol precipitation. Following treatment of the double stranded cDNA with *E. coli* polymerase large fragment (Klenow, BRL), Kpn I linkers were ligated to the double stranded cDNAs which were then digested with Kpn I and inserted into the Kpn I site of pUC 19. cDNA clones corresponding to DI-RNA G or DI1 were sequenced by a dideoxynucleotide chain termination protocol (Sequenase; US Biochemicals) using standard sequencing primers (Synthetic Genetics).

Primer-extension Sequencing

One *u*g of an oligonucleotide complementary to nucleotides 90-103 of DI-RNA G (oligo 12) was labeled with γ-^{32}P-ATP and polynucleotide kinase as described above. Approximately 0.5 *u*g of gel-purified DI-RNA G or DI1 was hybridized to oligo 12 in 10 *u*l of hybridization buffer for 10 min at 60°C, followed by slow cooling to 34°C. The

primer was extended using 20 units of MMLV reverse transcriptase (Bethesda Research Labs) under conditions suggested by the manufacturer. Full-length cDNA was isolated from 5% acrylamide, 50% urea gels and sequenced by the chemical modification method (Maxam and Gilbert, 1980).

Results

Identification of Low Molecular Weight RNAs Associated with the Berkeley Isolate of TCV

Collaborative studies were initiated in an effort to compare the satellite RNAs of Massachusetts and Berkeley isolates of TCV (TCV-M and TCV-B, respectively). Preliminary indications suggested that there were interesting biological differences between the isolates; TCV-B routinely produced less severe symptoms than TCV-M on cruciferous hosts (Fig. 4.1). Since the presence of sat-RNA C is correlated with the severe symptoms produced by TCV-M (Altenbach and Howell, 1981; Simon *et al.*, 1988; Chapter II, this thesis), the possibility existed that the low molecular weight RNAs associated with the isolates might be different. In order to determine whether or not sat-RNAs are also associated with TCV-B, RNA was isolated from turnip infected with TCV-B and subjected to electrophoresis on denaturing polyacrylamide gels (Fig. 4.2 A). Two low molecular weight RNAs not found in mock infected turnip were identified: one RNA which co-migrated

Fig. 4.1 Symptoms produced by TCV-B containing DI-RNA G, and TCV-M containing sat-RNA C. Mustard cv. Savanna (**A**) or Chinese cabbage cv. Pak Choi Joi Choi (**B**) were inoculated with either TCV-B or TCV-M and photographed 3 weeks post-inoculation.

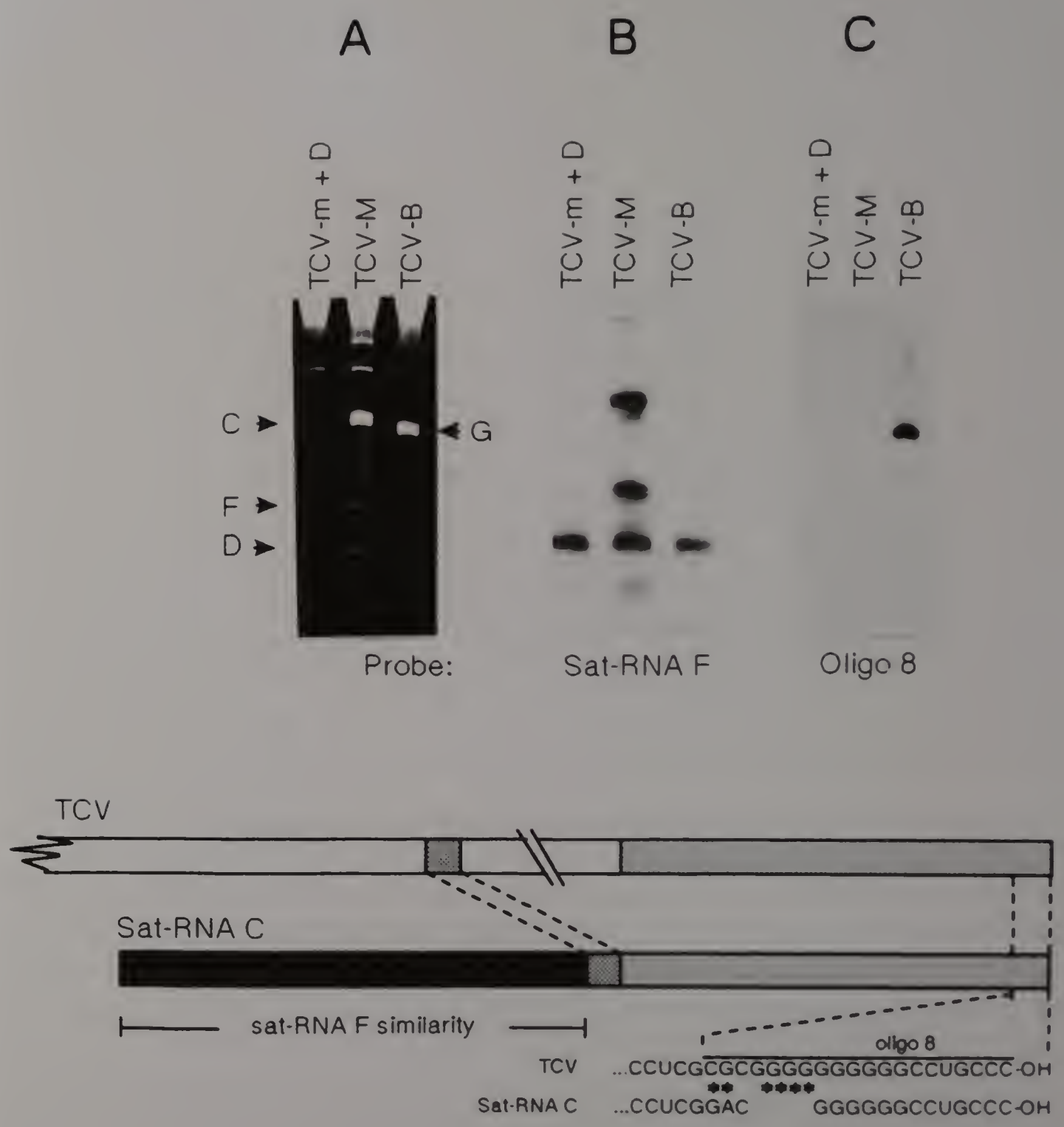
A Mustard

TCV-B TCV-M

B Cabbage

TCV-B TCV-M

Fig. 4.2 Hybridization blot analysis of small RNAs associated with two isolates of TCV. Total RNA was extracted from turnip infected with TCV-M, TCV-B or a strain derived from TCV-M which contains only genomic TCV and sat-RNA D (TCV-m + D), and subjected to electrophoresis on 4% acrylamide, 50% urea gels. (A) Ethidium bromide stained gel. (B and C) RNA was transferred to Nytran membrane and hybridized with a full-length cDNA clone of sat-RNA F labeled with α-^{32}P-dATP (B) or γ-^{32}P-ATP end-labeled oligonucleotide (oligo 8) complementary to the 3' terminal 20 nucleotides of TCV (C). TCV genomic RNA does not transfer efficiently under our conditions and cannot be detected after oligo 8 hybridization. 'C', 'F' and 'D' denote previously characterized TCV-M satellite RNAs. 'G' specifies the newly identified RNA associated with TCV-B. Sequence relationship within the 3' termini of TCV and sat-RNA C are shown below the blots. Astericks (*) denote nucleotide differences between TCV genomic RNA from isolate TCV-B and sat-RNA C.


A
B
C
TCV-m + D
TCV-M
TCV-B
C
F
D
G
Probe:
Sat-RNA F
Oligo 8
TCV
Sat-RNA C
sat-RNA F similarity
oligo 8
TCV ...CCUCGCGCGGGGGGGGGGCCUGCCC-OH
Sat-RNA C ...CCUCGGAC GGGGGGCCUGCCC-OH

with sat-RNA D, and a second RNA of slightly faster mobility than sat-RNA C. To determine whether or not these two RNAs were related to the similarly sized RNAs from TCV-M, RNAs from TCV-M and TCV-B infected plants were transferred to Nytran membranes following electrophoresis on denaturing polyacrylamide gels and then hybridized with either of two probes: a full-length cDNA of sat-RNA F, which shares substantial sequence similarity with both sat-RNA D and the 5′ 200 nucleotides of sat-RNA C (Simon and Howell, 1986), or an oligonucleotide (oligo 8) which is completely complementary to the 3′ terminal 20 nucleotides of genomic TCV-B RNA, but only weakly complementary to the corresponding portion of sat-RNA C (Fig. 4.2 B, C). The results of the hybridization analysis indicated that TCV-B contained a sat D-like RNA and a unique associated RNA which migrated slightly faster than sat-RNA C but which did not contain any of the characteristic 5′ satellite sequences. Furthermore, the slightly smaller RNA from TCV-B hybridized strongly to oligo 8 implying a 3′ sequence more similar to TCV genomic RNA than to sat-RNA C. This new RNA was termed RNA G.

RNA G Is Composed of Sequence Derived from TCV Genomic RNA

In order to determine the sequence of RNA G, cDNA clones were generated using oligo 8 as a primer for first strand cDNA synthesis. Following treatment with RNase H and *E. coli* DNA polymerase to produce the second DNA

strand, linkers were added and the cDNA cloned into the Kpn I site of the plasmid vector. The sequences of five nearly full-length clones of RNA G cDNA are presented in Figure 4.3. All five cDNA clones varied in sequence with the majority of nucleotide differences occurring within two hypervariable regions between bases 71-74 and 108-132. To determine the sequence at the 5' end of RNA G, an oligonucleotide (oligo 12) complementary to bases 90-103 was hybridized to gel-purified RNA G and then extended with reverse transcriptase. Fully extended cDNA was gel purified and the nucleotide sequence obtained by chemical modification methods (Maxama and Gilbert, 1980). The sequences of RNA G cDNA clones were compared with the sequence of genomic TCV (Carrington *et al.*, 1989) and the 3' domain of sat-RNA C which shares sequence similarity with TCV (Fig. 4·3). All of RNA G, with the exception of the 5' 21 nucleotides, was derived from genomic TCV. RNA G was contiguous with TCV beginning at TCV base 43 to base 140, then resuming at base 3863 until the 3' terminal nucleotide of TCV. The RNA G clones also contained a direct repeat of TCV sequences 3863-3898. Since RNA G is composed almost exclusively of helper virus segments and requires the helper virus for infectivity (data not shown), RNA G meets the definition of a defective interfering RNA. I henceforth refer to RNA G as DI RNA G. In DI RNA G, the 5' 21 nucleotides were diverged from the the 5 ' end sequence of TCV. About 50% of nucleotides within this

Fig. 4.3 Nucleotide sequence of RNA G. cDNAs were generated by extension of oligo 8 on gel purified RNA G by reverse transcriptase as described in Materials and Methods. Nucleotides following the brackets are the 5′ end points of the individual cDNAs. The 5′ sequence of RNA G was determined by chemical modification sequencing of primer extended cDNA as described in Methods. "." indicates a missing nucleotide. Only bases which differ from RNA G1 are shown. The nucleotide at position 10 in the terminal 5′ 21 bases was heterogeneous in the population of RNA G molecules. The 5′ terminal two nucleotides could not be determined. The sequence of TCV is from the Berkeley isolate (TCV-B) (Carrington *et al.*, 1989), and the sequence of sat-RNA C is from the cDNA clone 2-47 (Simon and Howell, 1986). An asterisk (*) indicates the point of divergence between the 5′ end of sat-RNA C and RNA G, and only that portion of the sat-RNA C sequence that is similar to RNA G is shown. Base numbering in italics corresponds to TCV (Carrington *et al.*, 1989).

```
                                1
TCV                      5'- NGTAATCTGCAAATCCCTGCACCC  CT  A TTGCC
RNA G                                                5'- NNTGCTAAANAGGAGGCT   18


TCV          C T                                                C      .
RNA G1       TACCAACCTTCTCTCTATTCACGATGCCTCTTCTACACACACTCAAAACAGCG.CTCG   75
RNA G3         [                                                      A  .
RNA G4                                          [                   T.
RNA G5                                        [                      .
RNA G9         [                                                     CA T

                                                          141/3863
TCV                        A                    T  .          ..   A        .
RNA G1       CAGTGGGACTCCTTGGAGCCAGGTACTACCCCGAAGGTTCAAAA.CCAAGACCCCCAAGT   134
RNA G3                          oligo12                              T    G      G
RNA G4                                                               T.          T
RNA G5                                                 ..  .         T.          T
RNA G9                                                     .         .T.    G     G

RNA C                                                                        •G
TCV                  T     CC          .....................................G
RNA G1       CGCTTTACTTTGAGATGTGTTAGAAAGCCCCAAGGTCATTTTACTTTGACCTGTGTTAGA   194
RNA G3                     AT
RNA G4                     AT
RNA G5                     AT
RNA G9                     AT

RNA C            A C     C        C                    C
TCV              A
RNA G1       GACCCAAAACGGTGGCAGCACTGTCTAGCTGCGGGCATTAGACTGGAAAACTAGTGCTCT   254
RNA G3
RNA G4
RNA G5
RNA G9

RNA C        T                                    .T   G              T   T
TCV          T                                    .    T    T             T
RNA G1       CTGGGTAACCACTAAAATCCCGAAAGGGTGGGCTAGTGGCGACCCTCCGAACTAAAAGAC   314
RNA G3                                            .    G              G   T
RNA G4                                            .
RNA G5                                            .
RNA G9                                            .    G              G   T

RNA C                       GAC....
TCV                          GC
RNA G1       AGCCTCCCTCCTCGC..GGGGGGGGGGCCTGCCC   346
RNA G3                          oligo 8
RNA G4
RNA G5
RNA G9
```

region were found in the same positions at the 5′ end of sat-RNA F. In a later experiment where DI RNA G dimer was cloned (see Chapter V for detailed information), I found that the 5′ 10 nucleotides of DI RNA G were accually identical to the 5′ 10 nucleotides of sat-RNA F. Problems in interpreting the primer extended sequence due to variable amount of adenosine residues near the 5′ end (see Fig. 5.9) may have contributed to the differences.

DI RNA G Intensifies the Symptoms of TCV

The observations that clonally pure inoculum resulted in slightly milder infections (Fig. 4.4) suggests that DI RNA G might be responsible for same symptom intensification in wild type TCV-B. To confirm this possibility, DI RNA G was gel purified following electrophoresis in denaturing acrylamide gels, added to an inoculum containing TCV-m + D, then inoculated on turnip, Chinese cabbage, or mustard. This isolate of TCV was used because of the extensive previous investigations on the symptom producing properties of TCV-M (Altenbach and Howell, 1981; Simon *et al.*, 1988; Chapter II, this thesis). Sat-RNA D was present with the helper virus since it has not been possible to maintain a stock of TCV-M free of this satellite (Simon and Howell, 1987). The results (Fig. 4.5) showed that addition of DI RNA G correlated with greater symptom production. Young leaves of plants accumulating DI RNA G had a darker green coloration and older leaves were chlorotic. Furthermore,

Fig. 4.4 Symptoms comparison among plants inoculated with buffer (M), TCV-B virus derived from pTCV-P1 transcripts (TCV-b) and TCV-B. (**A**) Mustard cv. Savanna and (**B**) Chinese cabbage cv. Pak Choi Joi Choi plants were photographed three weeks after inoculations.

A Mustard

B Cabbage

Fig. 4.5 Symptoms produced by DI RNA G. (A) Turnip cv. Just Right; (B) Chinese cabbage cv. Pak Choi Joi Choi or (C) Mustard cv. Tendergreen were photographed three weeks after the following inoculations: 1, buffer; 2, TCV-m + D; 3, TCV-m + D + gel purified DI-RNA G; 4, TCV-M.

A


Turnip
Mock
Turnip
TCV + D
Turnip
Turnip

B


Cabbage 5215
Mock
Cabbage 5215
TCV + D
Cabbage 5215

C


Mustard 5192
TCV+D

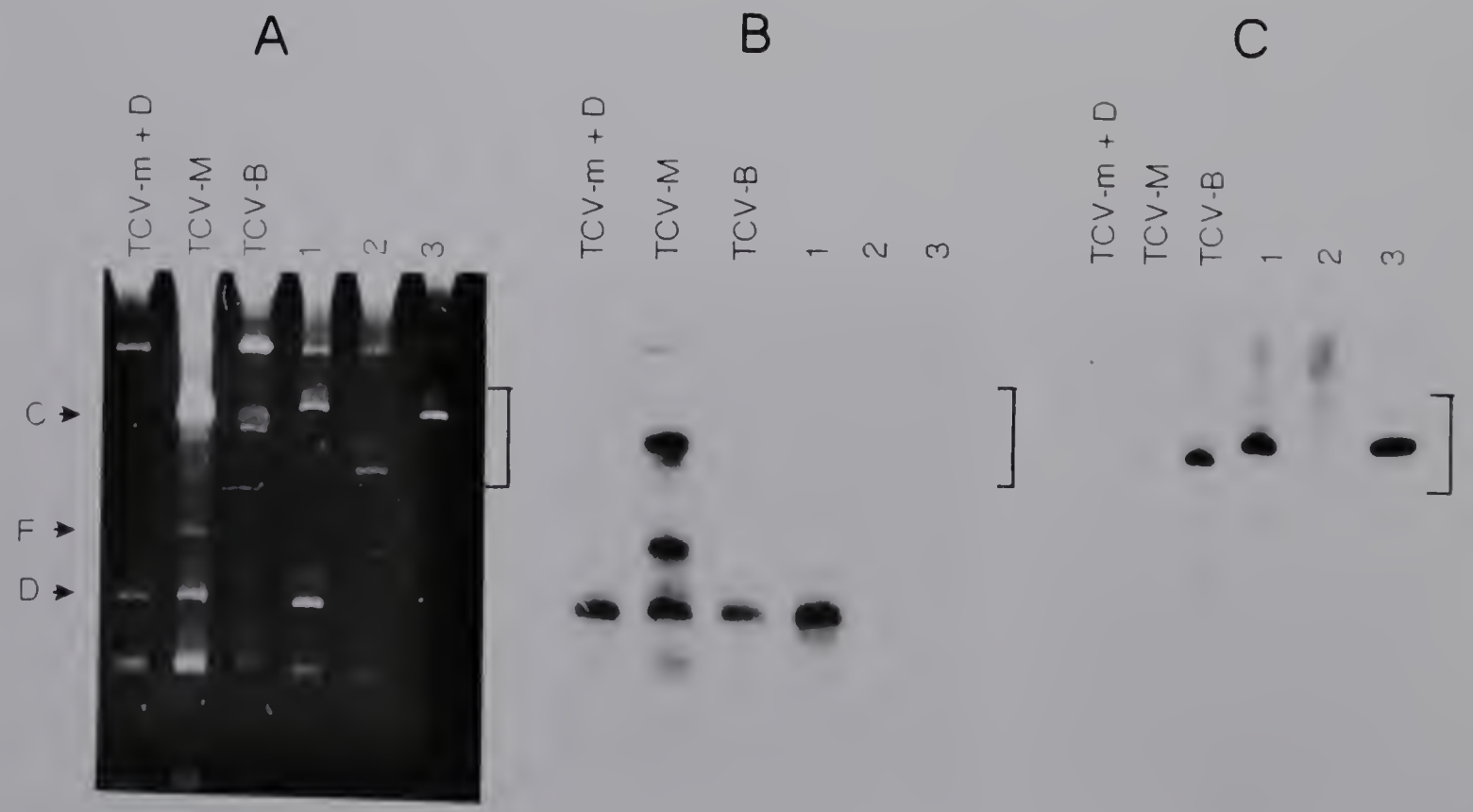
1 2 3 4

presence of DI-RNA G was associated with greater stunting and leaf crinkling. The symptoms attributed to the accumulation of DI-RNA G resembled those previously reported for sat-RNA C which accumulates in TCV-M infected plants (Chapter II, this thesis). These experiments confirmed that DI RNA G was capable of intensifying symptom severity in the absence of sat-RNA C.

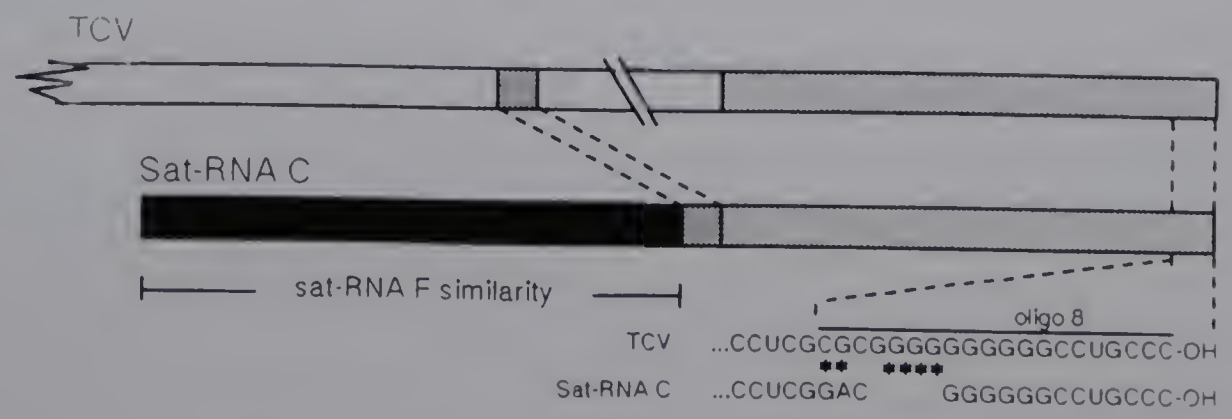
De Novo Generation of a DI RNA and Its Correlation with a Severe Symptom Expression

The TCV-B isolate has been maintained for many years and through many passages in turnip in the greenhouse. The association of low molecular species with the isolate has been long recognized and was assumed to be the sat-RNA C described previously (Altenbach and Howell, 1981; Simon and Howell, 1986). The demonstration that the species associated with this isolate was a DI RNA raised the speculation that its origin directly from the helper virus could be demonstrated. To evaluate this possibility, cabbage and mustard plants inoculated with virions derived from the infection with TCV full-length cDNA clone pTCV-P1 (TCV-B-P1) were evaluated for the presence of low molecular weight RNA species. Approximately 10% of inoculated cabbage and mustard plants accumulated new RNAs of approximately 300-390 nucleotides in size. Three representatives of which are shown in Fig. 4.6 A. Most of these new RNAs resembled DI-RNA G by hybridizing strongly to the TCV-specific probe

Fig. 4.6 Hybridization blot analysis of new RNAs accumulating in plants infected with virus derived from *in vitro* synthesized TCV (TCV-B-P1). Total RNA was extracted from plants inoculated two weeks previously with buffer (mock), TCV-m + D, TCV-M, TCV-B or TCV-B-P1 and subjected to electrophoresis on 5% polyacrylamide/50% urea gels. (**A**) Ethidium bromide stained gel. (**B** and **C**) RNA was transferred to Nytran membrane and hybridized with a full-length cDNA clone of sat-RNA F labeled with α-^{32}P-cATP (B) or a γ-^{32}P-ATP end-labeled oligonucleotide (oligo 8) complementary to the 3′ terminal 20 nucleotides of TCV (C). TCV genomic RNA does not transfer efficiently under the conditions and cannot be detected after oligo 8 hybridization. TCV-B-P1 were virions isolated from turnip inoculated with *in vitro* synthesized transcripts of genomic TCV (TCV-B isolate). Square bracket indicates RNA species of approximately 300-390 nucleotides including sat-RNA C in lane TCV-M and DI-RNA G in lane TCV-B. Sequence relationship within the 3′ termini of TCV and full length sat-RNA C is shown below the blots. Asterisks (*) denote nucleotide differences between TCV genomic RNA from isolate TCV-B and sat-RNA C.



Probe: Sat-RNA F Oligo 8



(oligo 8) and by not hybridizing to the sat-RNA-specific probe (sat-RNA F cDNA) (Fig. 4.6 B, C). To maintain a RNA stock for further cloning, and at the same time, to monitor symptoms caused by these new generated RNAs, RNA samples from three plants that had been infected previously with virus derived from cloned transcripts, TCV-B-P1, containing RNA species of approximately 380, 300 and 370 nucleotides ('First Passage') were used to infect mustard cv. Tendergreen or mustard cv. Savanna, cabbage cv. Pak Choi and turnip cv. Just Right. Virions derived from infection of pTCV-P1 transcripts were also used to infect these plants for symptom comparison. A summary of accumulation of RNA species and symptom production in these plants are listed in Table 4.1. Surprisingly, many plants inoculated with RNA containing 380, 300 and 370 nucleotide RNA species accumulated new RNAs with different sizes ('Second Passage', Table 4.1, upper part). Again, one out of 8 plants inoculated with the virions of cloned TCV-B-P1 generated a new RNA molecule (Table 4.1, lower part). The RNA species from "*" plant in Table 4.1 was cloned, and the sequence of three cDNAs indicated that the new RNA (DI1) was a co-linear deletion mutant of genomic TCV, containing both the 5' and 3' ends of TCV as well as one interior segment (Fig. 4.7). Unlike DI-RNA G, DI1 began with the 5' terminal nucleotide of TCV. The sequence of DI1 RNA was more similar to genomic sequence than DI RNA G, which has been maintained with the virus after prolonged passage,

Table 4.1 Symptoms and accumulation of DI RNA-like molecules in some cruciferous plants in the 2nd passage of infection with virus derived from pTCV-P1 transcripts

Initail Inoculum	1st Passage RNA	2nd Passage Plant		RNA (base)	Symptom
TCV-B-P1	Pak Choi (380 b)	Tendergreen	I	380	+
			II	380 + 350	+
		Pak Choi	I	380 + 350	+
			II	380*	+
		Savanna	I	380	+
			II	380 + 350	+
		Just Right	I	380	+
	Pak Choi (300 b)	Tendergreen	I	360	+
			II	350	+
		Pak Choi	I	350	+
			II	380 + 360	+
		Savanna	I	360	+
			II	360	+
		Just Right	I	360	+
			II	No band	-
	Savanna (370 b)	Tendergreen	I	350	+
			II	360	+
		Pak Choi	I	380	+
			II	380	+
		Savanna	I	No band	-
			II	No band	-
		Just	I	No band	-
			II	No band	-

Inoculum (Control)	Plant		RNA (base)	Symptom
TCV-B-P1	Tendergreen	I	No band	-
		II	360	+
	Pak Choi	I	No band	-
		II	No band	-
	Savanna	I	No band	-
		II	No band	-
	Just Right	I	No band	-
		II	No band	-

* The RNA from which DI-1 was cloned.
+/- With/without symptom intensification comparing with plants infected with TCV-B-P1 that did not accumulated any DI-like molecules

Fig. 4.7 Nucleotide sequence of DI1. DI1 was isolated from turnip previously inoculated with virus derived from pTCV-P1 (TCV-B-P1). Total RNA was isolated from these plants, hybridized to oligo 8 (see Fig. 4.2), and cDNA was generated by extension of the oligonucleotide with reverse transcriptase as described in Materials and Methods. Nucleotides following the brackets are the 5′ end points of the cDNA. The sequence 5′ of the brackets was determined using primer extended cDNA as described for DI-RNA G. "." indicates a missing nucleotide. Only nucleotides which differing from DI1-1 are shown. Numbering of TCV genomic RNA is in italics.

```
          1
TCV
DI1-1     NGTAATCTGCAAATCCCTGGCACCCGCCTAAAATTGCCCTCAT...CAAC    47
DI1-2                              [  G
DI1-3                         [                      CAT

TCV                                    T        T     C  C
DI1-1     CTTCTCTCTATTCACGATGCCTCTTCCACACACACCCAACAAAGTGCTCG    97
DI1-2
DI1-3

                                                 135/3707
TCV                                                  |
DI1-1     CAGTGGGACTCCTAGGAGCCAGGTACTACCCTGAGGTTCCAGGAGTGAAG   147
DI1-2
DI1-3

TCV                                    A                    .A
DI1-1     GTAGCAGAAAGGGGACAAGGCGTGAGAATGGTCACAACTGAGGAGCATGC   197
DI1-2
DI1-3                                                       .A
                                                            .A

                                              3797/3898
TCV           GGG   A T      AC                  .
DI1-1     CAAATTATAATTGGCAAGCTATCAGAATTTAGGTACCAAAAACGGTGGCA   247
DI1-2
DI1-3                                          .

TCV
DI1-1     GCACTGTCTAGCTGCGGGCATTAGACTGGAAAAC.TAGTGCTCTTTGGGT   296
DI1-2
DI1-3      .              .     .          A GT XT

TCV                    G
DI1-1     AACCACTAAAATCCCAAAAGGGTGGGCTGTGGTGACCTTCCGAACTAAAA   346
DI1-2
DI1-3                  G

                                          4051
TCV                                        |
DI1-1     GATAGCCTCCCTCCTCGCGCGGGGGGGGGGCCTGCCC    383
DI1-2
DI1-3
```

lending support to the *de novo* origin of DI1. Also, by carefully monitoring symptoms on each of the plant, a perfect correlation of symptom expression and generation of new RNAs was found. All the plants that did not generate any detectable DI-like RNAs showed milder symptoms than those that did accumulate DI-like RNA including the plant ("*" in Table 4.1) from which DI-1 was actually cloned. Plants representing different symptoms are shown in Fig 4.8. Therefore, I conclude that TCV *de novo* generated DI RNA also intensifies TCV symptoms.

Discussion

Thus far, TCV is unique among plant viruses in its association with three different types of small RNAs: 1) satellite RNAs (sat-RNA D, sat-RNA F) which share no sequence homology with the helper virus (Francki, 1985); 2) chimeric molecules composed of satellite sequences at the 5′ end and virus related sequence at the 3′ end (sat-RNA C and sat-RNA CX); 3) DI RNAs, composed entirely or nearly entirely of virus segments (DI-RNA G, DI1). A schematic representation of the relationship between TCV, its DI RNAs, and sat-RNA C is presented in Fig. 4.9. Although there is no single consensus sequence at the various junction points, both DI1 and sat-RNA C contain the same 3′

Fig. 4.8 Symptoms caused by DI RNA-like molecules on mustard cv. Tendergreen plants. Plants were photographed 2 to 3 weeks after inoculated with buffer (M), virus derived from pTCV-P1 transcripts (TCV-b), or the first passage RNA from TCV-P1 infection (DI-1 or DI-2). (A) Mustard cv. Tendergreen "DI-1" plant was the "Tendergreen I" plant in Table 4.1 (2nd passage) infected with RNA (1st passage) harboring a 380 b molecule. (B) Mustard cv. Tendergreen plant was the "Tendergreen II" plant in Table 4.1 (2nd passage) infected with RNA (1st passage) harboring a 300 b molecule.

A

M

TCV-b

DI-1

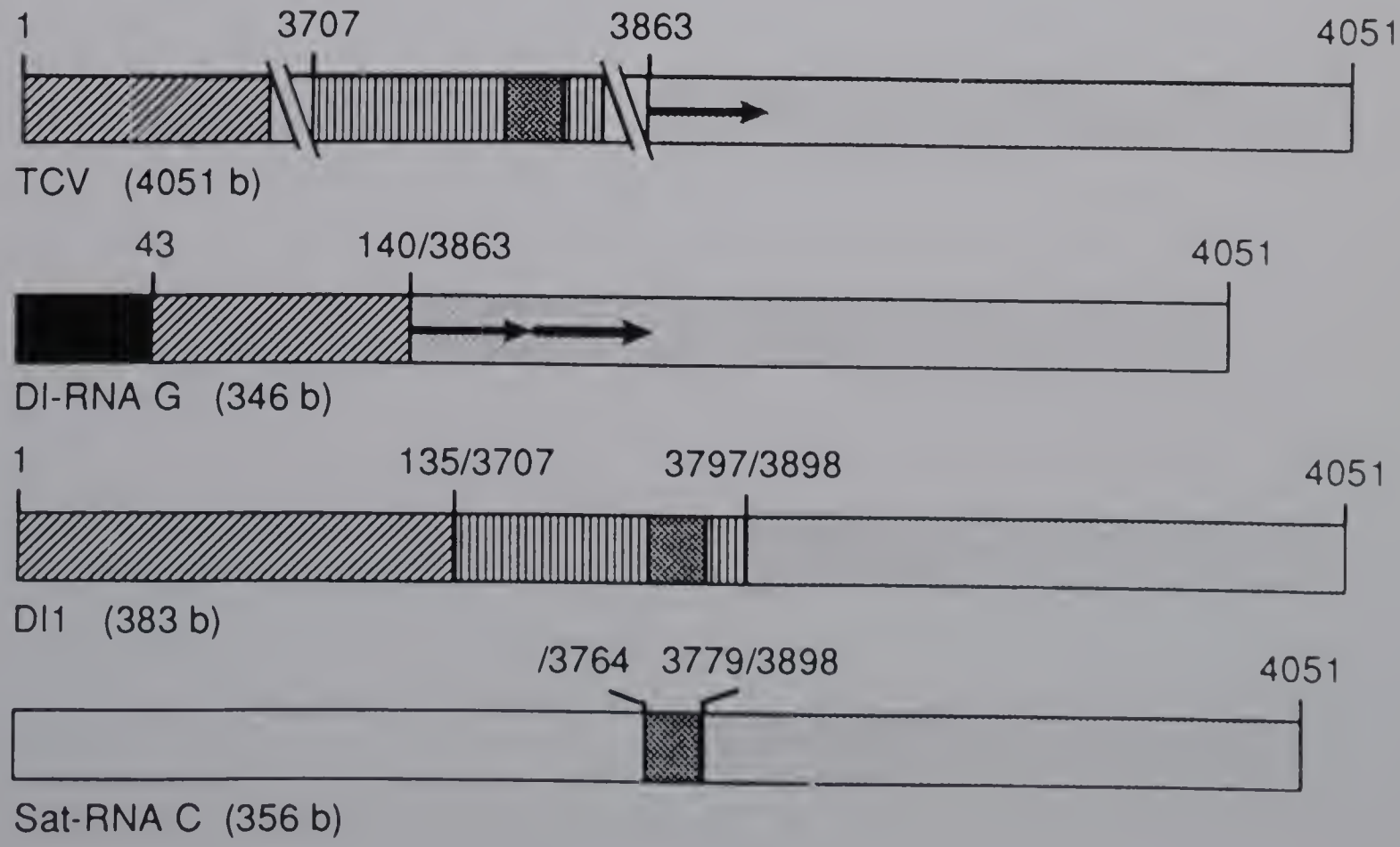
B

M

TCV-b

DI-2

Fig. 4.9 Schematic representation of the sequence similarity between TCV and DI-RNA G, DI1, and sat-RNA C. The arrow represents a 36 b repeated sequence in DI-RNA G corresponding to nucleotides 3863 to 3898 of TCV. Numbering in the DIs and sat-RNA C refer to TCV nucleotides involved in junction sequences.


1
3707
3863
4051
TCV (4051 b)
43
140/3863
4051
DI-RNA G (346 b)
1
135/3707
3797/3898
4051
DI1 (383 b)
/3764
3779/3898
4051
Sat-RNA C (356 b)

end segment of TCV, from 3897 to 4051. The end of the repeated sequence in DI-RNA G is also near the same junction sequence (3898).

The origin of the terminal 5′ 21 nucleotides of DI-RNA G is obscure. A search of the EMBL data bank did not reveal any sequences that closely match the 21 nucleotides. This sequence differs from the corresponding sequence of TCV by at least 11 bases. However, the 5′ 10 nucleotides of DI RNA G are homologous to the 5′ 10 nucleotides of sat-RNA F. The origin of these 21 nucleotides may be due to: 1) divergence from TCV as a result of prolonged passage in association with the helper virus; 2) the result of a recombination event between sat-RNA D and TCV which might have occurred during the original formation of DI RNA G; 3) the presence of a non-viral segment. Several alphavirus DIs also have 5′ sequences which have diverged from original viral sequences. DIs derived from Sindbis virus have been found with 5′ end sequences nearly identical to rat $tRNA^{asp}$ (Monroe and Schlesinger, 1983), and Semliki Forest virus DIs also contain a heterogeneous 5′ end (Petterson, 1981).

DI RNAs, although commonly associated with animal viruses, have only recently been identified in plant virus infections (Anzola *et al.*, 1987; Hillman *et al.*, 1987; Morris and Hillman, 1989; Ismail and Milner, 1988; Burgyan *et al.*, 1989). The best characterized plant virus DI is the symptom modulating DI associated with the tombusvirus

(TBSV, Hillman *et al.*, 1987). This 0.4 kb DI RNA is a mosaic composed of the 5′ non-coding region, an internal segment from the RNA replicase gene and a 3′ proximal domain composed of the last 130 bases of the extreme 3′ non-coding sequence. The features of the TBSV DIs are similar to those of DI RNA G and DI1 of TCV in that they have retained similar lengths of sequence from both ends of the helper genome.

The discovery of DI1 in a TCV isolate derived from cloned inoculum is the first definitive demonstration of *de novo* generation of a viral DI RNA from a plant RNA viral genome. The demonstrated origin of other animal and plant viral DI RNAs is not as rigorous. The identification of distinct size classes of DI RNAs upon high multiplicity passage of DI-free isolates of animal RNA viruses is generally interpreted as strong evidence for *de novo* generation. Such evidence has been presented in support of *de novo* generation of DI RNAs for plant tombusviruses as well (Morris and Hillman, 1989; Morris, unpublished). The tombusviruses are thought to be closely related to the carmoviruses (Morris and Carrington, 1988), the group to which TCV belongs. It may be that the ability to generate DIs is a common feature of their member virus replicases.

DI-like molecules associated with TCV-B seemed to evolve rapidly during passages. Evolution of naturally occurring DI RNAs from small to large has been observed in Sindbis virus DIs and was thought to be due to an increase

in the number of repeats in the molecules (Levis *et al.*, 1986). This might be also an explanation for rapid evolution of DI-like molecules in TCV-B. The discovery of a direct repeat sequence in DI RNA G molecule supports this speculation. On another hand, recombination might take place frequently among these small RNAs as has been found between sat-RNA C and sat-RNA D (Cascone *et al.*, 1990).

Oligo 8, which is complementary to the last 20 nucleotides of the 3′ end of TCV genomic RNA, hybridized to most, but not all, DI RNA-like molecules accumulating *de novo* (Fig. 4-6 C). This leads to the speculation that some DI RNAs might have diverged sequence in the 3′ portion. Alternatively, sat-RNAs might be associated with TCV-B and generated *de novo* as well.

DI-RNA G is responsible for intensified symptoms when inoculated together with TCV on mustard and other cruciferous hosts. Plants in which the DIs are replicating exhibit increased stunting and leaf crinkling. The TCV-similar domain of sat-RNA C was previously found to be at least partially responsible for the dark green, severely stunted and crinkled leaves associated with the satellite (Simon *et al.*, 1988). DI RNA G also contains this 3′ end domain which further supports the evidence that this region is involved in symptom production. In addition, DI1, as well as other uncharacterized DI RNA-like molecules, also intensify TCV symptoms. Although DI RNA G and DI1 RNA are the first plant virus DIs observed which intensify viral

symptoms, defective forms of animal retroviruses are also thought to have a role in pathogenesis (Overbaugh *et al.*, 1988; Aziz *et al.*, 1989). However, defective retroviruses are not simply deleted versions of their helper virus; many have acquired new cellular genes which are implicated in disease production.

CHAPTER V

TURNIP CRINKLE VIRUS DEFECTIVE INTERFERING RNA INFECTIVITY AND GENERATION STUDIES

Introduction

In general, there are three classes of DI RNAs (Schlesinger, 1988) based on alignment of genomic and DI RNA sequences: (1) Internal deletions; (2) 5′ conserved molecules; and (3) 3′ conserved molecules. In the internal deletion class, both the 5′ and the 3′ terminal sequences are retained. This type of DI RNA has been found associated with both positive and negative strand animal viruses. Some additional modifications such as repeats, rearrangement, or nucleotide changes can also be found in these DIs. Plant DI RNAs associated with tomato bushy stunt virus (TBSV) (Hillman *et al.*, 1987) and TCV (Chapter IV, this thesis) also belong to this group. DIs classified as 5′ conserved molecules retain the 5′ but not the 3′ terminus of the viral genome. The 3′ sequences are the inverted complement of the 5′ terminus. This type of DI RNA has only been found associated with a few negative strand RNA animal viruses (Perrault, 1981). DIs which are 3′ conserved molecules retain the 3′, but not the 5′ terminus of the virus genome.

This type of DI RNA has only been found in the Sindbis virus group of positive strand RNA animal viruses (Schlesinger and Weiss, 1986).

Early studies on animal DI RNA generation (for a review, see Schlesinger, 1988) have suggested that the formation of detectable DI RNAs in a virus population involves two major events. The first is the generation of defective molecules from a viral genome by aberrant replicative events. The second involves an amplification of the aberrant molecules, which requires that the molecules be recognized by the viral replicase and other proteins involved in the encapsidation process.

Studies on Sindbis virus DI RNAs have suggested that only 162 nucleotides at the 5′ terminus and 19 nucleotides at the 3′ terminus are specifically required for replication and packaging of the DI molecule (Levis *et al.*, 1986). The 19 nucleotides at the 3′ terminus are highly conserved among other viruses in the same group (Ou *et al.*, 1981). Thus, these nucleotides are thought to be necessary and sufficient to define the recognition site for initiation of negative strand synthesis. Studies of animal DI RNAs have also suggested that although many nucleotides in a DI molecule are not specifically involved in DI replication, they may be important in keeping DIs a certain size to meet the packaging constraints of the icosahedral structure of the viral nucleocapsid (Re and Kingsbury, 1988).

At present, knowledge of DI RNA generation and replication has been obtained almost exclusively from studies in animal virus DI RNA systems. Since DI RNAs have only recently been found associated with plant viruses, studies of plant virus DI RNA replication and infection processes are just now beginning. In this chapter, I will report my research on plant DI RNA generation and infection studies using TCV and its DI RNAs. These studies include making artificial DI RNA molecules from a full length cDNA clone of the TCV genome and mutagenesis of a full length cDNA copy of DI RNA G from which infectious RNA transcripts can be made *in vitro*. I have also localized important domains that are necessary for DI RNA G infection and I have identified sequences that are not required for DI RNA G infection.

Materials and Methods

Virus Isolates and Plant Inoculations

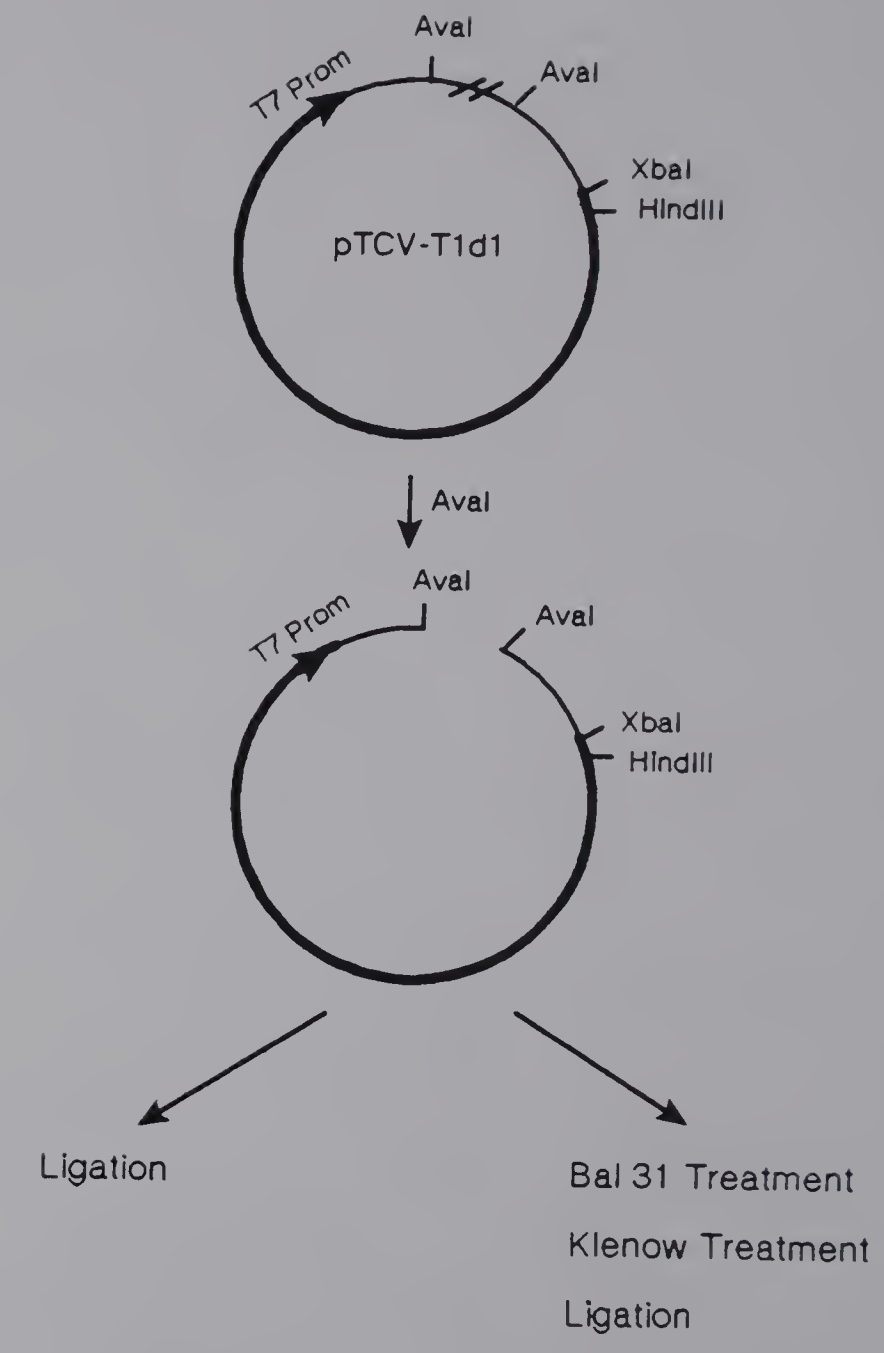
Methods for plant inoculations, plant growth conditions and TCV strains, TCV-M and TCV-B, have been described in previous chapters in this thesis. A full length infectious cDNA clone (pTCV-T1d1, Heaton *et al.*, 1989) of the Berkeley strain (TCV-B) used for making synthetic DI RNAs was described in detail in Chapter IV. The helper virus used for infectivity assay was a Massachusetts TCV strain (TCV-M) that had been previously

freed of sat-RNAs C and F (TCV-m + D) as described in Chapter II. Turnip cv. Just Right was used for infectivity tests of synthetic DIs or DI RNA G mutants.

Artificially Synthesizing DI RNAs from a Full Length cDNA Clone, pTCV-T1d1, of the Berkeley TCV Strain, TCV-B

Plasmid pTCV-T1d1 containing a full length cDNA copy (4051 b) of the Berkeley TCV strain (TCV-B) positioned downstream of a T7 promoter (Heaton *et al.*, 1989) was used to construct synthetic DIs (Fig. 5.1). Ten *u*g of pTCV-T1d1 was completely digested with 25 units of *Ava* I (BRL) at 37°C overnight. The largest fragment (about 3 kb) was electroeluted onto a piece of DE-81 paper from a 2% agarose gel. The DE-81 paper was washed twice with 100 *u*l each of low salt buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA (pH 8.0) and 100 mM LiCl. To collect DNA, the DE-81 paper was incubated with 100 *u*l of high salt buffer containing 20% ethanol, 1 M LiCl, 10 mM Tris (pH 7.5) and 1 mM EDTA (pH 8.0) at 65-80°C for at least 10 min. Following a quick spin, supernatant was collected and pooled with subsequent 100 *u*l of high salt wash supernatant. DNA was then precipitated with 400 *u*l of 95% ethanol and resuspended in 10 *u*l of ddH_2O. To generate clones with a 700-base insert, 1 *u*l of above DNA was treated with 0.5 unit of T4 DNA ligase (Bethesda Research Laboratories, BRL) in 10 *u*l of reaction mixture overnight at room temperature and 3 *u*l of the ligation mixture was used to transform bacterial strain

Fig. 5.1 Schematic used to construct artificial DIs from a full length cDNA copy of TCV-B genomic RNA, pTCV-T1d1. Thick lines represent the vector sequence. Thin lines represent cDNA insert sequence. The large portion of internal TCV cDNA sequence is omitted.


AvaI
AvaI
T7 Prom
XbaI
HindIII
pTCV-T1d1
AvaI
AvaI
T7 Prom
AvaI
XbaI
HindIII
Ligation
Bal 31 Treatment
Klenow Treatment
Ligation

DH5a. To generate clones with smaller inserts, 5 *u*l of above *Ava* I fragments was treated with 0.75 units of fast-acting form of Bal 31 (IBI) in 20 *u*l of 1X Bal 31 buffer supplied by IBI at 37°C for 5 min, 8 min, 11 min and 14 min. The reactions were terminated by removing 4 *u*l of reaction solution at each time point into 0.5 *u*l of 0.2 M EGTA in an eppendorf tube that had been pre-cooled on ice, followed by heating at 70°C for 15 min and ethanol precipitation in the presence of 5 *u*g of yeast tRNA. Deletions were confirmed by visualization of band smears around 460 bases on a 2% agarose gel after digestion of half the amount of DNA from each time point with *Hind* III. The rest of the DNA from the various time points were mixed, treated with 6 units of *E. coli* DNA polymerase large fragment (Klenow, BRL) and 0.2 mM of each of four dXTPs in 20 *u*l Klenow buffer supplied by the manufacturer for 15 min at room temperature. Following phenol/chloroform extraction in the presence of 1 *u*l of 10% SDS in a total valume of 200 *u*l of volume and ethanol precipitation, the DNA was resuspended in 10 *u*l of ddH_2O and 2 *u*l of an aliquot was self-ligated using 0.5 units of T4 DNA ligase in 10 *u*l of final reaction mixture. After incubation overnight at room temperature, 3 *u*l of the ligation mixture was used to transform bacterial strain DH5a. The precise junctions of the inserts were identified by dideoxynucleotide chain-termination (T7 DNA polymerase, BRL) sequencing using

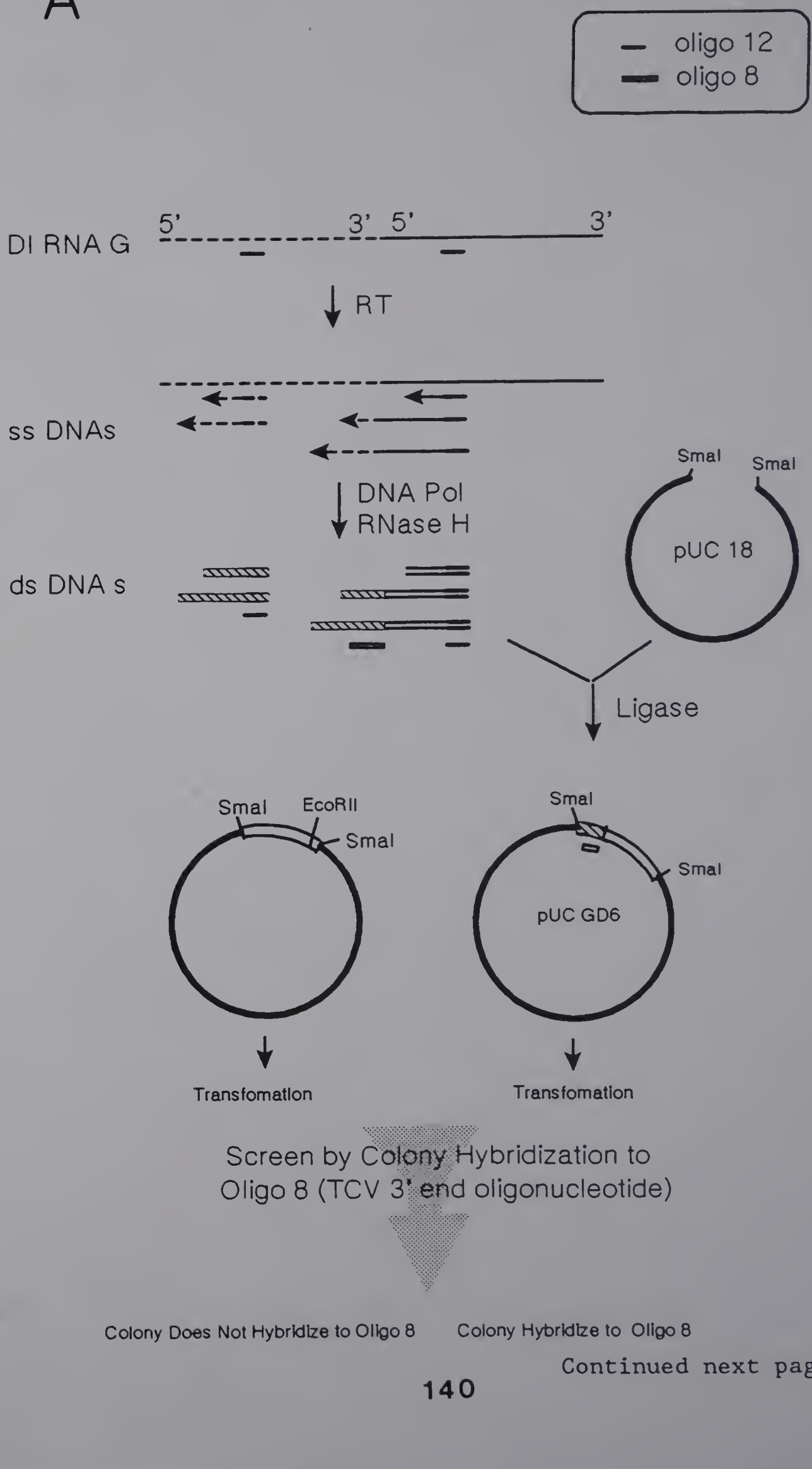
standard sequencing primers (Synthetic Genetics) as well as a 17-base synthetic oligonucleotide complementary to nucleotides 3921 to 3937 of TCV genomic RNA (oligo 15).

Cloning of a Full Length Infectious DI RNA G Molecule

In Chapter IV, I described the cloning of monomeric DI RNA G. All the clones (Fig. 4.3 in Chapter IV) obtained were missing bases at the 5′ end. In order to obtain a full length cDNA copy of DI RNA G, the junction region of DI RNA G dimers was cloned (Fig. 5.2 A). Three *u*g of oligo 12, a 11-base oligonucleotide complementary to bases 90 to 100 of DI RNA G, was hybridized to approximately 5 *u*g of gel-purified DI RNA G dimer in 20 *u*l of hybridization buffer (0.4 M NaCl/10mM Pipes, pH 6.4) at 68°C for 10 min followed by slow cooling to 34°C. The synthesis of first and second strand cDNAs was performed as previously described (Chapter IV, this thesis; Simon and Howell, 1986). Following treatment with Klenow, the double stranded cDNA was ligated into *Sma* I-digested, calf-alkaline-phosphatase-treated pUC18, followed by transformation into a dcm^- strain of *E. coli*. The cDNA clones extending through the 3′ and 5′ terminal junction of DI RNA G were selected by colony hybridization (Grunstein and Hogness, 1975) with a γ-^{32}P-ATP end-labeled oligonucleotide (oligo 8) which is complementary to 20 nucleotides at the 3′ termini of DI RNA G. cDNA clones corresponding to partial DI RNA G dimers were sequenced by

Fig. 5.2 Cloning of full length DI RNA G. **(A)** Diagram of the cloning of a DI RNA G partial dimer. Small open bars and small solid bars represent oligo 8 and oligo 12, respectively. RNA and first strand cDNA of 2 monomeric units are differentiated by dashed and solid lines. Double strand cDNA of 2 monomeric units are differentiated by hatched or open bars. Thick lines represent vector sequences. **(B)** Flow chart of procedure used to generate full length cDNA clones of DI RNA G (pPM2-G). Single hatched and open bars represent different monomeric units of a cDNA copy of partial DI RNA G dimer. Double hatched bars represent a partial cDNA copy of monomeric DI RNA G. Thick lines represent vector sequences. **(C)** Complete nucleotide sequence of the full length DI RNA G cDNA. Sequence complementary to Oligo 8, the oligo 12, and the *EcoR* II sites are indicated. **D.** Restriction map of the full length DI RNA G cDNA.

A



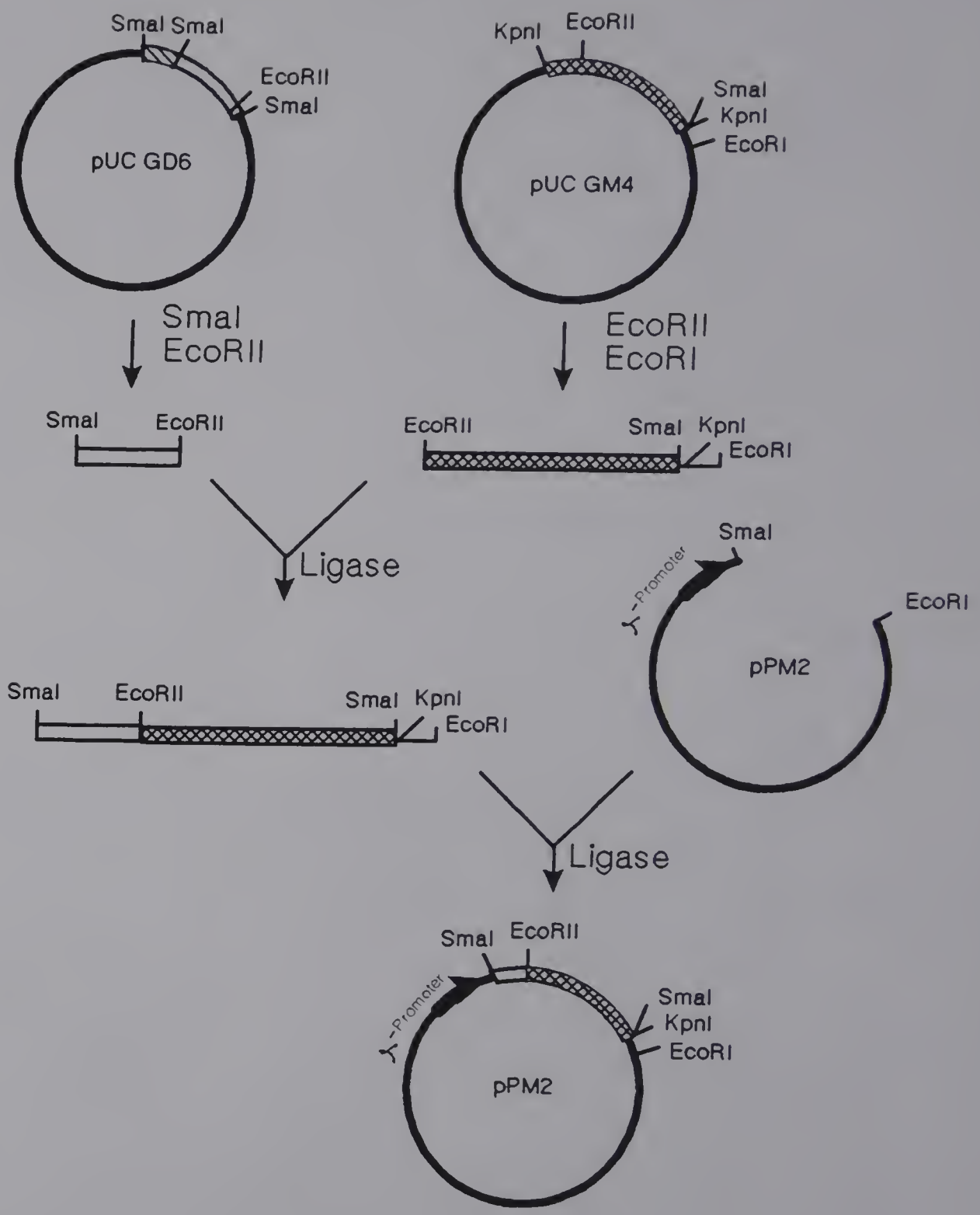
Screen by Colony Hybridization to
Oligo 8 (TCV 3' end oligonucleotide)

Colony Does Not Hybridize to Oligo 8

Colony Hybridize to Oligo 8

Continued next page

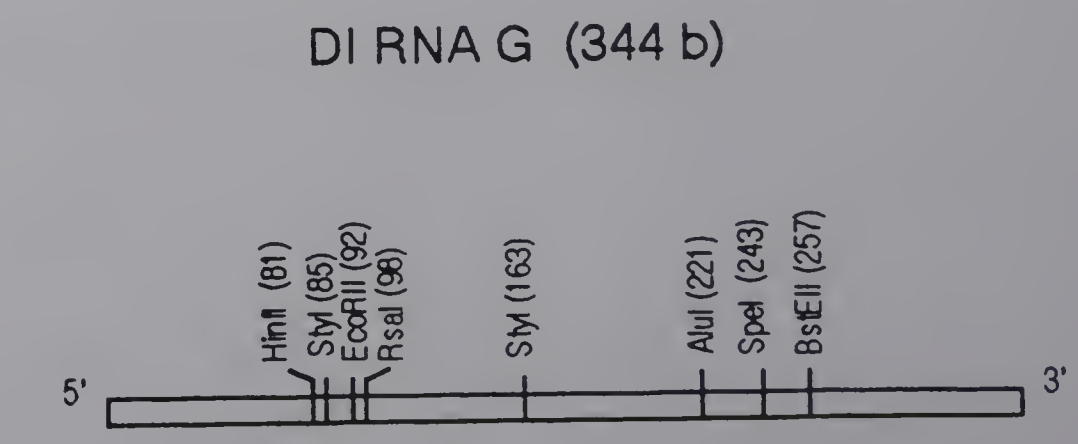
B


SmaI SmaI
EcoRII
SmaI
pUC GD6
SmaI
EcoRII
KpnI EcoRII
SmaI
KpnI
EcoRI
pUC GM4
EcoRII
EcoRI
SmaI EcoRII
EcoRII SmaI KpnI EcoRI
Ligase
SmaI
λ-Promoter
EcoRI
pPM2
SmaI EcoRII
SmaI KpnI EcoRI
Ligase
SmaI EcoRII
λ-Promoter
SmaI
KpnI
EcoRI
pPM2

C

```
                                                          5'
  1 GGGATAAAAA GGAGGCTTAC CAACCTTCTC TCTATTCACG ATGCCTCTTC
                                                  EcoRII
 51 TACACACACT CAAAACAGCG CTTGCAGTGG GACTCCTTGG AGCCAGGTAC
                                                oligo12
101 TACCCCGAAG GTTCAAAATC AAGACCCCCA TGTCGCTTTA CTTTGAATTG
151 TGTTAGAAAG CCCCAAGGTC ATTTTACTTT GACCTGTGTT AGAGACCCAA
201 AACGGTGGCA GCACTGTCTA GCTGCGGGCA TTAGACTGGA AAACTAGTGC
251 TCTCTGGGTA ACCACTAAAA TCCCGAAAGG GTGGGCTGTG GCGACCCTCC
301 GAACTAAAAG ACAGCCTCCC TCCTCGCGGG GGGGGGCCT GCCC
                          oligo 8
```

D



dideoxynucleotide chain-termination (T7 DNA polymerase, BRL) using standard sequencing primers (Synthetic Genetics).

Full length DI RNA G was obtained by linking the 3′ region of DI RNA G supplied by a partial monomer cDNA clone (DI RNA G4 cDNA, or pUC-GM4, Fig. 4.3 in Chapter IV) to the 5′ region of DI RNA G supplied by the partial dimer cDNA clone (pUC-GD6) (Fig. 5.2 B). To do this, the 92-base *Sma* I/*EcoR* II fragment corresponding to the 5′ region of DI RNA G was excised from pUC-GD6 and ligated to the 252-base *EcoR* II/*EcoR* I fragment previously isolated from pUC-GM4. The resulting 344-base *Sma* I/*EcoR* I fragment was then ligated into pPM2 (Simon *et al.*, 1988) that had been previously digested with *Sma* I and *EcoR* I. Clones containing a full length DI RNA G cDNA copy were identified by DNA sequencing using standard sequencing primers and named pPM2-G. Complete sequence of this full length DI RNA G is shown in Fig. 5.2 C and its restriction map is shown in Fig. 5.2 D.

Insertion of a Unique Restriction Site into a Full Length DI RNA G cDNA at Base 98

Twenty *u*g of pPM2-G was partially digested with 5 units of *Rsa* I (BRL) for 3 min at 37°C. After terminating the reaction at 65°C for 10 min, *Rsa* I linearized plasmid was electroeluted from a 2% agarose gel, ethanol precipitated and resuspended in 10 *u*l of ddH_2O. 0.5 *u*g of 8-bp *Apa* I linkers (GGGGCCCC, Bio Lab) were ligated to the

linear DNA using 4 units of T4 DNA ligase and conditions suggested by the manufacturer. After incubating overnight at room temperature, DNA was ethanol precipitated, then digested with 40 units of *Apa* I at 30 °C for 3 h. Following electroelution and ethanol precipitation, The plasmid was self-ligated using 3 units of T4 DNA ligase overnight at room temperature. Following transformation, clones harboring an *Apa* I site in the cDNA insert were identified by restriction mapping following *Apa* I and *EcoR* I digestion and named pPM2-GA. A diagrammatic representation of the entire procedure is diagramed in Fig. 5.3.

Gapped Duplex Mutagenesis

In order to create an unique *Nco* I site in pPM2-GA, two point mutations were made at residues 212 and 213 by substituting the DNA sequence between residues 201 and 224 (numbered according to DI RNA GA sequence) with a synthetic oligonucleotide (oligo XH-Nco, Genosys). Procedure for constructing point mutations was performed according to Morinaga *et al* (Morinaga *et al*., 1984). A brief scheme is shown in Fig. 5.4. Plasmid fragments were prepared as follows: 10 *u*g of pPM2 (backbone) and 10 *u*l of pPM2-GA (backbone + insert) were completely digested with *EcoR* I and *Sca* I, respectively. Both linear plasmids were treated with 1 unit of calf-alkaline-phosphatase (Boehringer Mannheim) in a 20 *u*l solution containing 50 mM Tris (pH 8.0) and 0.1 mM EDTA for 1 h at 50°C, followed by 45 min

Fig. 5.3 Flow chart of procedure used to insert an *Apa* I linker into the *Rsa* I site of a full length DI RNA G cDNA clone, pPM2-G. Thick lines represent vector sequence. Thin lines represent cDNA insert.

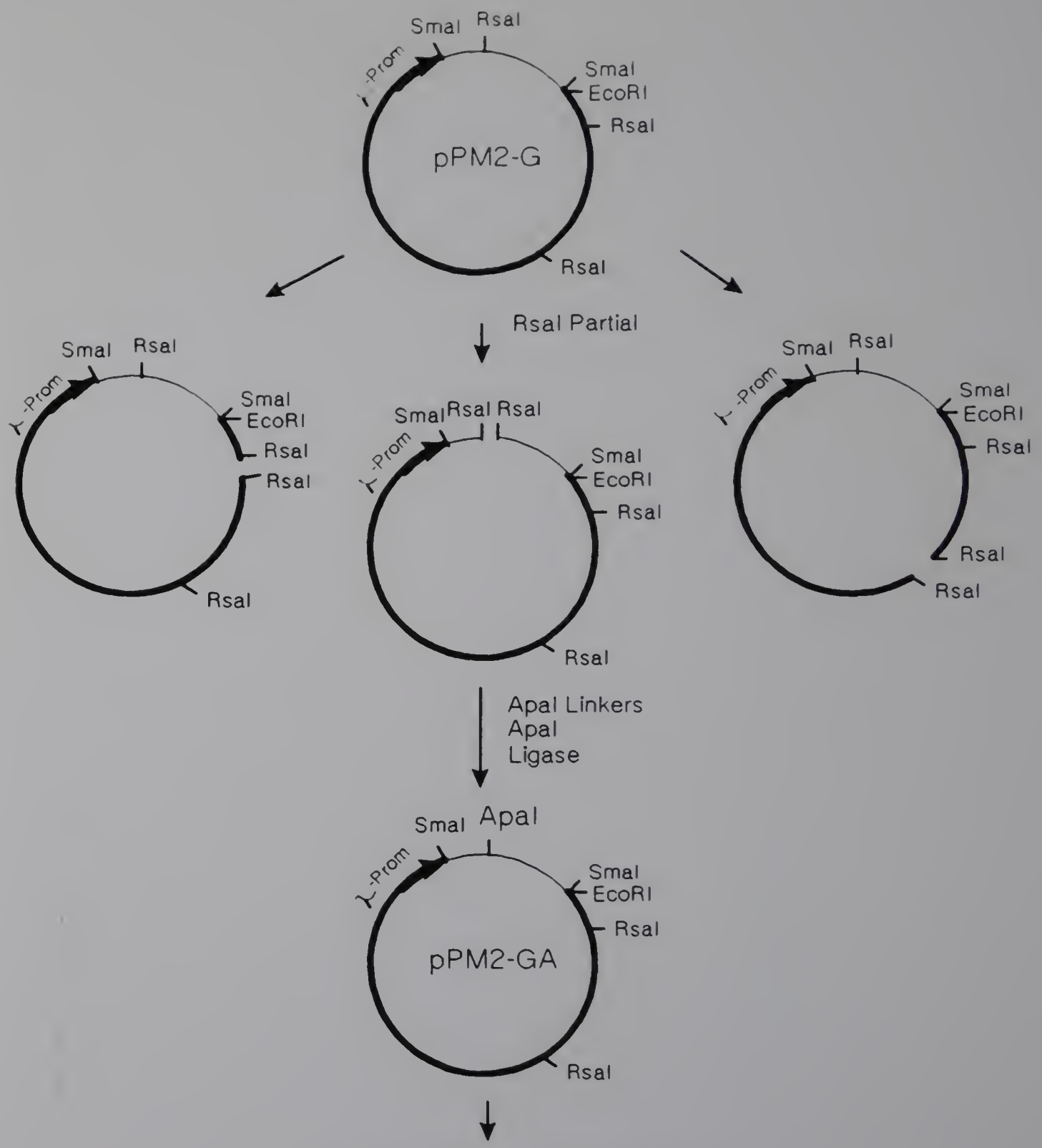
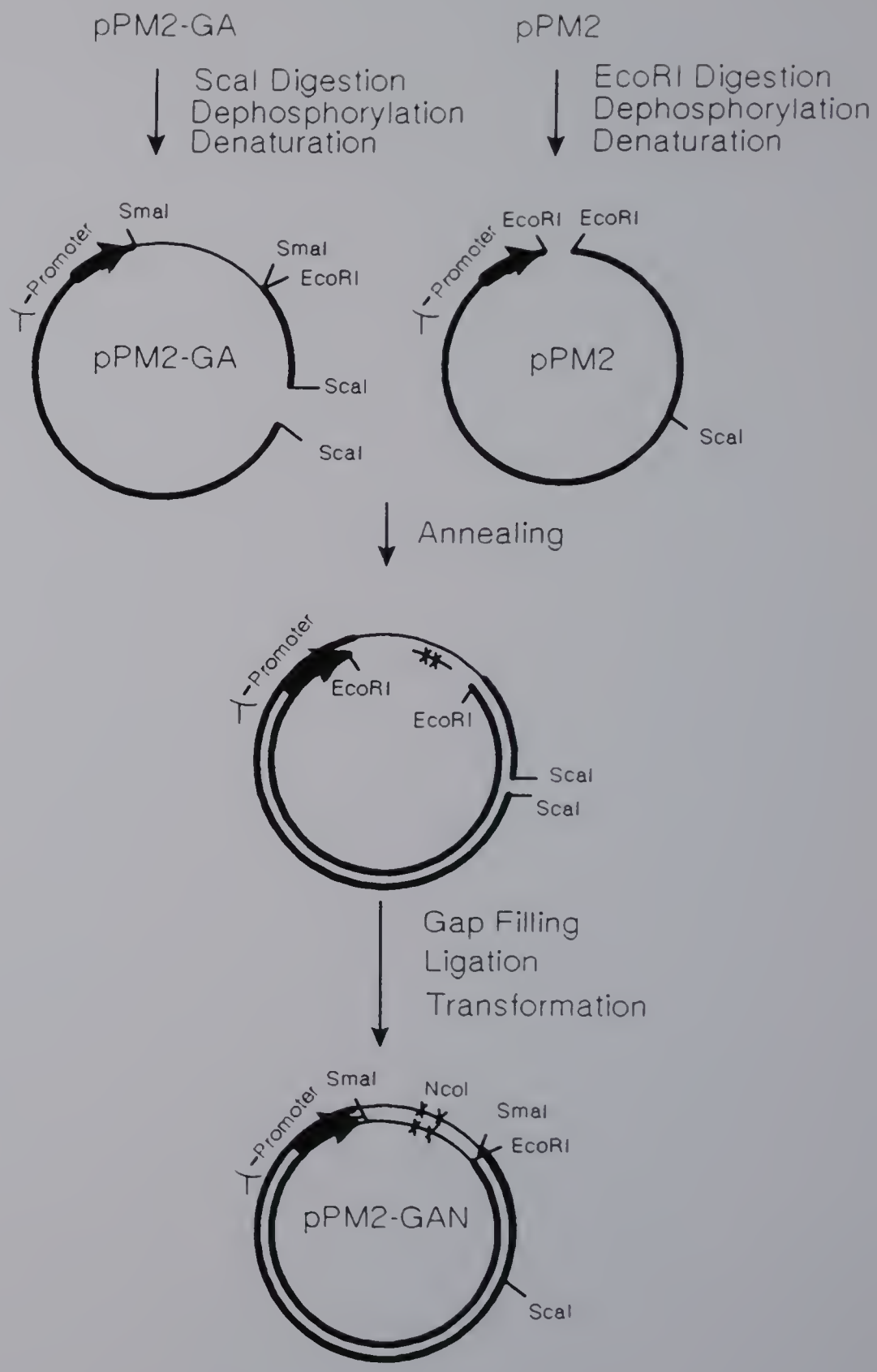

SmaI
RsaI
λ-Prom
SmaI
EcoRI
RsaI
pPM2-G
RsaI
RsaI Partial
SmaI
RsaI
λ-Prom
SmaI
EcoRI
RsaI
RsaI
RsaI
SmaI
RsaI
RsaI
λ-Prom
SmaI
EcoRI
RsaI
RsaI
SmaI
RsaI
λ-Prom
SmaI
EcoRI
RsaI
RsaI
RsaI
ApaI Linkers
ApaI
Ligase
SmaI
ApaI
λ-Prom
SmaI
EcoRI
RsaI
pPM2-GA
RsaI
Selection by ApaI/EcoRI Digestion

Fig. 5.4 Schematic of protocol used to generate two point mutations in DI RNA GA cDNA copy. Thick lines represent single strand vector sequences. Thin lines represent single strand insert cDNA to be mutated. Synthetic oligonucleotide harboring changed bases is indicated as a short line. Crosses ("x") represent changed bases.


pPM2-GA
ScaI Digestion
Dephosphorylation
Denaturation
pPM2
EcoRI Digestion
Dephosphorylation
Denaturation
Promoter
SmaI
SmaI
EcoRI
pPM2-GA
ScaI
ScaI
Promoter
EcoRI
EcoRI
pPM2
ScaI
Annealing
Promoter
EcoRI
EcoRI
ScaI
ScaI
Gap Filling
Ligation
Transformation
Promoter
SmaI
NcoI
SmaI
EcoRI
pPM2-GAN
ScaI

incubation at 65°C. DNA fragments were purified by phenol/chloroform extraction, followed by ethanol precipitation, and resuspended in 0.1X TE buffer [1 mM Tris-HCl (pH 7.5), 0.1 mM EDTA]. 0.5 *ug* (64 pmoles) of oligo XH-Nco was phosphorylated using 5 units of T4 polynucleotide kinase in 20 *ul* of 1X kinase buffer supplied by the manufacturer and 1 mM ATP. The reaction was incubated at 37°C for 10 min in an eppendorf tube. 12.5 pmoles of the phosphorylated oligo XH-Nco was mixed with 0.1 *ug* of the *Sca* I fragments of pPM2GA, 0.1 *ug* of the *EcoR* I fragments of pPM2 and 2 *ul* of 10 X annealing buffer [65 mM Tris-HCl (pH 7.5), 1 M NaCl, 80 mM $MgCl_2$ and 10 mM *B*-mercaptoethanol] in a total volume of 11.6 *ul*. The final mixture was heated in a boiling water bath for 3 min to denature the DNA, and then incubated at 30°C for 30 min. The tube was further incubated at 4°C for 30 min, and 0°C for additional 10 min. For filling in the gaps, 2 *ul* (2 U) of T4 DNA ligase, 4 *ul* of each of four dXTPs (2.5 mM each), 1 *ul* of 20 mM ATP, 0.4 *ul* (2 U) of Klenow and 1 *ul* of ddH_2O (final volume, 20 *ul*) were added to the plasmid/oligonucleotide annealing mixture and incubated overnight at 15°C. Following gap filling and transformation, clones that harbored a *Nco* I restriction site were identified by *Nco* I digestion of plasmid minipreps and further confirmed by DNA sequencing using oligo 15 as a sequencing primer. The DI RNA GA cDNA with the new *Nco*I site was named pPM2-GAN.

Sub-cloning of DI RNA G cDNA into pT7E19 Vector

In order to synthesize more abundant RNA transcripts via a stronger promoter (T7 promoter), cDNA inserts in pPM2-G, pPM2-GA and pPM2-GAN were sub-cloned into pT7E19 (Petty, 1988; Fig.5.5). pT7E19 vector used was obtained from Ian Petty (Department of Plant Pathology, University of California, Berkeley). pT7E19 was digested with *Sst* I, followed by Klenow (BRL) and calf-alkaline-phosphatase treatments. Treatment with Klenow removed 4 nucleotides of the 3′ over-hanging sequence of *Sst* I and left one "G" residue immediate downstream of the promoter. DI RNA G cDNA copies were precisely excised from pPM2-G, pPM2-GA and pPM2-GAN by complete digestion of 5 *ug* of each plasmid with 20 units of *Sma* I (BRL) for 1 h at 30°C. DNA fragments around 350 nucleotides were electroeluted from a 2% agarose gel. Approximately 1/10 of fragments recovered was ligated overnight at room temperature to 1 *ug* of *Sst* I-linearized, Klenow and calf-alkaline-phosphatease treated pT7E19 using 1 unit of T4 DNA ligase. After transformation, insertion and correct orientation of the cDNAs were confirmed by *BstE* II and *Hind* III restriction mapping. Resulting positive clones were named pT7-G, pT7-GA and pT7-GAN.

Deletion Mutations in the 5′ Region of DI RNA G

Stepwise deletions at *the Apa* I site towards either the 5′ end of DI RNA G, towards the 3′ end of DI RNA G, or spanning the *Apa* I site were generated in pT7GA (Fig. 5.6).

Fig. 5.5 Schematic drawing of the procedure used to sub-clone DI RNA G (or DI RNA GA/DI RNA GAN) cDNA into vector pT7E19. Open bars represent cDNA inserts. Thick lines represent vector sequence. Location of *Sst* I site in the vector plasmid is indicated.

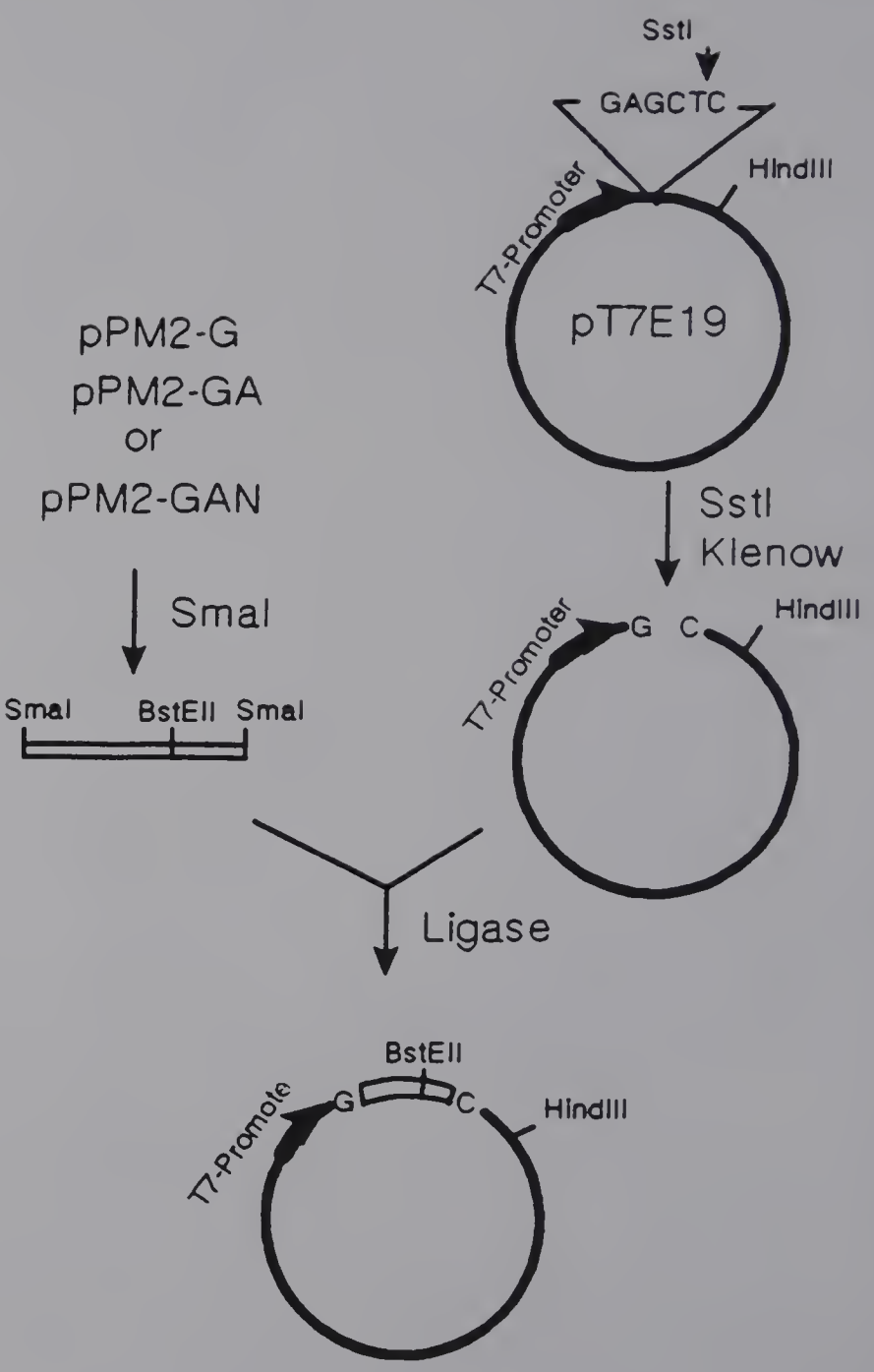
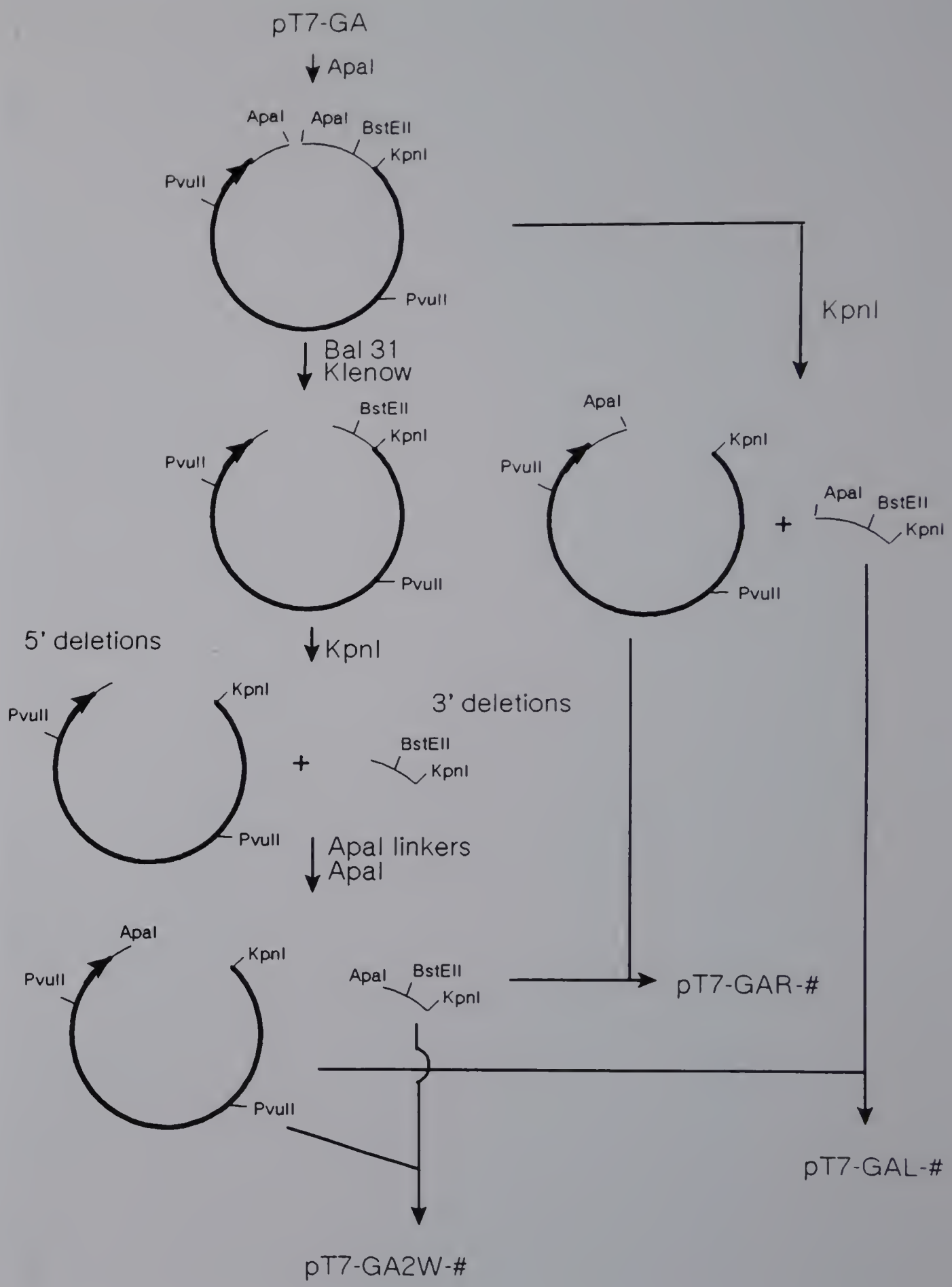

SstI
GAGCTC
HindIII
T7-Promoter
pT7E19
pPM2-G
pPM2-GA
or
pPM2-GAN
SmaI
SstI
Klenow
SmaI
BstEII
SmaI
T7-Promoter
G
C
HindIII
Ligase
BstEII
T7-Promote
G
C
HindIII

Fig. 5.6 Flow chart of the procedure used to generate step-wise deletions at the *Apa* I site. After Bal 31 treatment, *Apa* I linkers were re-ligated such that this unique site was retained in all deletion mutants. Thick lines represent vector sequence and thin lines represent insert sequence.


pT7-GA
ApaI
ApaI
ApaI
BstEII
KpnI
PvuII
PvuII
Bal 31
Klenow
BstEII
KpnI
PvuII
PvuII
KpnI
5' deletions
KpnI
PvuII
PvuII
+
BstEII
KpnI
ApaI linkers
ApaI
ApaI
KpnI
PvuII
PvuII
ApaI
BstEII
KpnI
pT7-GA2W-#
KpnI
ApaI
KpnI
PvuII
PvuII
+
ApaI
BstEII
KpnI
3' deletions
pT7-GAR-#
pT7-GAL-#

Nine *ug* of *Apa* I-digested pT7-GA was treated with 1 unit of the slow-acting form of Bal 31 in 160 *ul* of Bal 31 buffer supplied by IBI for 10 min, 20 min, 40 min and 60 min at 37°C. Reactions were terminated by removing 40 *ul* of the reaction mixture at each time point and adding 2 *ul* of 0.2 M EGTA (pH 8.0) in an eppendorf tube that had been prechilled, followed by heating at 70°C for 15 min. Size of the deletions was checked by visualizing the small smear fragments in 6% polyacrylamide gels after digesting half of the DNA from each of the time point with *BstE* II which cleaves pT7-GA 161 bases downstream of the *Apa* I site. The remaining plasmid from each time point was treated with 2.5 units of Klenow (BRL) and 0.5 mM dXTPs in 20 *ul* of 1X Klenow buffer supplied by the manufacturer, followed by phenol/chloroform extraction and ethanol precipitation. To re-insert the unique *Apa* I site, 0.5 *ul* of *Apa* I linkers was ligated to each of the Bal31/Klenow treated plasmids from the various time points using 1 unit of T4 DNA ligase. DNA was purified by phenol/chloroform extraction, followed by ethanol precipitation, and then completely digested with 15 units of *Apa* I for 1.5 h at 30°C. DNA was further digested with *20 units of Kpn* I for 1 h at 37°C. To construct deletions upstream of the *Apa* I site (towards the 5′ end of DI RNA G), the large *Kpn* I/*Apa* I fragment containing the deletions was gel purified and ligated to the small *Apa* I/*Kpn* I fragment previously excised from pT7-GA. To construct deletions downstream of the *Apa* I

site (towards the 3′ end of DI RNA G), the small *ApaI/Kpn* I fragment harboring deletions was gel purified and ligated to the large *Kpn* I/*Apa* I fragment previously excised from pT7-GA. Random deletions in both directions were generated by ligating the large fragment containing deletions to the small fragment containing deletions. Plasmids with deletions of different sizes were identified by visualization of *Pvu* II/*Apa* I restriction fragments (for deletions upstream of the *Apa* I site) or *Apa* I/*BstE* II restriction fragments (for deletions downstream of the *Apa* I site) on 6% polyacrylamide gels. The exact nucleotides deleted were identified by sequencing the DNA in the region of the deletions using oligo 15 and standard sequencing primers. Plasmids harboring deletions upstream or downstream of the *Apa* I site of DI RNA G were referred to as pT7GA<u>L</u>-# (<u>L</u> = left of *Apa* I) or pT7GA<u>R</u>-# (<u>R</u> = right of *Apa* I). The '#' indicates numbers of deleted bases. Similarly, clones harboring random deletions towards both directions from the *Apa* I site were referred to as pT7GA<u>2W</u>-# (<u>2W</u> = 2 way).

Insertion of Foreign Sequences into the Deletion Mutants

Five *ug* of pT7GAL-31 (31 base deletion), pT7GAL-47 (47 base deletion), pT7GAL-50 (50 base deletion), pT7GAL-53 (53 base deletion) or pT7GAR-32 (32 base deletion) were digested with 30 units of *Apa* I. Ends of the DNA were made flush by Klenow and dephosphorylated by

calf-intestine-alkaline-phosphatase treatment. The Klenow treatment removed 4 nucleotides of the 3′ overhanging sequence of *Apa* I. Foreign sequences excised from different positions in pUC19 or PUC8 by respective restriction digestions were gel purified, treated with Klenow and ligated into each of the above linearized plasmids. Foreign sequences used were as follows: "SH" (GGGGATCGATCCGTCGACCTGCAGCCAAGCT) was the 31-base *Sma* I/*Hind* III polylinker fragment of pUC8 into which 4 nucleotides had been previously inserted at *the BamH* I site previously by *BamH* I-digestion, Klenow-treatment and subsequent ligation. "EH" was the 51-base *EcoR* I/*Hind* III polylinker fragment of pUC19 in which the *Kpn* I site had been previously destroyed by removing 4 nucleotides following Klenow treatment. "KH" was the 39-base *Kpn* I/*Hind* III polylinker fragment of pUC19. "HH65" was the 68-base *Hinf* I/*Hinf* I (nucleotides 642-706) fragment of pUC19. "HH75" was the 78-base *Hinf* I/*Hinf* I (nucleotides 707-781) fragment of pUC19. All these fragments were treated with Klenow before ligation to deletion constructs. Insertions were confirmed by DNA sequencing.

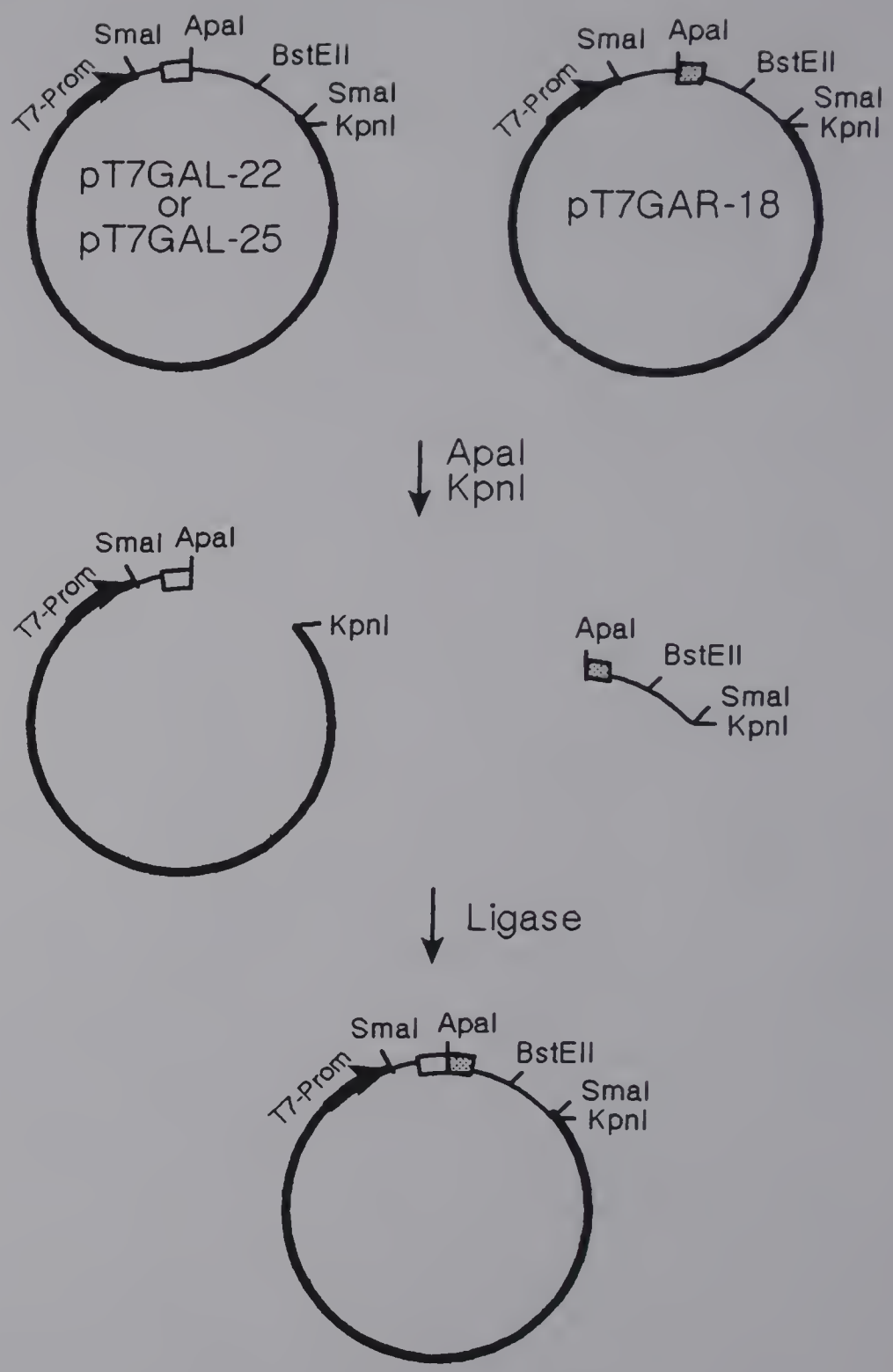
Insertions of foreign sequence at the *Sty* I sites of pT7-GA were made as follows: 5 *ug* of pT7-GA was completely digested with 50 units of *Sty* I overnight at 37°C. The larger fragment, which contained the entire vector sequence and DI RNA G bases 1-89 and 172-352 (numbering according to DI RNA GA sequence) at the two ends, was electroeluted.

Ends of the fragment were made flush by Klenow treatment and ligated to "HH". Following transformation, clones harboring a 78-base foreign sequence that replaced a 82-base fragment (including 8 bases of *Apa* I linker) between the two *Sty* I sites in pT7GA were identified by DNA sequencing using oligo 15 as a primer and named pT7GAStyHH75.

Combination of Deletions Upstream and Downstream of the *Apa* I Site

To delete the desired number of bases in both directions from the *Apa* I site, plasmids (5ul of each) harboring a 22-base or a 25-base deletion upstream of the *Apa* I site (pT7GAL-22 or pT7GAL-25), and a 18-base deletion downstream of the *Apa* I site (pT7GAR-18) were completely digested with 30 units of *Kpn* I and 30 units of *Apa* I. The large *Apa* I/*Kpn* I fragment of pT7GAL-22 or pT7GAL-25 and the small *Kpn* I/*Apa* I fragment of pT7GAR-18 were electroeluted and ligated to form intact plasmids. Following transformation of bacterial strain DH5a, positive clones containing inserts were identified by restriction mapping. Selected positive plasmids, pT7GA(L-22)(R-18) and pT7GA(L-25)(R-18), contained 40- and 43-base deletions around the *Apa* I site, respectively (Fig. 5.7).

Fig. 5.7 Schematic drawing of the procedure used to combine deletions upstream and downstream of the *Apa* I site. Open boxes represent 22-base or 25.base deletions upstream of the *Apa* I site and shaded boxes represent a 18-base deletion downstream of the *Apa* I site. Thick lines represent vector sequence and thin lines represent cDNA inserts.



pT7GA(L-22)(R-18)
or
pT7GA(L-25)(R-18)

Generation of Mutations in the Central and the 3′ Portions of DI RNA G

To make deletions in the 3′ region of DI RNA G, 20 *ug* of pT7-G was digested with *Spe* I or *BstE* II, followed by Bal 31 treatment at various time points from 5 to 20 min. This procedure for Bal 31 treatment was similar to the one described earlier for making stepwise deletion at the *Apa* I site (this chapter), except that, following Bal 31 treatment, ends of DNA were ligated directly without insertion of *Apa* I linkers, and deletions were made randomly in both directions from the respective restriction sites. Four nucleotide insertions at *Spe* I and *Sty* I sites were generated by completely digesting pT7-G with the respective restriction enzymes, treatment with Klenow, and subsequent ligation. One mutant (pT7Sp+1) with a 1-base insertion at the *Spe* I site was unexpected, probably resulting from an aberrant Klenow reaction. Deletions and insertions were all confirmed by DNA sequencing using oligo 8, a 20-base oligonucleotides complementary to the 3′ end of DI RNA G, as a sequencing primer.

In Vitro RNA Synthesis

Copy RNA transcripts were synthesized *in vitro* from various cDNAs inserted into pT7E19 (Petty, 1988) as well as from mutated cDNAs of pTCV-T1d1 (Heaton *et al.*, 1989). Plasmids (5*u*l) were linearized by *Kpn* I (for cDNA in pT7E19) or *Xba* I (for cDNAs mutated from pTCV-T1d1)

digestion, followed by phenol/chloroform extraction and ethanol precipitation. Linear plasmids were incubated in 46 *u*l of reaction solution including 1.25 *u*l of each of four rXTPs (20 mM each), 5 *u*l of 100 mM DTT, 1 *u*l of RNasin (Promega, 40 U/*u*l) and 10 *u*l of 5X T7 RNA polymerase buffer (BRL) for 5 min at 37°C, followed by the addition of 2 *u*l of T7 RNA polymerase (BRL, 50 U/*u*l). The reaction mixture was incubated at 37°C for 45 min, followed by the addition of 5 *u*l of 0.5 M EDTA (pH 8.0). This final mixture was combined with the same volume of 2X RNA infection buffer (see Chapter II, this thesis) to which 12 *u*g of TCV-m + D helper virus inoculum had been added for use in infecting 6 turnip plants.

Results

Synthetic DIs Derived from TCV-B Genomic RNA Were Not Infectious

Previous research showed that virus accumulated following infection using cloned TCV-B genomic RNA transcripts generated *de novo* DI RNA(s) (Chapter IV, this thesis). To understand the sequences required for DI RNA formation, a plasmid containing a full length cDNA copy of TCV-B genomic RNA downstream of the T7 promoter (pTCV-T1d1), was used to construct artificial DI molecules *in vitro*. A diagram of these DI constructs as well as the junction sites corresponding to TCV sequence is presented

in Fig. 5.8. *In vitro* synthesized transcripts from pT7SDI-291, pT7SDI-554, pT7SDI-589 and pT7SDI-700 were used to infect turnip cv. Just Right plants along with the helper inoculum TCV-m + D. Two to three weeks post-inoculation, no RNA molecules corresponding to synthetic DIs were recovered from plants, although all the plants were systemically infected with TCV-m + D as revealed from 5% acrylamide/50% urea gel electrophoresis (data not shown). The results indicate that all these synthetic DIs were not infectious on turnip plants, at least for the first passage.

Ten Nucleotides at the 5′ Ends of DI RNA G and Sat-RNA F Are Identical

Cloning of full length DI RNA G required a cDNA corresponding to the 5′ end of DI RNA G. To obtain such a sequence, the region surrounding the junction of DI RNA G dimers was cloned. The first strand cDNA was generated by extending on oligo 12 (complementary to bases 90-100) using gel purified DI RNA G dimer as a template and the second strand synthesized using RNase H and *E. coli* DNA polymerase I. Five clones were identified that included the junction between monomeric units (Fig. 5.9). The sequence of the partial dimer cDNA was almost identical to sequence of DI RNA G monomer previously determined (see Fig. 4.3, Chapter IV); however, 4 to 5 nucleotides at the 5′ ends of DI RNA G dimer and monomer were different. Interestingly,

Fig. 5.8 Illustration of artificial DIs made from a full length cDNA copy of TCV-B genomic RNA, pTCV-T1d1. Numbering in blocks represent the joining points of DIs according to TCV sequence. Size of each DI construct is indicated. The constructs were named according to their sizes. Shaded regions indicate sequences upstream of base 282. Open region represent sequences downstream of base 3634.

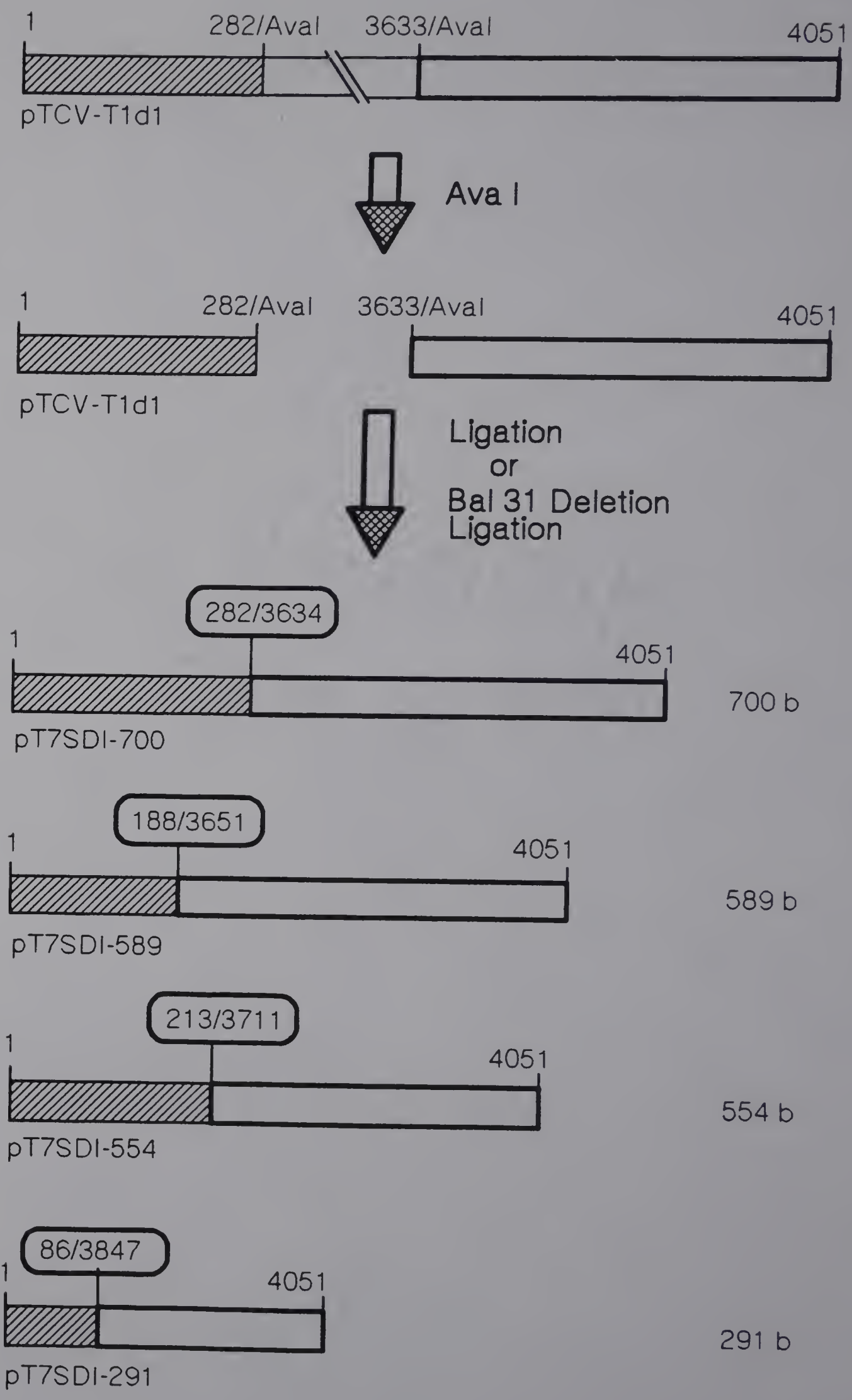

1
282/Aval
3633/Aval
4051
pTCV-T1d1
Ava I
1
282/Aval
3633/Aval
4051
pTCV-T1d1
Ligation
or
Bal 31 Deletion
Ligation
282/3634
1
4051
700 b
pT7SDI-700
188/3651
1
4051
589 b
pT7SDI-589
213/3711
1
4051
554 b
pT7SDI-554
86/3847
1
4051
291 b
pT7SDI-291

Fig. 5.9 Nucleotide sequence of DI RNA G partial dimer cDNAs. cDNAs were generated by extension of oligo 12 on gel-purified DI RNA G dimer using reverse transcriptase. Brackets indicate the 5′ and the 3′ end points of the cDNAs. Only bases that differ from pUCGD3 cDNA are shown. Dots represent missing nucleotides. The first nucleotide at the 5′ of the second monomeric unit is indicated as "1". The sequence is oriented 5′ to 3′. Position of oligo 12 complementary sequence is underlined.

```
pUCGD3                        [CTCCGAATTAAGAGATAGCCTCCC
pUCGD5                                                 [
pUCGD6              [
pUCGD7    [GGTGGGCTGTGGGGACC
pUCGD8
```

```
                                                  1
                                              ------>
pUCGD3    TCC.TCGCGGGGGGGGGGGGCCTGCCCGGGGGATAAAAAAGG
pUCGD5       C                              ..      ..
pUCGD6       .                              ..       .
pUCGD7       .                              ..       .
pUCGD8    [  .                              CA       .
```

```
pUCGD3    AGGCTTACCAACCTTCTCTCTATTCACGATGCCTCTTCTAC
pUCGD5
pUCGD6
pUCGD7
pUCGD8
```

```
pUCGD3    ACACACTCAAAACAGCGCTTGCAGTGGGACTCCTTGGAGCC
pUCGD5                                             ]
pUCGD6
pUCGD7                                        ]
pUCGD8
```

```
pUCGD3    AGGTAC]
pUCGD5    oligo12
pUCGD6          TA]
pUCGD7
pUCGD8         ]
```

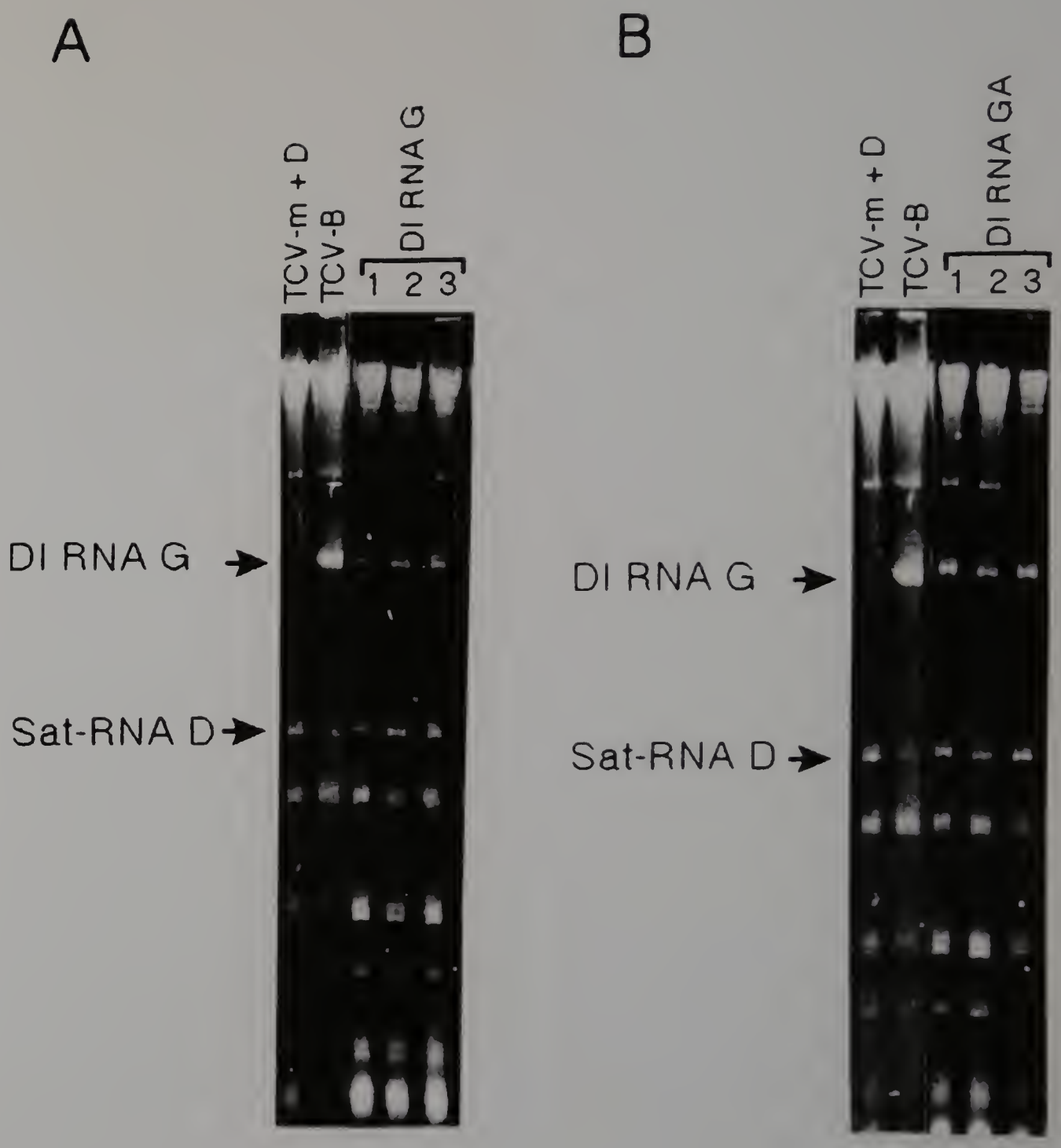
DI RNA G sequence in almost all of the partial dimer clones had a 10-nucleotide stretch at the 5′ end of a monomeric unit that was completely homologous to the 5′ end of Sat-RNA F (Cascone *et al* ., 1990). Also, there were 2 extra nucleotides (GG or CA) at the junction sites between monomeric units in two clones and a variable number of adenoine resifues 6 bases from the 5′ end.

In Vitro Transcripts of pT7-G, a Full Length cDNA Clone of DI RNA G, and Its Derivative, pT7-GA, Were Infectious on Turnip cv. Just Right

A full length DI RNA G cDNA was constructed by joining bases 1-92 of a partial DI RNA G dimer cDNA to bases 93-344 of a partial DI RNA G monomer cDNA (Fig. 5.2 B). *In vitro* run-off transcripts from this plasmid (pT7-G) were used to infect turnip plants along with the helper inoculum (TCV-m + D). All inoculated plants accumulated similar levels of DI RNA G two to three weeks post-inoculation (Fig. 5.10 A) and showed the same level of symptom severity as naturally occurred DI RNA G (date not shown).

To construct DI RNA G cDNA with an unique restriction site in the 5′ portion of the molecule for further mutagenesis studies, pT7-G was linearized at base 98 by partial *Rsa* I digestion which cleaves the plasmid at any one of three positions. A 8-base pair *Apa* I linker (GGGGCCCC) was inserted into the *Rsa* I-cleaved sites in the plasmid (Fig. 5.3). A clone that harbored a *Apa* I linker at

Fig. 5.10 Accumulation of RNAs in plants inoculated with *in vitro* synthesized full length DI RNA G or DI RNA GA along with the helper virus. *In vitro* RNAs were made from pT7-G (**A**) or pT7-GA (B) using T7 RNA polymerase. Total RNA was extracted from plants 3 weeks post-inoculation and subjected to electrophoresis on 5% polyacrylamide/50% urea gels, and stained with ethidium bromide. Different inocula are indicated above the lanes. Brackets indicate RNAs isolated from plants inoculated with *in vitro* made DI RNA G (A) or DI RNA GA (B). Lanes 1, 2 and 3 represent RNAs extracted from different infected plants.


A
TCV-m + D
TCV-B
1
2
3
DI RNA G
DI RNA G
Sat-RNA D
B
TCV-m + D
TCV-B
1
2
3
DI RNA GA
DI RNA G
Sat-RNA D

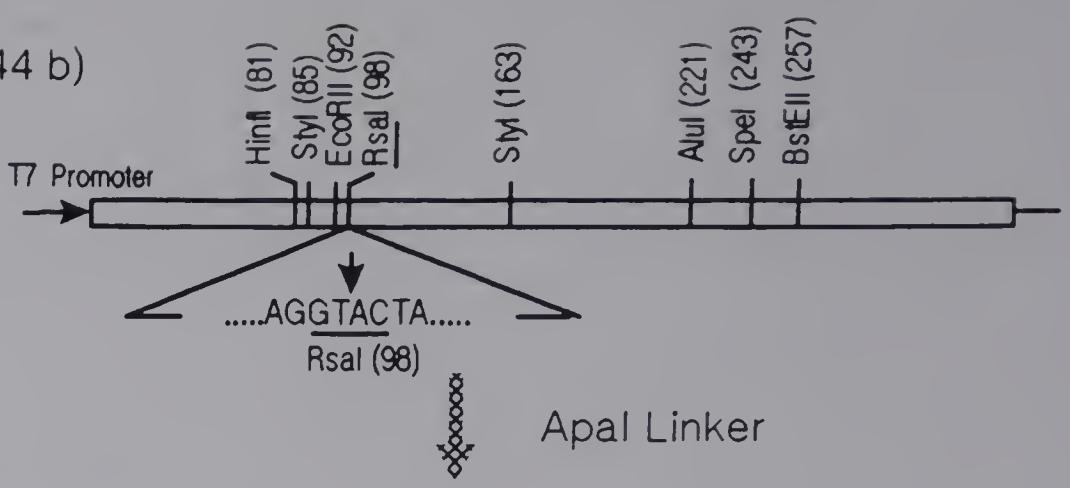
base 98 of DI RNA G cDNA was identified and named pT7-GA. Plants infected with RNA synthesized *in vitro* from pT7-GA (DI RNA GA) along with the helper inoculum (TCV-m + D) developed symptoms characteristic of DI RNA G (data not shown) and accumulated similar levels of RNA as wild type DI RNA G (Fig. 5.10 B). Therefore, pT7GA was used as the template for the generation of mutations in further studies.

Deletions Greater than 26 Nucleotides in the 5′ Region of DI RNA GA Abolish RNA Infectivity.

To identify sequences required for DI RNA GA infectivity, a series of mutations were generated within the molecule and infectivity of mutants was monitored on turnip cv. Just Right. Stepwise deletions in both directions from the *Apa* I site were generated in pT7-GA. By re-inserting an *Apa* I linker, this unique site remained in the deletion mutants (Fig. 5.11). DI RNA GA harboring a 25-nucleotide deletion (bases 74-98) or less were infectious. However, deletion of bases 73-98 (26 bases upstream of the *Apa* I insertion) or more abolished RNA infectivity (Fig. 5.12). Deletions were also constructed from the *Apa* I site towards the 3′ end of the molecule (Fig. 5.13). DI RNA GA with deletions of bases 107-124 (numbered according to DI RNA GA, 18 bases downstream of the *Apa* I insertion) or less were infectious. However, DI

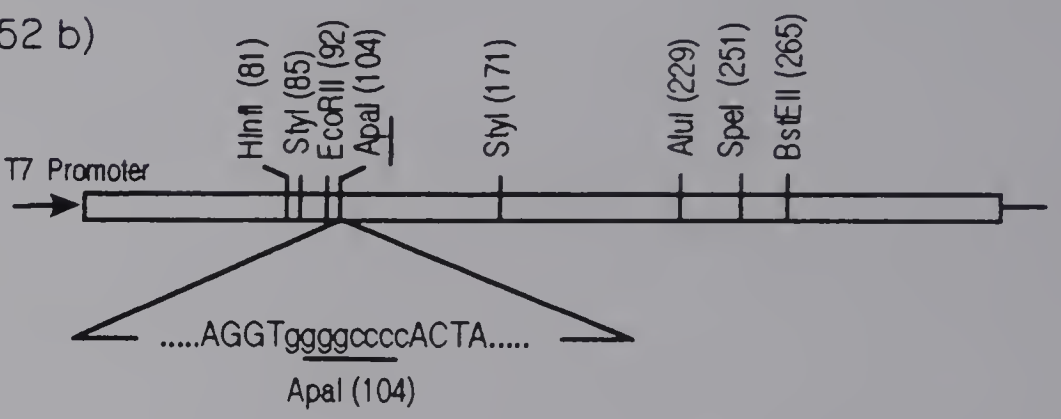
Fig. 5.11 Insertion of an *Apa* I linker into the *Rsa* I site of DI RNA G cDNA and deletions generated at the *Apa* I site. Note that numbering in DI RNA G is different from that in DI RNA GA because of the *Apa* I linker insertion. Small open bars represent deletions. Plasmids with deletions were named according to the direction of the deletions as well as number of bases deleted.

pT7G (344 b)

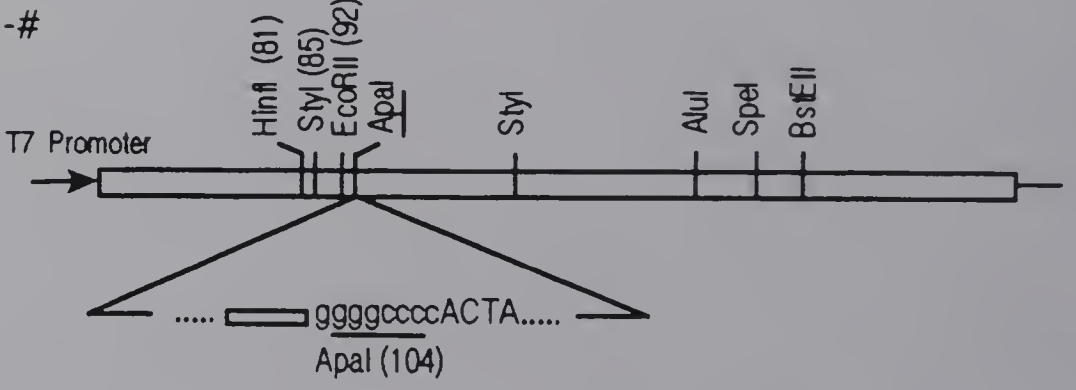


ApaI Linker

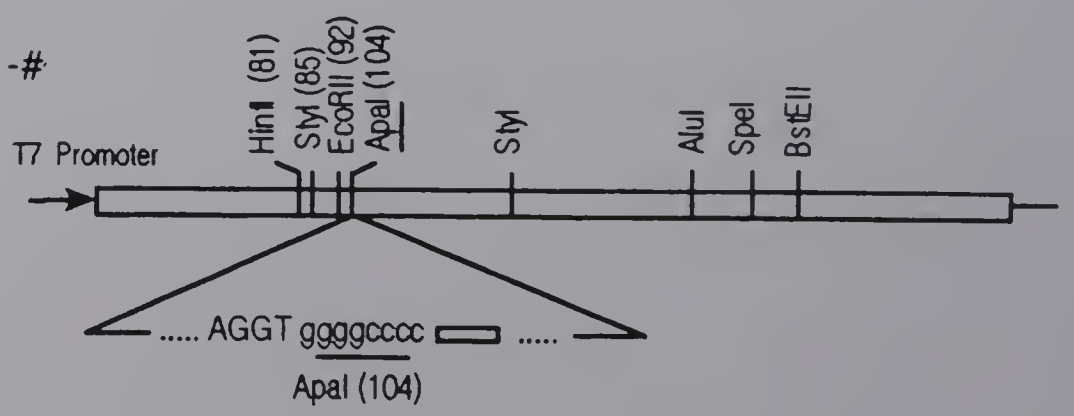
pT7GA (352 b)



pT7GA L-#



pT7GA R-#



pT7GA 2W-#

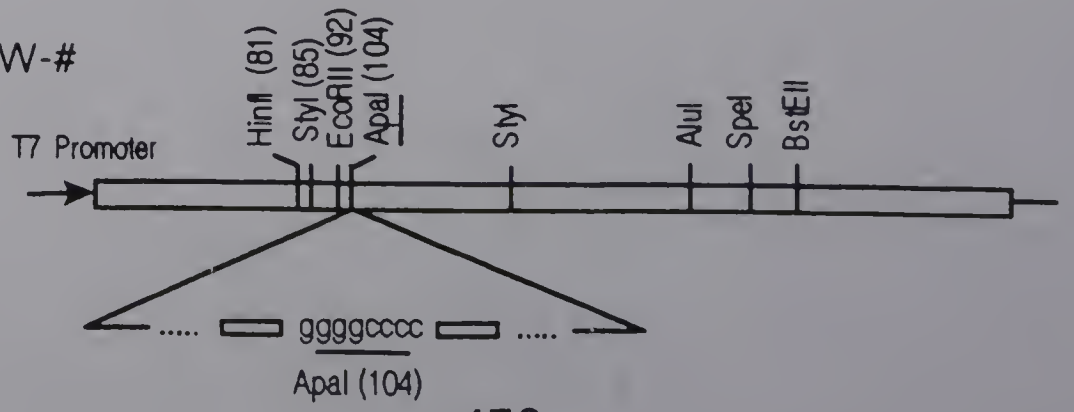
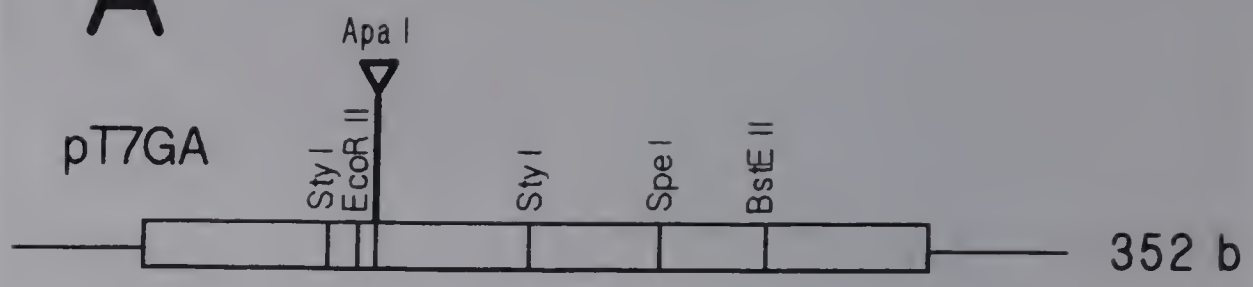


Fig. 5.12 Infectivity of DI RNA GA harboring progressive deletions towards the 5′ end. (**A**) Illustration of deletions made towards the 5′ end of DI RNA GA. An *Apa* I linker was ligated back into each of the deletion mutants. Size and infectivity are indicated for each mutant. Black underlined indicate relative positions of deletions. (**B**) Accumulation of selected DI RNA GA mutants in inoculated plants. Total RNA was extracted 3 weeks post-inoculation and was subjected to electrophoresis on 5% acrylamide/50% urea gels. RNA inocula are indicated above each lane.

A



			Infectivity
pT7GAL-11	-11	341 b	+
pT7GAL-14	-14	338 b	+
pT7GAL-17	-17	335 b	+
pT7GAL-22	-22	330 b	+
pT7GAL-25	-25	327 b	+
pT7GAL-26	-26	326 b	-
pT7GAL-31	-31	321 b	-

B

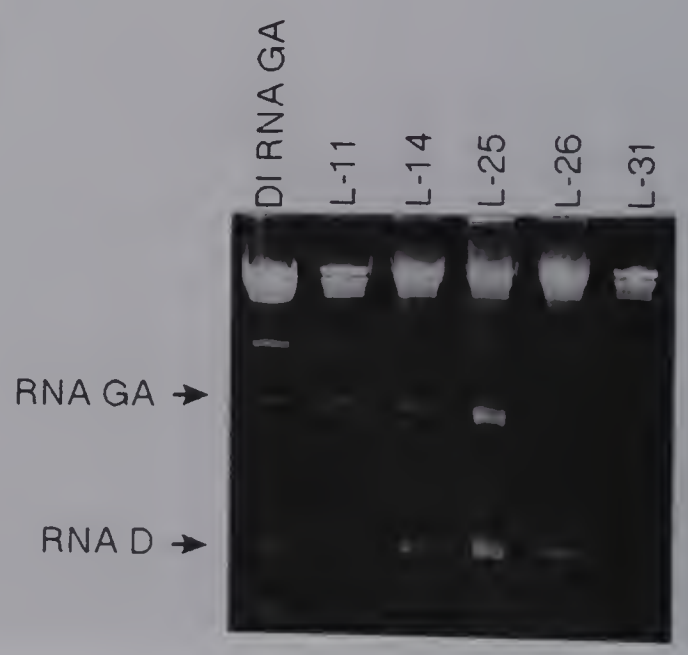
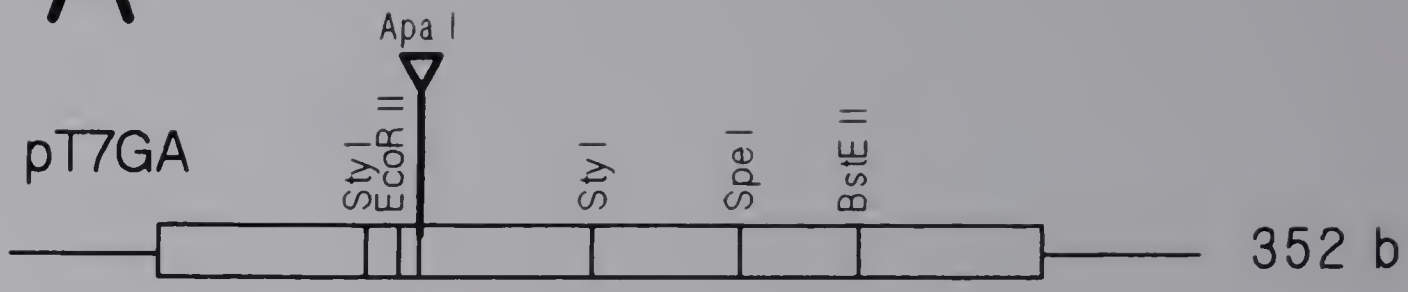


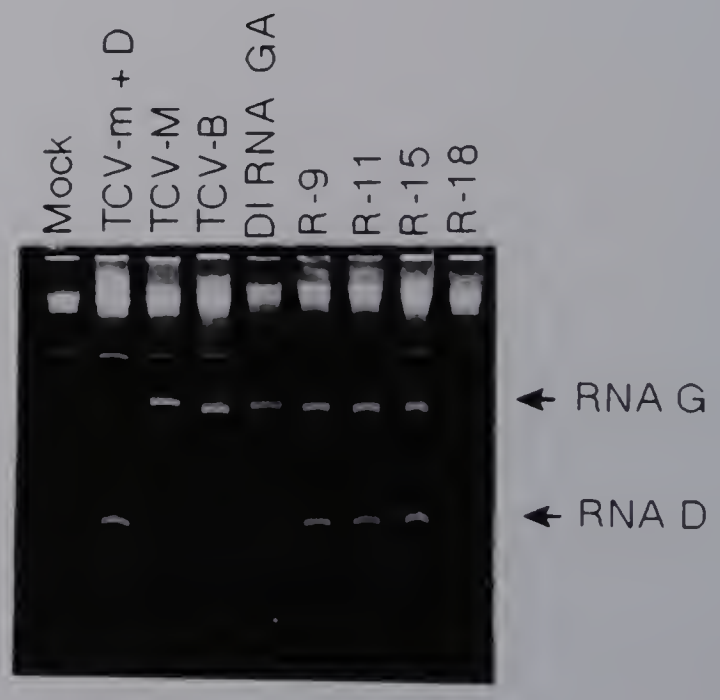
Fig. 5.13 Infectivity of DI RNA GA harboring progressive deletions towards the 3′ end. (A) Illustration of deletions made towards to the 3′ end of DI RNA GA. *Apa* I linkers were ligated back into each of the deletion mutants. Size and infectivity are indicated for each mutant. Black underlines represent relative positions of deletions. (B) Accumulation of selected DI RNA GA mutants in inoculated plants. Total RNA was extracted 3 weeks post-inoculation and subjected to electrophoresis on 5% acrylamide/50% urea gels. RNA inocula are indicated above individual lanes.

A



			Infectivity
pT7GAR-9	-9	343 b	+
pT7GAR-11	-11	341 b	+
pT7GAR-15	-15	337 b	+
pT7GAR-18	-18	334 b	+
pT7GAR-32	-32	320 b	-
pT7GAR-53	-53	299 b	-
pT7GAR-67	-67	285 b	-

B



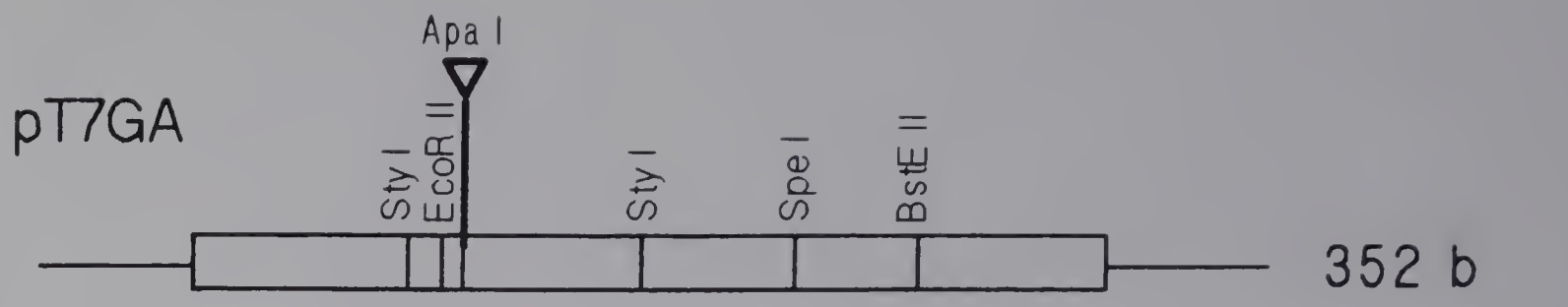
RNA GA harboring deletions of bases 107-138 (32 bases downstream of the *Apa* I insertion) or more were not infectious (Fig. 5.13).

To gain more information on sequence requirements for infectivity of the DI RNA in this region, deletions of random sizes spanning the *Apa* I site were generated. DI RNA GA with deletions up to 21 bases around the *Apa* I site (bases 95-98 and bases 107-113) were infectious. DI RNA GA harboring deletions of 41 bases or more (bases 86-126, pT7GA2W-33) were not infectious (Fig. 5.14). pT7GA2W-33 differed from other deletion mutants by lacking the *Apa* I linker at the deletion site. Therefore, the 41 deleted bases include 33 bases of DI RNA G sequence and 8 bases of *Apa* I linker. These results along with the single direction deletions suggest that DI RNA GA harboring deletions of 30 or more nucleotides around base 98 did not accumulate in turnip.

At Least 71 Bases in DI RNA GA (Bases 68-138, Including the 8-base *Apa* I Linker) Are Not Required for RNA Infectivity --- Revealed by Insertion of Foreign Sequences into DI RNA GA Deletion Mutants

The results above indicate that deletions of about 30 to 40 nucleotides near the 5′ end of DI RNA GA were enough to abolish RNA infectivity. However, it was not known if the deleted sequence itself and/or the size of the RNA was the factor determining infectivity. To determine specific

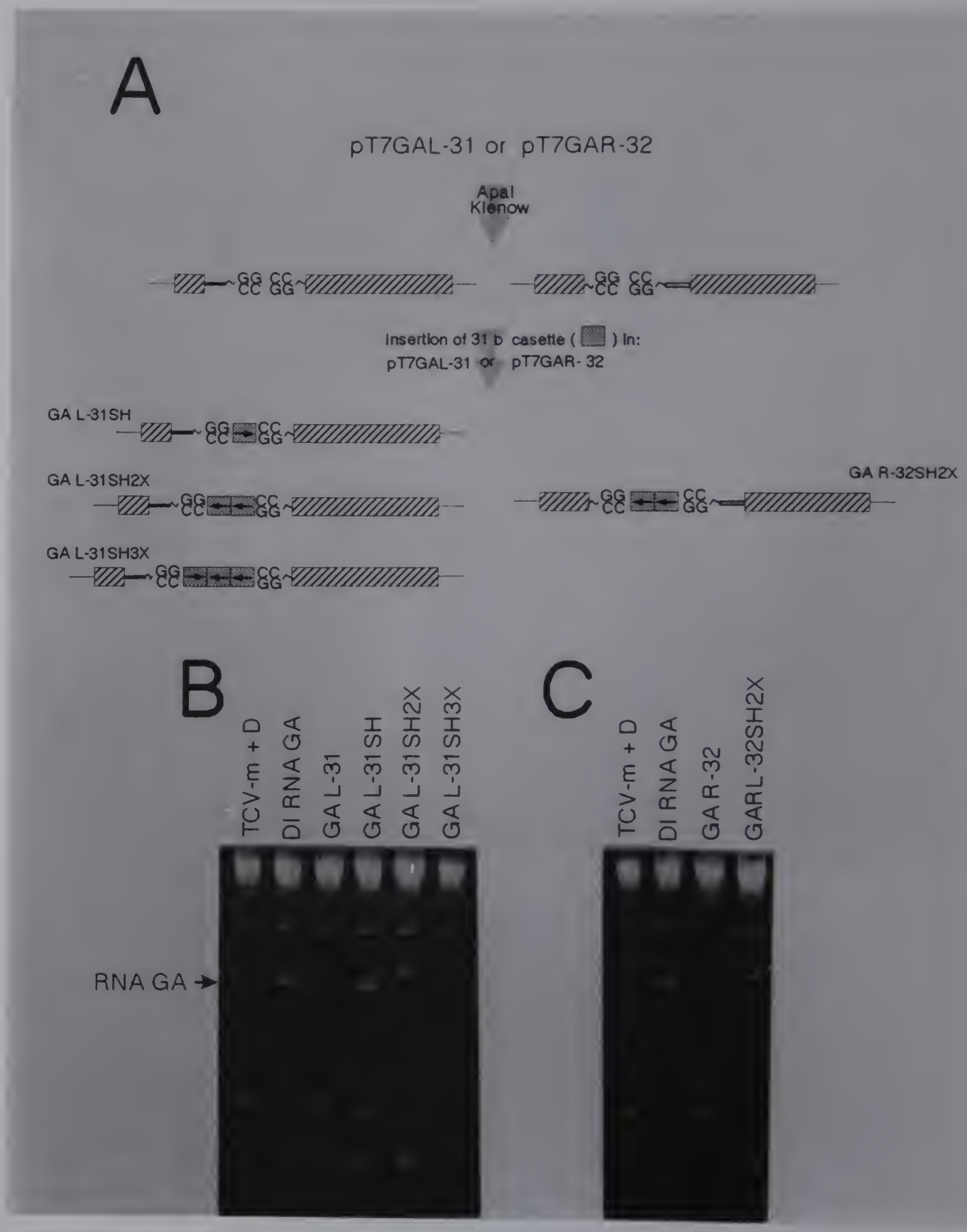
Fig. 5.14 Deletions generated randomly around the *Apa* I site in DI RNA GA. Black lines represent relative positions of deletions. A "*" represents an unusual clone (pT7GA2W-33) that was missing the 8-bp *Apa* I linker sequence. Exact positions of deleted bases relative to the original *Apa* I insertion site (base 98) are indicated as "L-# R-#". Size and infectivity of each mutant are also indicated.



				Infectivity
pT7GA2W-11	-11	L-4 R-7	341 b	+
pT7GA2W-14	-14	L-3 R-11	338 b	+
pT7GA2W-20	-20	L-10 R-10	332 b	+
pT7GA2W-21	-21	L-11 R-10	331 b	+
pT7GA2W-33	-33 (-8 b)	L-13 R-20	311 b *	-
pT7GA2W-49	-49	L-4 R-45	303 b	-

involvement of some of the deleted sequences that had abolished DI RNA GA infectivity, a 31-base sequence ("SH") derived from a modified *Sma* I/*Hind* III fragment from the pUC 8 polylinker was inserted into the Klenow-treated *Apa* I site of pT7GAL-31 (31-base deletion upstream of the *Apa* I site) and pT7GAR-32 (32-base deletion downstream of the *Apa* I site) (Fig. 5.15 A). The resulting plasmids were named pT7GAL-31SH and pT7GAR-32SH, respectively. Turnip plants inoculated with helper virus TCV-m + D and *in vitro* synthesized RNA from pT7GAL-31SH showed the same level of symptom severity (data not shown) and accumulated similar levels of RNA as the transcripts made from pT7-GA (Fig. 5.15 B). Insertion of two copies of the 31-base foreign sequence casette (pT7GAL-31SH2X) also restored infectivity of the RNA (Fig. 5.15 B). However, infectivity was only restored when two copies of the 31-base casette were arranged "head-to-tail". "Head-to-head" arrangement of the two inserted casettes did not restore infectivity (data not shown). Furthermore, insertion of three copies of the casette, where two copies were arranged head-to-tail and another copy was in reverse orientation, did not restore RNA infectivity (Fig. 5.15 B). Insertion of two copies of "SH" in a head-to-tail arrangement into pT7GAR-32 at Klenow-treated *Apa* I site also restored infectivity of the RNA (Fig. 5.15 A, C). These results demonstrate that at least 71 bases residing in DI RNA GA from base 67 to base 138 are not specifically involved in DI RNA GA infectivity.

Fig. 5.15 Restoration of infectivity of a 31-base (DI RNA GA L-31) or a 32-base (DI RNA GA R-32) deletion mutant of DI RNA GA by insertion of a 31-base foreign sequence. (**A**) Insertion of a foreign sequence into the deletion site of pT7GAL-31 or pT7GAR-32. Hatched regions represent non-mutated DI RNA GA L-31 or DI RNA GA R-32 cDNA sequences. Four nucleotides were removed at the insertion site resulting from Klenow treatment of *Apa* I 3′ overhanging ends as illustrated. Solid and open bars represent the 31-base deletion in pT7GAL-31 and the 32-base deletion in pT7GAR-32, respectively. Orientations of the inserted pUC 8 sequence from *Hind* III to *Sma* I sites are indecated as arrows. (**B**) Accumulation of RNA extracted from plants inoculated with: A 31-base deletion mutant (lane GAL-31), the deletion mutant with a single copy of the foreign 31-base casette (lane GAL-31SH), deletion mutant with two copies of the 31-base casette (lane GAL-31SH2X) and deletion mutant with three copies of the 31-base casette (lane GAL-31SH3X). (**C**) Accumulation of RNA extracted from plants inoculated with: a 32-base deletion mutant (lane GAR-32) and deletion mutant with two copies of the 31-base casette (lane GAR-32SH2X).


A
pT7GAL-31 or pT7GAR-32
ApaI
Klenow
GG CC
CC GG
Insertion of 31 b casette ( ) In:
pT7GAL-31 or pT7GAR- 32
GA L-31SH
GA L-31SH2X
GA R-32SH2X
GA L-31SH3X
B
TCV-m + D
DI RNA GA
GA L-31
GA L-31SH
GA L-31SH2X
GA L-31SH3X
RNA GA
C
TCV-m + D
DI RNA GA
GA R-32
GARL-32SH2X

Foreign sequences were also inserted into larger deletion mutants of DI RNA GA. A summary of these deletion/insertion mutants is shown in Table 5.1. RNA species harboring foreign sequences replacing deletions of 47 bases (bases 52-98), 50 bases (bases 49-98) or 53 bases (bases 46-98) in DI RNA GA were not infectious. Similarly, RNA harboring a 78-base bacterial sequence ("HH75") replacing a 82-base deletion of bases 86-171 in DI RNA GA was not infectious. Together with the finding that bases 68-98 and 107-138 of DI RNA GA could be replaced by foreign sequences, I conclude that sequences located within bases 52-67 and within bases 139-171 (base numbered according to RNA GA) are necessary for DI RNA GA infectivity. Bases 67-138 of DI RNA GA are more likely involved in maintaining the RNA molecule at the necessary size rather than having a specific function in the infection process.

Larger Deletions Generated by Combination of Smaller Deletions of Infectious RNAs Abolished RNA Infectivity

To further determine possible size effects on maintenance of DI RNA GA infectivity, infectious mutants harboring deletions towards either the 5′ or the 3′ termini from the *Apa* I insertion were combined to generate larger deletions. As shown above, RNA transcripts harboring a 22-base (pT7GAL-22) or a 25-base (pT7GAL-25) deletion upstream of the *Apa* I site, and a 18-base (pT7GAR-18) deletion downstream of the *Apa* I site were infectious (Fig.

Table 5.1 Summary of extensive deletion/insertion mutations of DI RNA GA. Deleted bases included DI RNA G sequence as well as sequence derived from the *Apa* I linker. Numbering referring to deletion position is presented according to DI RNA G (not GA) sequence.

Constructs	Deleted Bases		Position In RNA GA		Inserted Bases
	RNA G	ApaI	RNA G	ApaI	
pT7GAL-47KH	47 b	4 b	52-98	101-104	39 b
pT7GAL-50KH	50 b	4 b	49-98	101-104	39 b
pT7GAL-53HH65	53 b	4 b	46-98	101-104	68 b
pT7GAL-53EH	53 b	4 b	46-98	101-104	51 b
pT7GAL-53KH	53 b	4 b	46-98	101-104	39 b
pT7GAStyStyHH75	74 b	8 b	90-171		78 b

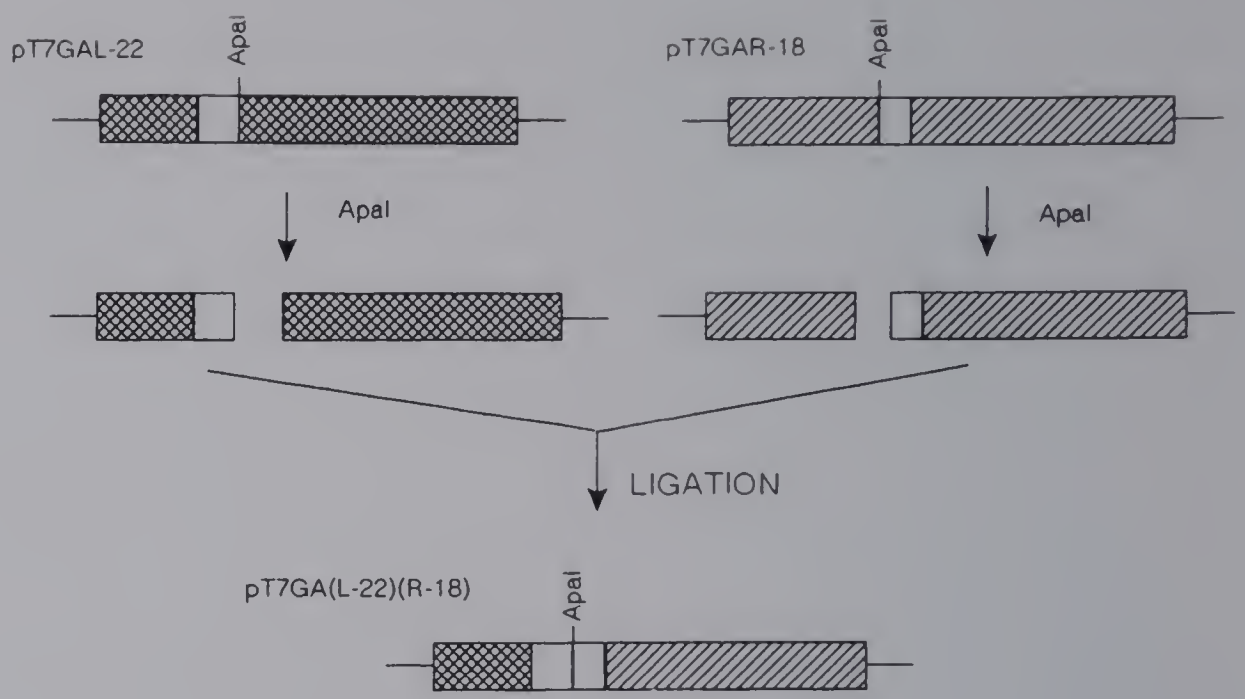
5.12; Fig. 5.13). RNA transcripts made from combination clones, pT7GA(L-22)(R-18) (40-base deletion) and pT7GA(L-25)(R-18) (43-base deletion), were used to infect turnip plants along with the helper inoculum, TCV-m + D. Neither of these two RNAs were infectious (Fig. 5.16). These results further indicate that size, or distance between the 5′ and the 3′ termini, is important in either replication, packaging or movement of DI RNA GA.

Mutations Generated in the Central and the 3′ Portions of DI RNA G or DI RNA GA

Several mutations were generated at the *Spe* I and *BstE* II sites as well as the *Sty* I sites of pT7-G to study 3′ sequences required in DI RNA infectivity. A summary of the results is shown in FiG. 5.17. With the exception of a 1-base insertion in the *Spe* I site, RNA transcripts harboring all deletions or insertions ranging from 4-base insertion to 17-base deletion in either *Spe* I or *BstE* II sites were not infectious on turnip cv. Just Right. However, RNA harboring 4-base insertions in the two *Sty* I sites located at bases 85 and 171 in DI RNA GA were infectious. Over 10 plants tested for the 1-base insertion at the *Spe* I site of DI RNA G (pT7Se+1) accumulated wild type level of the DI RNA, and in every single case, infected plants showed slightly more intense symptoms than plants infected with DI RNA G (data not shown).

Fig. 5.16 Infectivity of combined infectious deletion mutants. (A) Generation of large deletion(s) by combining deletions from two infectious clones. Double hatched bars represent a clone that harbors a deletion upstream of the *Apa* I site. Single hatched bars represent a clone that harbors a deletion downstream of the *Apa* I site. Open bars represent deleted nucleotides. The direction of deletions is indicated by position of the *Apa* I site. (B) Accumulation of RNAs extracted from plants inoculated with different mutants along with the helper virus inoculum. The inoculum of each plant is indicated above each lane.

A

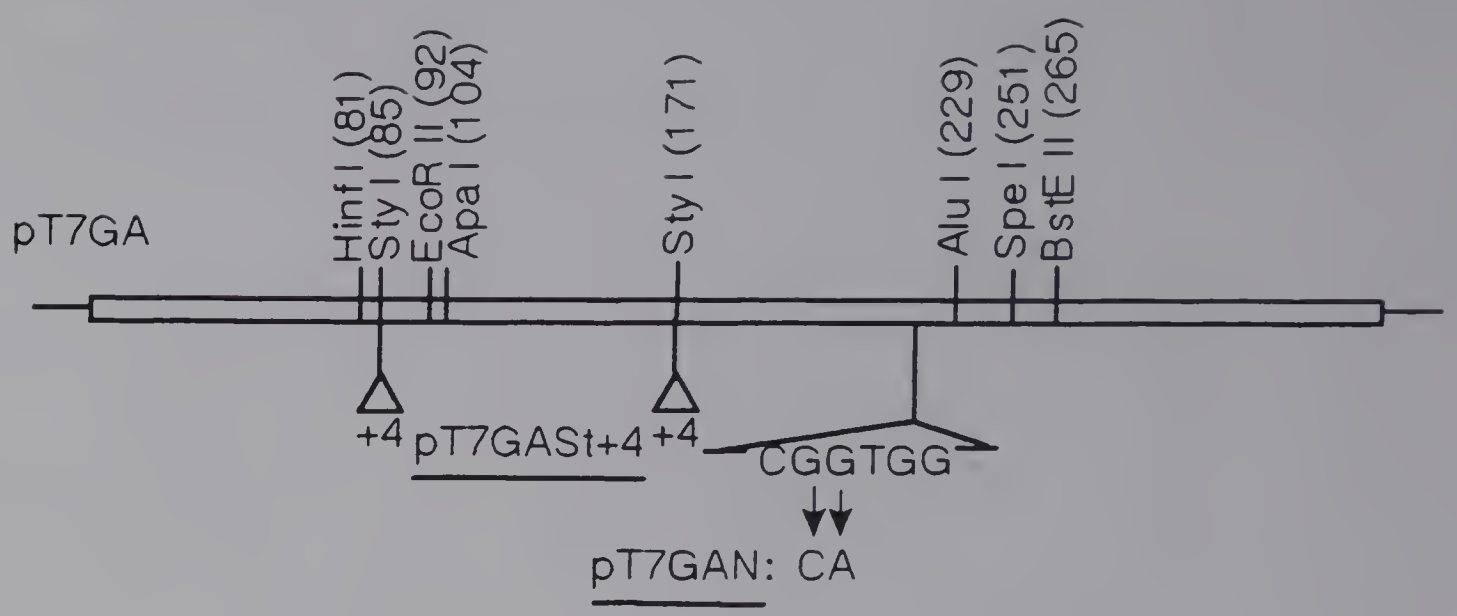

pT7GAL-22
ApaI
pT7GAR-18
ApaI
ApaI
ApaI
LIGATION
pT7GA(L-22)(R-18)
ApaI

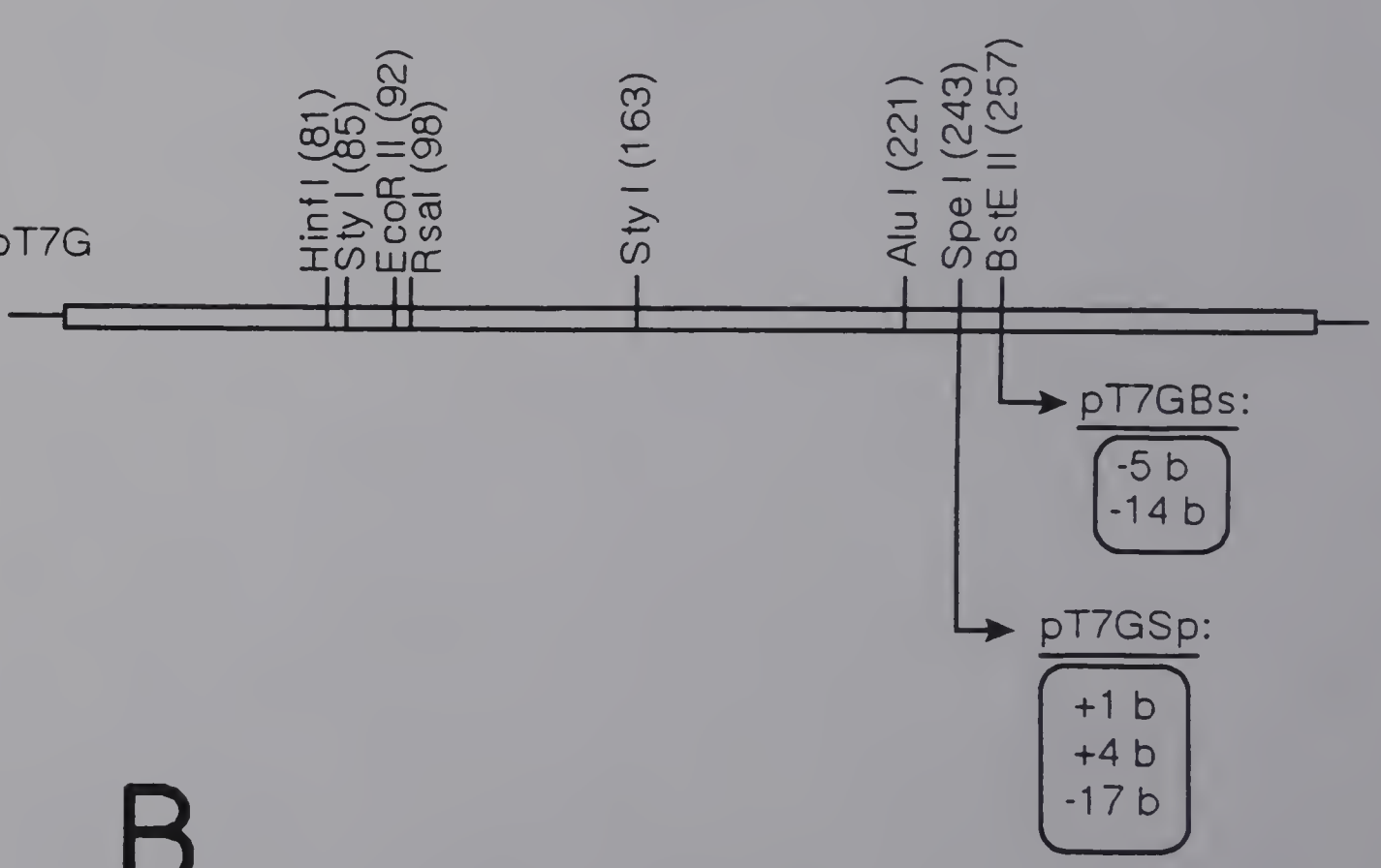
B


DI RNA GA
GA L-22
GA R-18
GA (L-22)(R-18)
RNA GA

Fig. 5.17 Infectivity of RNAs harboring mutations in the 3′ regions of DI RNA G and DI RNA GA. (A) Position of mutations in the 3′ region of DI RNA G and DI RNA GA. (B) Summary of the infectivity of these mutants.

A





B

Constructs	Mutation Site	Infectivity
pT7GAN	Bases 212 and 213	-
pT7GSp+1	SpeI	+
pT7GSp+4	SpeI	-
pT7GSp-17	SpeI	-
pT7GBs-5	BstEII	-
pT7GBs-14	BstEII	-
pT7GASt+4	StyI (2 Sites)	+

Attempt to replace two bases at positions 212 and 213 from "GG" to "CA" at bases 212 and 214 in DI RNA GA in order to create a *Nco* I site in cDNA copy for further studies resulted in non-infectious RNA (Fig. 5.17). These results seem to indicate that the 3′ half of DI RNA G or DI RNA GA is more sensitive to mutations than the 5′ half.

Discussion

The 5′ End of DI RNA G Is Relatively Tolerant to Large Mutations and the Large Section of Sequence in the 5′ Region Is Probably Important in Keeping the RNA a Proper Size

The purpose of this study was to begin to map the sequences required for DI infectivity and generation. Deletion and insertion analysis of the 5′ half of DI RNA G have revealed that replacement of at least 71 bases (nucleotides 68 to 138) of DI RNA GA by unrelated foreign sequence resulted in accumulation of the RNA *in planta*. However, no RNA accumulated if the deletions were greater than 26 bases. Combining two infectious clones which harbored deletions in opposite direction from the *Apa* I site, abolished RNA infectivity. These results imply: 1) the nucleotide sequence in a segment of DI RNA GA or DI RNA G near the 5′ end is not specifically involved in RNA infectivity; 2) size appears important in making DI RNA G an infectious unit. The importance of such so called "size

effects" has been demonstrated in certain animal DI RNAs (Levis *et al.*, 1986; Re and Kingsbuty, 1988). Presumably, non-specific nucleotides may function in DI infectivity by "holding" the molecule in such a way that recognition and initiation signals for replication and encapsidation are fully exposed. Alternatively, these non-specific nucleotides may also function as "stuffer" to physically keep DI molecules certain sizes to meet possible encapsidation constraints. Synthetic DIs, although containing the major portions of TCV genomic RNA from which the DI RNA G and DI1 sequence were derived, were not infectious. This probably also indicates size effects on DI infectivity since all these artificial DIs were made either larger or smaller compared with naturally occurred DI RNA G and DI1 RNA.

The 3′ Region of DI RNA G Is Not Tolerant to Perterbations

Inoculating DI RNA G transcripts containing mutations in the 3′ half of the molecule revealed that even small insertions and deletions within this region abolished RNA infectivity, indicating a specific involvement of this portion of the DI RNA in the infection process. These results were analogous to previous finding for sat-RNA C (Simon *et al.*, 1988). Although the 5′ end of sat-RNA C could harbor relatively large deletions and insertions, the 3′ end was intolerant to small insertions or deletions. Working on the TCV encapsidation process, Wei *et al* (1990)

recently found a possible encapsidation initiation site in TCV DI RNAs using an RNase protection technique. A 29 nucleotide fragment (CACUAAAAUCCCGAAAGGGUGGGCUGUGG) was specifically protected by TCV coat protein in DI molecules. Although the viral genome also contains this sequence, it is not protected by the coat protein. They suggested that the coat protein binding sites in TCV-B genomic RNA are different from that in TCV DI RNAs, presumably because the region folds differently in genomic RNA and in the small RNAs due to differences in the surrounding context. This 29-base TCV coat protein protected fragment is highly conserved among all the clones of sat-RNA C, DI RNA G and DI1 RNA with at most one nucleotide difference, indicating a possible universal involvement of this site in infection or packaging of TCV subviral RNAs. However, the 29-nucleotide fragment is not the only site involved in the packaging process of all TCV subgenomic RNAs. Since the TCV-similar 3′ region is entirely missing in sat-RNA D and sat-RNA F, TCV coat protein binding site(s) must be different in these molecules. Although, in DI RNA G mutagenesis studies, no deletion was made direcly in the 29 base region, changing of surrounding sequence may influence the secondary or teriary structure of this region, which may lead to failure of the RNA being packaged. Studies of positive RNA animal virus DIs have implied that DI RNAs

which have an advantage in packaging have advantages for survival, replication and infection, and vice versa (Makino *et al.*, 1990).

The importance of the 3′ end of positive strand RNA viruses has been also suggested in other unrelated virus systems. Using *in vitro* experiments, Nishihara *et al* (Nishihara *et al.*, 1983) found that when the 5′ terminus of bacteriophage *QB*, was missing, the RNA still bound to the viral replicase. However, the RNA was inactive if the 3′ terminus was deleted. Furthermore, *In vitro* studies of brome mosaic viral replication have shown that deletion of a 130-base fragment at the 3′ terminus inhibited viral replication (Miller *et al.*, 1986; Bujarski *et al.*, 1986).

TCV DI RNAs Are Presumably Generated by a "Replicase-driven Copy Choice" Mechanism --- a Model

In animal virus systems, a replicase "copy choice" model has been suggested to be a likely mechanism for the formation of mosaic type DI molecules (Holland, 1985; Makino *et al.*, 1988a; Makino *et al.*, 1988b). As mentioned earlier in this thesis, this model has been previously proposed for the generation of TCV chimeric sat-RNAs and sat-RNA recombinants (Cascone *et al.*, 1990) based on the sequence similarity among the right side junctions in these discontinuous molecules, the 5′ ends of the sat-RNAs, and the 5′ end of TCV genomic RNA (Fig. 1 2, Chapter I). In this chapter, along with Chapter IV, I have described

another type of discontinuous sub-viral RNA, DI RNAs, that are naturally associated with TCV. These RNA molecules retain both the 5′ end and the 3′ end of TCV genomic RNA sequence as well as internal sequences. All of the DI RNA right side junction sequences and 5′ ends fall into three motifs previously proposed to be replicase recognition sequences (Fig. 5.18; Cascone *et al.*, 1990; Cascone *et al.*, in preparation). No such alignment exists for sequences at the left sides of the junctions. Based on these results, TCV DI RNAs are proposed to be generated by the same mechanism as other discontinuous RNAs associated with TCV. While replicating a (-) strand of viral genome, the replicase dissociates from the (-) strand template by some unknown mechanisms and reinitiates at internal replicase recognition sequences (motifs) on the same template before releasing the nascent (+) strand (Fig. 5.19). In addition, DI RNA G dimers also contain extra non-template nucleotides at the joining points of monomeric units (Fig. 5.9), similar to sat-RNA multimers (Carpenter *et al.*, manuscript submitted) and sat-RNA recombinants (Cascone *et al.*, 1990), which is characteristic of non-homologous recombination. This observation further supports the hypothesis that the generation of TCV DI RNAs is driven by a replication event rather than a breakage and joining process, characterized by precision at the junction site.

Fig. 5.18 Comparison of motif sequences found among TCV sub-viral RNAs and TCV genomic RNA. **(A)** Comparison of sequence between the 5′ end of TCV (Carrington *et al.*, 1989), the junctions of sat-RNA D/C recombinants (Cascone *et al.*, 1990) and the second junction of DI RNA G (this thesis). **(B)** Comparison of sequences among the 5′ end of sat-RNA F (sat-RNAs C and D are similar; Simon and Howell, 1986), the 5′ end of DI RNA G (this thesis), the first and second junctions in sat-RNA C (Simon and Howell, 1986) and DI1 RNA (this thesis), and a sequence in the vicinity of the possible initiation site of the TCV subgenomic RNA encoding for the 8 KDa protein (Carrington *et al.*, 1989). C. Comparison of sequences among junctions of the newly identified chimeric sat-RNA CX (Cascone *et al.*, in preparation), the first junction in DI RNA G (this thesis), sequence downstream of promoter of the TCV subgenomic RNA encoding for the coat protein (Carrington *et al.*, 1989) and a junction of a double recombinant involving sat-RNA D, TCV and sat-RNA C (Cascone *et al.*, in preparation).

A

TCV 5' end 5'- NGUAAUCUGCAAAUCCCUGCACCCGCCUA ...

D/C Recombinant ... AUCCCACUCGAAAGAGUCGAAUCCC . AGACCCUCCAG ...

DI-RNA G ... UACUACCCCGAAGGUUCAAAAUCCA . AGACCCCCAAG ...

Motif I

B

Sat-RNA F 5' end 5'- GGGAUAAAAAUAAGGGAUUCAUAC ...

DI-RNA G 5' end 5'- GGGAUAAAAAA . GGAGGCUUACCA ...

Sat-RNA C (2) ... CCAGCCAAAGGGUAAAUGGGACC AAAAACGGCGGCAGCACC ...

Sat-RNA C (1) ... AAAGAAUCCCAGACCCUCCAGCC AAAG . . GGUAAAUGGGAC ...

DI1-RNA (1) ... AGGUACUACCCUGAGGUUCCAGGAGUGAAGGUAGCAGAAAG ...

DI1-RNA (2) ... CAAGCUAUCAGAAUUUAGGUACC AAAAACGGUGGCAGCACU ...

TCV sub-genomic (3) . . . GGGAUAUAAAGAAGAAGCUUAGTG...

Motif II

C

Motif III

SAT-RNA CX ... GAGUCCAAGACCCUGUCUUAGUAGUUAGCUCUCUCU ...

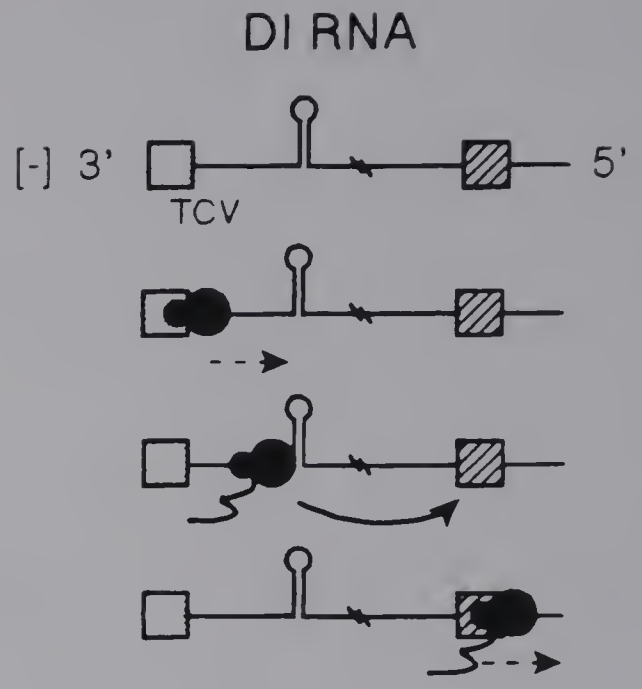
DI-RNA G 5' NNUGCUAAANAGGAGGCUUACCA . . ACCUUCUCUCU

TCV sub-genomic (2) . . GCUUUCUA . . CAACUCUCUCU ...

Double recomb. ... CCAAGACCCUGCCUCUUAGUAGUUCUCAAAAGAA .....

Motif I.....

Fig. 5.19 Model for the generation of TCV DI RNAs. Motifs I and II are indicated as shaded and hatched boxes, respectively. In this model, the replicase is thought to release from viral (-) strand as a replicase/partial (+) nascent strand complex and reinitiate replication at internal recognition sites (motifs) on the same template, resulting in a mosaic type DI RNAs.


DI RNA
[-] 3'
5'
TCV

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