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An investigation of tomato curly stunt virus in South Africa

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CHAPTER SIX

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CHAPTER FIVE

Conclusion and Future Direction

Though not conclusive, we have provided some evidence that ToCSV [99/0631] and ToCSV [01/2521] are associated with small DNA molecules that resemble either satellite DNA molecules or subgenomic defective DNA molecules. The infectious nature or biological role of these small DNA molecules identified in this study has not been established. However, the chimeric nature of the 842nt DNA molecule found in association with ToCSV [99/0631], and the fact that it appears to have originated partly from ToCSV [99/0631] suggests the 842nt DNA molecule to be a subgenomic defective DNA molecule that is derived from ToCSV-DNA-A. AYVV is an example of a monopartite begomovirus reported to be associated with a defective DNA molecule that originates from its single genomic molecule (Stanley *et al*., 1997). The full-length genome of ToCSV [01/2521] has not been established, therefore we were not able to confirm the nature and origin of the two small DNA molecules (1449bp and 755bp) found in association with ToCSV [01/2521]. All three small DNA molecule found associated with ToCSV[99/0631] and ToCSV [01/2521], were fortuitously amplified using PCR primers designed to amplify a region that excluded that part of the IR that includes the putative stem loop and the geminivirus conserved nonanucleotide. Thus it is imperative that the presence or absence of the stem-loop in these small DNA molecules is established. Futhermore, co-infection of the natural host plant and other indicator plants with these molecules and their associated virus is necessary in order to elucidate the biological nature of these molecules. It is necessary to repeat the host range study using a different inoculation method.

of ToCSV [99/0631]-infected tomato plants. No symptoms were observed in all but one of the test plants, and no viral DNA was detected in any of the test plants. The failure to successfully infect and detect viral DNA in bean (*Phaseolus vulgaris* L) and *Nicotiana spp*. in particular, was surprising as these plant species are reported by J.K Brown (2003) on the GeminiDetective website as experimental hosts of ToCSV [99/0631]. She does however not mention which method of inoculation was used in their infectivity study. In our study, biolistic inoculation of all test plants was conducted in a vacuum chamber, and many plants such as *Datura stramonium* L. did not survive. We therefore cannot rule out the possibility that, our unsuccessful attempt to infect these test plants could be due to the inefficiency of this biolistic delivery device rather than the lack of susceptibility of these test plants to ToCSV [99/0631]. Dicotyledonous plants have been successfully inoculated and infected by biolistic delivery of geminiviruses before (Briddon *et al*., 1998; Paximadis *et al*., 1999; Berrie *et al*., 2001; Rothenstein *et al*., 2005). But, in all of these cases, a hand-held particle gun, and not a vacuum chamber, was the biolistic device of choice. Rothenstein *et al*. (2005) used both the vacuum chamber and hand-held particle gun in their experiment and they report that they successfully infected cassava with *Indian cassava mosaic virus* using the hand-held particle gun, but none of the plants survived the inoculation process in the biolistic chamber. Agroinoculation of test plants with geminiviruses has been consistently reported as an efficient method of viral DNA delivery (Liu *et al*., 1997; Berrie *et al*., 2001). However, full-length DNA is needed to construct infectious dimers in order to utilize *Agrobacterium*-mediated transfer of viral DNA to test plants, hence we did not use this method. We therefore cannot conclude unequivocally that the test plants used in this host range study are not susceptible to infection with ToCSV [99/0631].

Putative Satellite DNA Molecules Associated with ToCSV [01/2521]

Based on BLAST search results and alignment of the 755bp putative satellite molecule to DNA sequences mentioned in section 2.8, we were unable to determine the origin of this putative satellite molecule found in association with ToCSV [01/2521] DNA. The only indication that the 1449bp putative satellite molecule might be partly derived from a geminivirus, is the presence of the 22nt sequence that showed 100% nucleotide sequence identity to a region within the IR of ToCSV[99/0631]. The region of the 1449bp molecule that aligns to ToCSV[99/0631] includes 5(TAATA) of the 9 (TAATATTAC) nucleotide sequences that make up the geminivirus conserved nonanucleotide sequence. The rest of the IR, including the putative stem loop, is missing. It is strongly suspected that due to the location of the primer binding sites, we failed to amplify a putative stem loop. The primers used, (AL1c2745 and PAR1v32), were originally designed to amplify a near-full length DNA-A of ToCSV, missing about 60 nucleotide sequences from the IR. Nucleotide sequences originating from the tomato host genome were identified in the 1449bp sequence. We suspect that both the 1449bp molecule and the 755bp molecule might possibly be defective chimeric DNA molecules derived possibly partly from ToCSV [01/2521] DNA. Because the DNA sequence of ToCSV [01/2521] has not been established, we could not align and compare these molecules to ToCSV [01/2521] DNA, therefore we were unable to confirm our suspicion. Abutting full-length primers need to be designed to amplify full-length ToCSV[01/2521] DNA in order to elucidate the origin and subsequently the biological role of both small DNA molecules found in association with ToCSV [01/2521]. As already discussed, identifying these molecules as defective DNA molecules, may possibly explain the less severe symptoms (Slight leaf yellowing and curling) observed on tomato plants infected with ToCSV[01/2521].

Host Range Study

In order to assess the host range of ToCSV [99/0631], a total of nine test plants were biolistically inoculated with total DNA extracted from the uppermost leaves

discovered small subgenomic defective DNA molecules associated with *Tobacco leaf curl Zimbabwe virus* (TbLCZWV). These molecules plus all previously reported subgenomic defective DNA molecules, appear to have a fairly uniform structure. In addition to the IR (containing the putative stem-loop), they always retain a large portion (5' end) of the BV1 or C1 (CP) ORFs, with a large portion of the BV1 or CP and remaining ORFs deleted. They usually have sequences of unknown origin inserted between the truncated BVI or C1 and the remaining virus-specific sequence. A similar structure was observed with the defective DNA molecule (842bp) associated with ToCSV [99/0631] in this study. However, a stem-loop structure with the conserved nonanucleotide sequence was not identified. If this 842bp DNA molecule does have a stem-loop, it is possible that it was missed as a consequence of the binding sites of the primers (AL1c2745 and PAR1v32) used to amplify these fragments. These primers were originally designed to amplify a near-full length DNA-A of ToCSV, missing about 60 nucleotide sequences from the IR. It is therefore imperative to search for the loop by PCR-amplification using primers that will amplify the IR of the 842bp molecule, if present. The biological or interfering role of this molecule has not been established experimentally. Defective DNA molecules associated with ToCSV have not been reported. The discovery of a suspected defective DNA molecule in ToCSV [99/0631]-infected tomato plants may possible explain the variable symptom severity observed from tomato plants infected with the second isolate, ToCSV [01/2521]. Paximadis and Rey (1997) noted that two defective molecules ("Mild" and "HG") from two different tobacco plants, one displaying severe leaf curl symptoms and the other displaying mild leaf curl symptoms, were different in structure. Based on the known biological function of defective DNAs, which is to cause symptom amelioration (Stanley *et al*., 1997), Paximadis and Rey (1997) consequently hypothesized that the defective DNAs could play a role in symptom modifications. The possibility that environmental stress experienced by an individual plant, may play a role in symptom severity as previously suggested (McClean, 1940) cannot be excluded. It is also possible that unidentified factors such as the satellite β molecule found in association with *Ageratum yellow vein virus* (AYVV) may contribute to symptom severity.

DNA1 molecule, we would have to demonstrate that a DNA β molecule is associated with ToCSV [01/2521] DNA.

The low levels of ToCSV [01/2521] DNA observed through southern blotting could also be possibly attributed to tissue tropism, e.g. the type of cell and tissues infected by this virus (Tyler and Fields, 1996). In order to mount a systemic infection, plant viruses need to move into and through the vascular system of its plant host. However, it is well documented that some begomoviruses, like the TGMV, exhibit phloem limitation, i.e. they remain confined to the vascular system, a phenotype that is reported to be at least in part, genetically determined by the virus (Morra and Petty, 2002). The confinement of a virus in the vascular system subsequently reduces viral accumulation in other parts of the infected host plant, thus complicating its detection. Based on the southern blot results, it was conclude that the isolate ToCSV [01/2521] is a begomovirus because the CCP gene (~550bp) was detected, though in very low concentration, through hybridization with a *Begomovirus*-specific probe.

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Putative Satellite DNA Molecule Associated with ToCSV [99/063]

Along with the ToCSV [99/0631] genomic DNA, a putative satellite DNA molecule, 842 bases long, was coincidentally detected following PCR amplification with primers AL1c2745 and PAR1v32. This small DNA molecule appears to have possibly derived from the co-infecting ToCSV [99/0631] DNA molecule, because it contains variable amounts of the coding regions of ToCSV [99/0631] genes AV1, AV2, AC2, AC3 and AC1. The rest of the genome is of unknown origin. The occurrence of contiguous regions of nucleotide sequences that seem to be of different origins (i.e. chimeric sequences) (Fig. 3.12), suggests that recombination might have been involved in the evolution of this DNA molecule. Similar DNA molecules have been identified before (Stanley and Townsend, 1985; MacDowell *et al* 1986; Stanley *et al*., 1997; Paximadis and Rey, 1997; Paximadis and Rey, 2001). The majority of these molecules are half the size of their co-infecting begomovirus, but DNA forms less than 1000bp have also been reported elsewhere (Liu *et al*., 1998) Recently, Paximadis and Rey (2001)

ameliorating symptoms and reducing the accumulation of their co-infecting helper virus, have been reported for AYVV and CLCuD, respectively (Stanley et al., 1997; Liu et al., 1998). It is reported that each of these recombinant DNA molecules contain the origin of replication from their associated monopartite begomoviruses together with sequences of unknown origin.

Southern Blot Analysis

When PCR amplification of total DNA containing ToCSV [01/2521] DNA with primers AV1c1048 and AV1v514 (Wyatt and Brown, 1996), repeatedly failed to amplify the CCP region with begomovirus genus-specific primers, this prompted doubt as to whether this isolate was indeed a begomovirus as was initially suspected. To ascertain whether ToCSV [01/2521] was a geminivirus, southern blotting was employed, and the results confirmed the presence of the CCP gene (Fig. 3.5) from ToCSV [01/2521]-infected plants. Very low levels of ToCSV [01/2521] DNA were detected by southern blot, as compared to detected levels of ToCSV [99/0631] DNA. We suspect that this is the result of reduced ToCSV [01/2521] replication and subsequently, viral accumulation. Though the biological role of the small molecules (1449bp and 755bp) found in association with ToCSV [01/2521] DNA has not been determined, the southern blot results (very low concentration of isolate 01/2521 DNA) strongly suggest that these putative satellite molecules, act as satellites or defective interfering DNAs (DIs) by ameliorating the symptoms and reducing DNA accumulation of ToCSV [01/2521] in the host plant. Defective interfering DNAs, less than the genomic size, and associated with begomoviruses AYVV and CLCuMV, have been reported (Stanley *et al*., 1997; Liu *et al*., 1998; Briddon *et al*., 2000; Briddon *et al*., 2001). It is possible that the small DNA molecules are DNA1 molecules, even though a DNA1 molecule less than 1000bp has not yet been reported. DNA1 molecules are satellite-like molecules that are capable of autonomous replication but require a begomovirus for encapsidation and movement. All reported DNA1 molecules so far are always half the size of their co-infecting begomoviruses (Briddon *et al*., 2004). DNA1 molecules are found to be exclusively associated with monopartite begomovirus-DNA β complexes. Therefore if one of these molecules were a

failed attempt to amplify the core coat gene from ToCSV [01/2521] DNA. The biological role of these small DNA molecules has not yet been determined, but based on the southern blot results (Fig. 3.5), the observed low levels of ToCSV [01/2521] DNA, could be an indication that these small DNA molecules, found fortuitously in association with ToCSV [01/2521] DNA, could act as defective interfering DNAs, subsequently reducing viral DNA accumulation in the host plant. If this is the case, then we can attribute the failure to amplify the CCP gene from ToCSV [01/2521] –infected plants to viral DNA levels that were too low in the plant for detection with these primers. This would also explain the faint ~2700bp fragment (Fig. 3.3) and the inability to reproduce this fragment using the band stab PCR amplification technique (Fig. 3.4). Indeed it has been reported that tomato seedlings infected with ToCSV by grafting, often test positive with begomovirus CCP primers but then later test negative (Schalk van Heerden, Sakata Seeds, pers. Comm.). It is suggested that this may be due to partial host discovery and reduction in virus multiplication, leading to undetectable viral DNA level.

PCR amplification of ToCSV [01/2521] DNA with primers AV1707 and AC515 produced a DNA product of 1508bp in length. BLAST searches with this sequence showed no nucleotide sequence identity to any geminivirus posted in GenBank. Alignment of this sequence with ToCSV [99/0631] DNA also showed no nucleotide sequence identity between the two. It is unlikely that this is a DNA β satellite molecule because multiple alignment of the 1508bp to a number of chosen DNA β satellite molecule (section 2.8) showed that the 1508bp DNA sequence does not contain any of the conserved sequence motifs (stem loop structure with the loop sequence TAA/GTATTAC, the satellite conserved region and the A rich region) commonly present in all DNA β satellite molecules reported so far (Briddon et al., 2003). This sequence could possibly represent a defective DNA molecule derived from ToCSV [01/2521] DNA, but this, we will only be able to establish when the full-length ToCSV [01/2521] genome is available. DIs have been associated with monopartite begomoviruses. Circular recombinant components that behave as defective interfering DNAs by

Polymerase Chain Reaction (PCR) Amplifications

Primer pairs JSP001/JSP003 and JSP001/JSP002 amplify the full-length CP genes (~750bp) of EACMV and ACMV respectively. Based on nucleotide (nt) sequence homology within the coat protein gene, Pietersen and Smith (2002) have shown that ToCSV and EACMV share 77% nt sequence similarity. Since these primers were available in the lab, we decided to test them on ToCSV. We were unable to PCR amplify the full-length CP gene from ToCSV [99/0631]- and ToCSV infected plants using these primers. These results were not entirely surprising because it is reported that despite the high degree of CP gene conservation among begomoviruses, the full-length coat protein gene, is not readily accessible using a degenerate PCR-based approach. This is because the motifs that flank the CP gene, to which primers used during direct PCR amplification of this gene are usually based, are not conserved across the *Begomovirus* genus (Brown, 2000).

Direct PCR-based detection of begomoviruses was carried out using degenerate primers, AV1c1048 and AV1v514, known to amplify all whitefly-transmitted geminiviruses (Wyatt and Brown, 1996). The expected DNA product of ~550bp (CCP) was amplified from ToCSV [99/0631]-infected plants but not from ToCSV [01/2521]-infected plants. The failure to amplify the CCP gene from ToCSV [01/2521]-infected plants was unexpected and surprising because these CCP primers, as already mentioned, are known to amplify the core coat region of all whitefly-transmitted geminiviruses because they are designed to anneal to highly conserved sequences that flank the CCP gene. These results prompted doubt as to whether ToCSV [01/2521] was indeed a begomovirus. To ascertain whether ToCSV [01/2521] is a begomovirus, southern blotting was employed. *Begomovirus* CCP was detected from ToCSV [01/2521] total DNA through hybridization with digoxigenin-labeled ToCSV [99/0631] CCP (fig. 3.5), thus confirming that ToCSV [01/2521] is a begomovirus. When we later unintentionally amplified small DNA molecules (putative satellites or DI molecules) (1449bp and 755bp) from TNA containing ToCSV [01/2521] DNA with primers AL1c2745 and PAR1v32 (fig. 3.3), it became apparent that the presence of the small DNA molecules might somehow be responsible for our

CHAPTER FOUR

Discussion

Begomoviruses have been an emerging threat to tomato (*Lycopersicon esculentum* Mill.) for decades, with incidences increasing greatly in the last decade (Li *et al*., 2004). The large number of recognized and tentative *Begomovirus* species reported to naturally infect tomatoes worldwide (Varma and Malathi, 2003) suggest that tomato is an ideal host for these viruses. Africa has not been spared, reports of tomato-infecting begomoviruses in the African continent date back to the 1960s when TYLCD was first reported in the Sudan (Yassin and Nou, 1965). Tomato-infecting begomoviruses are threatening to become a major problem in South Africa. A new disease caused by *Tomato curly stunt virus* (ToCSV), designated ToCSV [99/0631] in this study, was reported to occur in the Strydomblok Distrik, close to the Mozambique border (Pietersen *et al*., 2000). The virus has since spread to Pongolo and Nkwalini, KwaZulu-Natal and Trichardtsdal, Limpopo (Pietersen and Smith, 2002). The recent outbreak of ToCSV disease in Maputo, Mozambique, was particularly devastating in the 2004/5 and 2005/6 seasons. Because of the close proximity of Maputo with some of the major tomato growing areas of South Africa, South Africa is now considered potentially at risk of a major outbreak of the disease. Tomato plants with slightly different and milder symptoms (slight leaf yellowing and downward curl of leaf margins) were observed near Pietermaritzburg, KwaZulu-Natal. Based on the milder symptoms and the initial unsuccessful attempts by Dr G. Pietersen to PCR amplify viral DNA from total DNA extracted from these plants using ToCSV [99/0631]-specific primers, it was putatively suggested that these plants could be infected with a different ToCSV isolate, designated ToCSV [01/2521] in this study. The aim of this study was to investigate and characterize ToCSV [01/2521] in South Africa.

3.8 Host Range Study

Among the plant species inoculated with ToCSV [99/0631] only one species, *Capsicum annuum* L, appeared possibly to be susceptible (Table 4), based on the phenotype. Symptoms observed after 21 days were slight upward leaf curl and yellowing. The rest of the test plants did not exhibit any geminivirus-like symptoms. All test plants, including those that did not exhibit any geminivirus-like symptoms were tested for systemic infection by PCR amplification, using the degenerate primer pair AV1c1048/AV1v514 that yields a ~550bp fragment of the *AV1* gene diagnostic for begomoviruses (Brown *et al*., 2001). Only tomato (*Lycopersicon esculentum*), the natural host and positive control in this experiment, tested positive with these primers, and the expected fragment of ~550bp was seen when samples were run on 0.8% agarose gel (results not shown). Although pepper (*Capsicum annuum* L.) exhibited geminivirus-like symptoms, we were not able to confirm systemic infection by PCR amplification with the core coat protein primers.

Table 5.1 Host Range Study by Biolistic Inoculation of ToCSV isolate 99/0631

Host	**Symptom Phenotype**	**PCR**
Capsicum annuum L.	Leaf curl, Leaf yellowing	**-**
Cucurbita pepo L.	Asymptomatic	**-**
Vigna unguiculata	Asymptomatic	**-**
Solanum melongena	Asymptomatic	**-**
Abelmoschus esculentus	Asymptomatic	**-**
Datura stramonium L	Asymptomatic	**-**
Gossypium hirsutum	Asymptomatic	**-**
Malva parviflora	Asymptomatic	**-**
Nicotiana tabacum	Asymptomatic	**-**
Phaseolus vulgaris L.	Asymptomatic	**-**
Lycopersicon esculentum	Slight leaf yellowing	**+**

3.7 Sequence Analysis of Two/Third DNA-A

Primers AC515 and AV1707, designed by DrA.M Idris to amplify 2/3 of ToCSV [99/0631] were tested. The region targeted by these primers include the whole IR, AC1 and AC4 ORFs and parts of AV2, AV1 and AC2 ORFs of the isolate 99/0631. These primers were used to PCR amplify ToCSV [01/2521] DNA, hoping to amplify a DNA product that could possibly be used to design virus specific full-length DNA primers to amplify full-length ToCSV [01/2521] DNA-A. A DNA product estimated to be ~1.8kbp was obtained following PCR amplification with these primers and subsequently cloned and sequenced. The complete nucleotide sequence was determined to be 1508 bases in length. BLAST searches revealed no nucleotide sequence identity to any of the geminiviruses available in the GenBank, DDJB and EMBL nucleotide sequence database. Pairwise alignment of this sequence to ToCSV [99/0631] showed no significant sequence homology to the virus. Multiple alignment of this sequence to the DNA β satellite molecules mention section 2.8 revealed that none of the conserved sequences present in all DNA β molecules reported so far were present.

```
ORIGIN
1       CGATTTAAAA TGAAAAAAAA TCCGCGAGGA AAAGAGTTTT AGATTTTAGG AAAATCTTAT
61      TTCCGCCCGT GAAAAGGGTA TTAAAATTTA AAGAAAGTAA ATTTATTTTT AATGAAAAAA
121     AAAATTAGTG AAAGAGGGAT TTGAACTCGG GTTTACATCA AAATAATTGA AGAAAAAAGA
181     AATACCCTTA TGGAGAAATC AAACCCGGGC AAAGAGTAGG ATATCGCCAG CTATTACCAA
241     CGGTGCTGCG CCTTATTTAC AATTTTTAGG AGTCCAAATT CTATAGTATG TCAATGTTAA
301     ATTAAAATTA TACGTATATA TATAGCATAA TTTTTCGATG AAGACTGTCC TGGAGCCTAC
361     GTGGCTCTTC CCATAACAAA AGGAAAAATG TATTTTAACA TATTCGTGGC AAGTTCATTT
421     ATCACACATT TACTGGTGCA AAGAGAAATT GAAGACCTTC CGTTTTCAGG ATGCATGCAC
481     ATAGATTCCA GGACCACTAC TTATAATACA AGGCCAAATA CCTTCTCAGT GTCCAGAAAA
541     GCCATTCACC GCGTATTACG TTCATTAACT TGTTACATTA TTCATAGAAA CCTTGAATAC
601     ATAACTGTTT TTAACACTCA ACTATAAGTG GATATGGAAT TATCTGACAT TTAAAGATGA
661     AACAGCAGAA GCCACACAAA CAAAAGAAGC TTCTGTAGTA CTGGCACACT GCATATCAGT
721     ATATATTTAT GTTTGTATAT GTGATATGTA CTTGGACTAG GGTATATCTG TGGGGTTGGA
781     TTGAGCTAGA CTGTGGATAT TATATGAGGA TCCACAGTCT AGGTCTTGCT CCATGACGTG
841     GTTAGTTAGT TTCATGACTT TGCTTAGCTT TCAATTACAT AAAAAAAAAA TCTTTCATTA
901     TTTCAGTATC CTGAAAAAGA GATTCCATAT ACTCACTATT TTCGTAATTC ACGTCCTTAT
961     AAGTAGAAAG ATGTAAAAGG ATGAGTATCC AATTCACGCT AAGAAATAGA CGGGATGGTC
1021    TTAAACTATC AATAAAATCG AGTTAACATG TAGCCATCGT AATATATTTG ATACTTTCTT
1081    GAAAACAACT AAAGCGTGAG AAAACCACAC TCTATTTCTC CACTCGTTTA CGTCAATGCC
1141    TAATACAGCA ATACATATGT GCTAGACGAA TCTCTACGTT GGTACCAAAA AAATATACTG
1201    TTGGTAAAAC ATCTTGATCC AGGAAGCTAG TACTTCCATA ATTTGTCTCG ACCTATGCAT
1261    ACATATGAAA AAATGAATGA ATAGTGAATA CCCACATATA TTAGATTCAC CTCTCACTGA
1321    TCTAACTCTT TCTCTAGTTG CAATTTAACG ACATCCATGA GTGAGTCCAG TGGCAGGAAT
1381    TTTATCAAGG GTGTCCAAAC TTTTAATAAT AATTCCTTTG TTTTTTAAAA AAAAAATGAT
1441    AATTTAAACC ATCAAAACCA ATAATTAAGT TAAACAATAT TTAATTAAAA TATAAGAAAT
1501    TTCTTAAA
```

Figure 3.16. Complete nucleotide sequence of the 1508bp DNA fragment amplified with primers AC515 and AV1707 was cloned and sequenced. The complete nucleotide sequence was determined to be 1508bp long.

```
ORIGIN
1       CGCGCTATTA ATCGGATGGC CGCTTTGAAT CTGTTAAGGA ACTTGATGAA TAATAGGGAC
61      AATAAGAACG GCATATTTGA GTTAGTACTT GCTGCAAAAA TTTGTGCAGA GTATCCTAAC
121     TTATTGGAAG AAGGGATGGG GATTCCCGGA AAGTGCTCCC GAAAATTGAA GGGAAATGTA
181     ACCAGATGGC TAGTGTTGCA AAATGCTTAC TTGGTCTTCT ACTCTCAGGC CGATCTCGCA
241     CCATTGTGTC TGATTCTGAG AGAACCTCAA GATTGTGTGA GGCTCTTGAA TCACTTGACT
301     CGGCACATAA GATGACCGGA GGAAGAAATC CTAATGTTCT TTTCTATCTT AGTTTGGAGA
361     ATGCAGAGCA GAGAAAGTTG GACATTGCTC TCTATTATGC AAAGCAGCTA TTGAAGTTGG
421     AGGGGGGTTC TACTGTCAAA GGATGGCTTC TTCTAGCTCG CATACTATCT GCTCAAAAGA
481     GGTATATAGA TGCGGAAAGC ATAATTAATG CTGCACTAGA CGAAACAGGA AAATGGAATC
541     AAGGAGAACT GCTCCGCACT AAAGCTAAAC TACAGATTGC CCAGGGTCAT CTGCGGGATG
601     CCGTGGAGAC ATATACTCAT CTTCTTGCAG ACTCCAGGTT CAGAGAAAAA GTTTGGAGTC
661     ACAAAAAGTG TTAAGGTATG GTTCCTTGCA ATAGAGTCTC TTCAGCCATT ACATTGTTGA
721     ATGTTCAATC TGTCTTCAAT TTCTGAGTTT AGTCTTTCAA TTTATCCTAA GTTTCCCATC
781     TTTCATCAGT TGCATTGTTC ATTTTTCCCT TTGACCCCAC AGAACACGAG AGACAACAGT
841     AGAAGTTTAG AAATGGAAAC GTGGCACGAT CTAGCAAATG TATACACAAA CTTGTCTCAG
901     TGGCGTGATG CAGAGGTTTG CCTAATCAAA TCCGAGGCTA TCAATCCTCA TTCTGCCTCG
961     AGATGCCACT CTGCAGGTAT TACTTGCTTG ATTCATTTAT CTTGGCTTAT GTTCCCAACT
1021    TTTCTTATAA GATGTCTCTT GGAGTGTCAA AAGAATTCAG CACCATCCAT CAAAGTTTGA
1081    GGAGACACAA CATTTTTTAG ATTGCAATAA CAGAAGCAAA GGAGGAGTGT TAAAAATTGC
1141    TGATGTTAAG ATTTTTTTTT TGAAATTAGT ATTGTTGTAA TCCTCAGCAT TAAGGATGTG
1201    CTGGTGACCT CCAAAAATTA GTACATAGAG GGACTAGGAA GGCTAATTGC AAAGATCCTA
1261    GCATATCTAC TAGTTGGTCT ACCTCCTCTA TACCTTCCTG TTTACACCAA AAATACAAAT
1321    ATGCTATACA ATTTTCTCTA ACTTTCTGTA TGGAGTTTGA TTTGTTCTCA TAGCATCTTC
1381    CGTTTCTTTC CTTCCATATT AACCACCATA TGCAAGCTGG AATAATTCTC CACCACTTTT
1441    TTTTGGGGG
```

Figure 3.14. Complete nucleotide sequence of the 1449bp DNA fragment found in association with ToCSV [01/2521] was cloned and sequenced. The complete nucleotide sequence was determined to be 1449bp long.

```
ORIGIN
1       TTAATCGGAT GGCCGCTTGG TTCTCACCGA GTTGTTGTAC CTCTATATAG GGCCCTATGT
61      TGGGAGCCAA CTCATTGGCC TCATCCCGAA TGAGGCTAAT ATCCAATGTG TCAGGTAGGG
121     TCTTGATCAC ATCAATGTGC CATACGGGCA ATCCTGATGA CCTGGACAAC TCAAGGACCA
181     TGCACGGGAA CGGGTAAGTA GTAGTGGCCT TAAAGTCCCT CTAATGTATC ACTGCCTGCA
241     ACATCCACGT GAAGTCCACC TAAAATCCCT AACCATGACA GCCATCAGCA CTGCACAATC
301     CAAGGTGACA ATGTTATCAG TACCACTGGG GAAAACGCAG TGTTGCACCA GTAGCCATAA
361     GAATTTGGCT GTGAAGGTGA GGTTGGACAT CTTGATAATC CCATTCGGCT CCAGCACCCA
421     ACCATGAGCC TCACCATACA GGGAGAGGTG TTGTGCAATC CACCACTTAG TGGTCTCTCT
481     CACATGCGGC TCATGCGGGA ACCGTTCATC TTTGATCGAC TTCCAACGTT AGTCAAACTC
541     GGCGGTTAAG GGGGTCCTAG TGGCATCAAC GTCCATGCCA TAGAGAATTC TGCTAATAGA
661     GGCACGATTT GGACGACCTG TCCAACTGTG ATTGTACCGT CACCACATAA GAAAGGTAGA
721     ACTCTTGCAC ATTTATTCTC CAAAGGACAA AAATT
```

Figure 3.15. Complete nucleotide sequence of the 755bp DNA fragment found in association with ToCSV [01/2521] was cloned and sequenced. The complete nucleotide sequence was determined to be 755bp long.

ToCSV putative stem loop sequence and includes 5nts, TAATA, of the nonanucleotide sequence, located within this loop. The missing 4 nts of the nonanucleotide, TT↓AC, include the origin of replication (shown with the downward arrow). The 1449bp sequence also have sequences from the host plant *Lycopersicon esculentum*, 67nts aligned to *L. esculentum* chromosome 11 and 72nts aligned to *L. esculentum* CTR1-like protein kinase gene. The origin of the remaining 1449bp nucleotide sequence is unknown. The 755bp putative satellite sequence showed no sequence identity to any geminivirus sequences posted on the nucleotide sequence databases. Multiple alignments of the putative satellite molecules with a number of chosen DNA β molecules and defective DNA β molecules (section 2.8), showed no significant sequence identity. Alignments with DNA β molecules and defective DNA β molecules also showed that both putative satellite molecules do not contain the conserved sequences that are present in all DNA β molecules and defective DNA β molecules reported thus far (Briddon *et al*., 2003; Zhou *et al*., 2003). Both putative satellite sequences were also reversed and aligned, and still showed no sequence identity to any geminivirus sequences. Because we did not screen for the presence of DNA-B components, and thus do not know yet whether isolate 01/2521 is a monopartite or a bipartite begomovirus, we also compared and aligned these molecules to both DNA-A and DNA-B components of begomoviruses mentioned in section 2.8, and no significant sequence identity was observed. The putative satellite molecules were also aligned and compared to defective DNA molecules derived from both begomovirus DNA-A and DNA-B components respectively (section 2.8), and once again there was no significant sequence identity between these begomovirus sequences and the sequences of the putative satellite molecules.

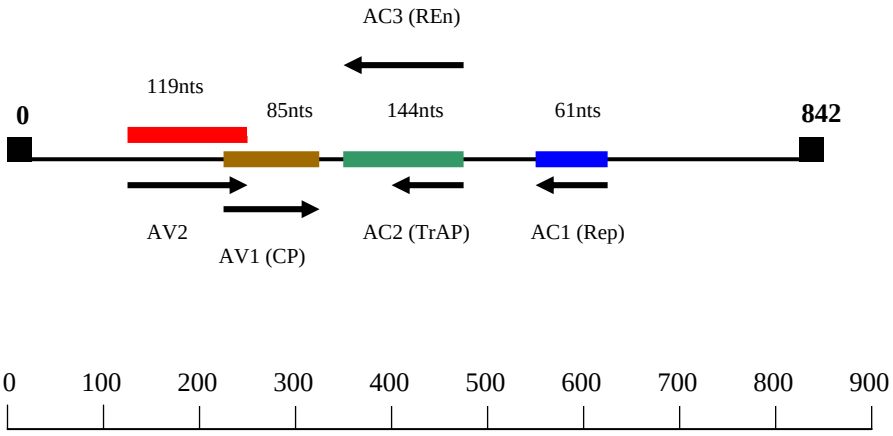

Figure 3.13. Graphical representation of the putative satellite DNA molecule, found associated with ToCSV [99/0631]. This chimeric molecule was determined to be 842nts in length, 408 nts are derived from ToCSV, the remaining nts are of unknown origin. The nts stretching from position 102-238 (red) are derived from ToCSV V2 gene, nts 236-321(brown) are derived from ToCSV V1 gene, nts 324-468 (green) are derived from the overlapping ToCSV C3 and C2 genes and nts 544-610 are derived from ToCSV C1 gene. The arrows denote the corresponding ToCSV ORFs.

3.6.2 Putative Satellite DNAs associated with ToCSV[01/2521]

The first evidence of the occurrence of unexpected DNA molecules in ToCSV [01/2521]-infected plants was obtained when DNA from tomato plants was amplified with primers AL1c2745 and PAR1v32, and estimated products of ~1.5kbp and 900bp were consistently obtained. These fragments were successfully cloned and sequenced in both orientations to obtain their full-length DNA sequences. The complete nucleotide sequence of the estimated ~1.5kbp band (Fig.3.4) was determined to be 1449 bases in length and the complete nucleotide sequence of the estimated ~900bp band (Fig.3.4) was determined to be 755 bases in length. Inspection of the 1449bp putative satellite molecule showed that it contains 22 nucleotide sequences between nucleotides 6 to 27, that showed 100% percent sequence identity to a region within the IR of ToCSV [99/0631], stretching from nts 2743 to 2764. Nucleotides 2743 to 2764 represent part of the

C

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Putative sat 1 ..................AAGGCCGCGCACCGGCCCCACA    565
ToCSV          caatactattgggtctccAAGGCCGCGCAgCGGCaCCACA   2000
Consensus                        aaggccgcgca cggc ccaca

Putative sat 1 CACATTCTCAAAGACCCACTTTTTCAGGTTCCTCAGGGAC    605
ToCSV          CACATTCTCAgAGACCCACTc.TTCAaGTTCCTCAGGGAC   2039
Consensus      cacattctca agacccact  ttca gttcctcagggac

Putative sat 1 TTGATTCAAAGGAGGATTGAGGAAAAGGGAGAATTTAACG    645
ToCSV          TTGATcaAAAGaAGa..TGAaGAAAAaGGAGAAaTatAaG   2077
Consensus      ttgat  aaag ag   tga gaaaa ggagaa t  a g
```

D

```
Putative sat 1 GTAGTGCGGA.TGTCAGAATGTGGGATCCACTGTTAAACG    123
ToCSV          GaAGTtaccAtTGTCAagATGTGGGATCCACTGTTAAACG    160
Consensus      g agt    a tgtca  atgtgggatccactgttaaacg

Putative sat 1 AATTCCCAGACTCTGTTCATGGGTTTCGTTGTATGCTTGC    163
ToCSV          AATTCCCAGACTCcGTcCAcGGtTTTCGTTGTATGCTTGC    200
Consensus      aattcccagactc gt ca gg tttcgttgtatgcttgc

Putative sat 1 TATAAAATACTTGCAGGTTATTGAGTCCACTTATGAGCCC    203
ToCSV          TgTtAAATAtcTGCAGtccgTTGAagCCACaTAcGAGCCC    240
Consensus      t t aaata  tgcag    ttga  ccac ta gagccc

Putative sat 1 AATACTTTGGGCCACGATCTTATACGAGATCT........    235
ToCSV          AAcACaTTGGGCCACGATCTTATACGAGATCTgattctgg    280
Consensus      aa ac ttgggccacgatcttatacgagatct
```

Figure 3.12B Nucleotide sequence alignment. Alignment of the nucleotide sequence of the 842bp putative satellite DNA molecule to ToCSV [99/031] DNA. Nucleotides of ToCSV [99/0631] that align to the putative satellite DNA are shown in uppercase. C: Shows alignment of the putative satellite DNA to a region within the C1 gene of ToCSV [99/063]. DNA-A. D: Shows alignment of the putative satellite DNA to a region within the V2 gene of ToCSV [99/0631].

all DNA β molecules and defective DNA β molecules identified and detailed in previous studies (Briddon *et al*., 2003; Zhou *et al*., 2003).

A

```
Putative sat 1   ..............ATGTGTCTCAACAACATCGATAGTG    348
ToCSV            attaaattttatttcATGTGTCTCAACAACATCGATAGTG   1120
Consensus                     atgtgtctcaacaacatcgatagtg

Putative sat 1   TTTACAAGTACATTATAAAGTACATGATCAACTGCTCTAA    388
ToCSV            TTTACAAGTACATTATAAAGTACATGATCAACTGCTCTAA   1160
Consensus        tttacaagtacattataaagtacatgatcaactgctctaa

Putative sat 1   TTACATTGTTAATTAAAATTACACCCAAATTATCTAAATA    428
ToCSV            TTACATTGTTAATTgAAATTACACCCAAATTATCTAAATA   1200
Consensus        ttacattgttaatt aaattacacccaaattatctaaata

Putative sat 1   CTTAAAAACTTGAAATTTAAATACGCTTAAGAAACGACCA    468
ToCSV            CTTAAAAACTTGAAATTTAAATACGCTTAAGAAACGACCA   1240
Consensus        cttaaaaacttgaaatttaaatacgcttaagaaacgacca

Putative sat 1   TCCCAAGGTTGTAAGGCCAACCAAACTGGGAATTTAAAAA    508
ToCSV            gtCtgAGGcTGTAAGGtCgtCCAgACTtGGAAgTTgAgAA   1280
Consensus          c  agg tgtaagg c  cca act ggaa tt a aa

Putative sat 1   AACATTTGGAATTCCCCATTTCCTTCCGGAAGCCA.....    543
ToCSV            AACATTTGtgAaTCCCCAgTTCCTTCCGGAgGttgtggtt   1320
Consensus        aacatttg  a tcccca ttccttccgga g
```

B

```
Putative sat 1   ....GATAGAAGGCCCTATGGAACAAGCCCAATGGACTTT    271
ToCSV            ccgaGATAGAAGGCCCTATGGAACAAGCCCAATGGACTTT    760
Consensus            gatagaaggccctatggaacaagcccaatggacttt

Putative sat 1   GGGCAGGTGTTTAACATGTTTGATAACGAGCCCAGAACAG    311
ToCSV            GGGCAGGTGTTTAACATGTTTGATAACGAGCCCAGtACAG    800
Consensus        gggcaggtgtttaacatgtttgataacgagcccag acag

Putative sat     CCACGGTGAATA............................    323
ToCSV            CCACGGTGAAgAacgatcttcgtgatcgattccaggtgtt    840
Consensus        ccacggtgaa a
```

Figure 3.12A Nucleotide sequence alignment. Alignment of the nucleotide sequence of the 842bp putative satellite DNA molecule to ToCSV [99/031] DNA. Nucleotides of ToCSV [99/0631] that align to the putative satellite DNA are shown in uppercase. A: Shows alignment of the putative satellite DNA to regions of the overlapping C2 and C3 genes of ToCSV [99/0631]. DNA-A. B: Shows alignment of putative satellite DNA to a region within the CP gene of ToCSV [99/0631].

3.6 Sequence Analysis of Putative Satellite DNA Molecules

3.6.1 Putative Satellite DNA Associated with ToCSV [99/0631]

Along with the genomic DNA, a putative satellite DNA molecule estimated to be ~900bp was amplified from diseased tomato plants with primers AL1c2745 and PAR1v32 located in the IR of ToCSV [99/0631] and was subsequently cloned into pBluescript (KS). The complete nucleotide sequence was determined to be 842 nts in length. BLAST search showed this sequence to have the highest nucleotide sequence identity to *Tomato curly stunt virus* (ToCSV, AF261885). Full optimal alignment of ToCSV with the 842nt molecule using DNAMAN version 4.0, showed the sequence identity between the two to be 48%. Eight possible ORFs on the plus strand and 7 possible ORFs on the minus strand were identified with DNAMAN. Pairwise alignment of ToCSV with the 842nt molecule revealed the highest percentage sequence identity to be over the following regions (Fig. 3.12A & B): nucleotides 1096 to 1242 (overlapping C3 and C2 genes) of ToCSV and nucleotides 324 to 468 of the putative satellite molecule, where 99% (144/145 nts) sequence identity occurs; nucleotides 725 to 810 (V1 gene) of ToCSV and nucleotides 236 to 321 of the putative satellite molecule, where 98% (85/86 nts) sequence identity occurs; nucleotides 1979 to 2044 (C1 gene) of ToCSV and nucleotides 544 to 610 of the putative satellite molecule, where 91% (61/67 nts) sequence identity occurs; nucleotides 139 to 275 (V2 gene) of ToCSV and nucleotides 102 to 238 of the putative satellite molecule, where 86% (119/137 nts) sequence identity occurs. Multiple sequence alignment of the putative satellite DNA with the DNA β molecules listed in section 2.8 showed no significant sequence identity. Multiple sequence alignment of this putative satellite DNA molecule with the defective DNA β molecules listed in section 2.8 also showed no significant sequence identity. Comparison of the putative satellite nucleotide sequences with the above mentioned DNA β molecules and defective DNA β molecules showed that the putative satellite molecule did not have the three absolutely conserved features (stem loop structure with the loop sequence TAA/GTATTAC, the satellite conserved region and the A rich region) present in

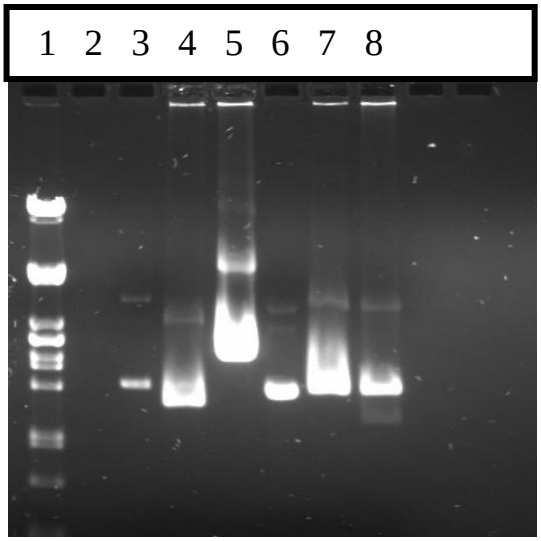


Figure 3.10 0.8% agarose gel of 5 clones (lanes 4-8) suspected of harboring the estimated ~900bp insert after transformation. Clones 3 (lane 5) appears to be migrating slower than the undigested pBS plasmid control (lane 3) and was chosen for further analysis. Lane 1: DNA molecular weight marker (HyperLadder I, BIOLINE)

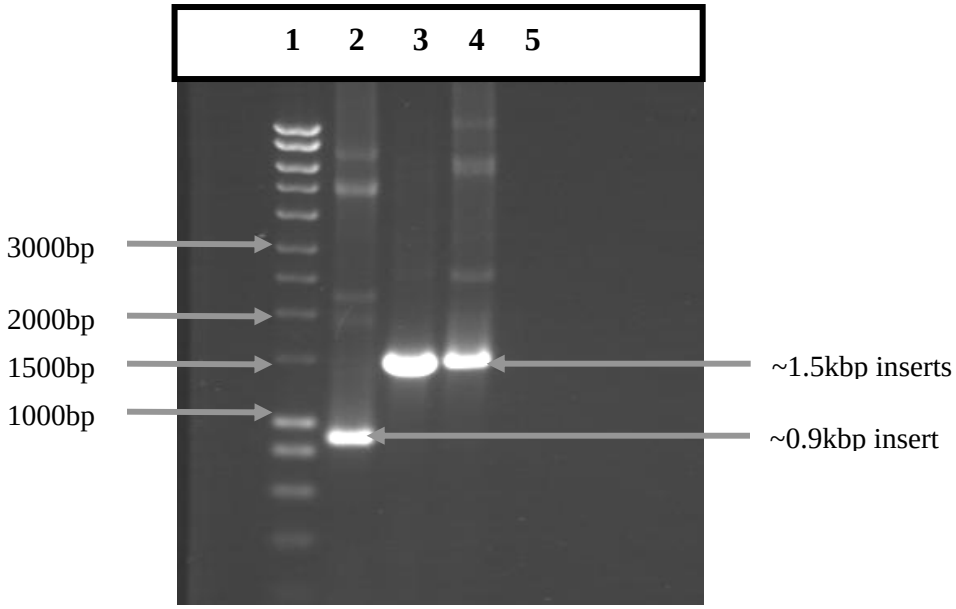


Figure 3.11. 0.8% agarose gel of PCR amplification product of clones suspected of harboring ~0.9kbp insert and ~1.5kbp insert respectively. DNA fragments of expected size were amplified from the different clones with the primer pair AL1c2745/PAR1v32. Lane 1: DNA molecular weight marker, HypeLadder I (BIOLINE). Lane 2: Fragment amplified from a clone suspected of harboring the ~0.9kbp insert. Lanes 3 and 4: Fragments amplified from clones suspected of harboring the ~1.5kbp insert. Lane 5: Negative control.

3.5.1 Screening of Recombinant Clones

Following transformation, 9 white colonies were selected for plasmid extraction to test which clones harbor the estimated ~1.5kbp DNA insert. Two clones (lanes 5 and 6), figure 3.9, appeared to be harboring inserts as they were migrating slower than the pBluescript (KS) vector (lane 2), figure 3.9 These clones were selected for further screening by PCR amplification with primers AL1c2745 and PAR1v32. The insert, ~1.5kbp (figure 3.11) was amplified from both clones with these primers.

Following transformation, 5 white colonies were selected white colonies were selected for plasmid extraction to test which clones harbor the estimated ~900bp DNA insert. When analyzed on 0.8% agarose gel, only one of the selected clones (lane 5), figure 3.10, appeared to be harboring the 900bp insert, as it was migrating slower than the undigested pBluescript (KS) vector (lane 3), figure 3.10. This clone was selected for further screening by PCR amplification with primers AL1c2745 and PAR1v32. The insert, ~900bp was amplified from clone 2 (figure 3.11) with these primers.

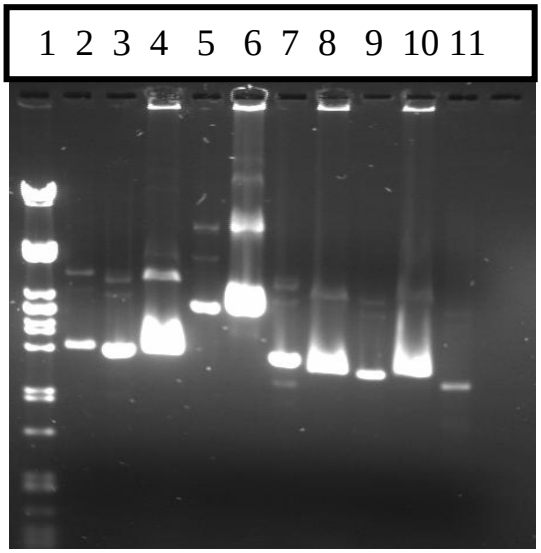


Figure 3.9 0.8% agarose gel of 9 clones(lanes 3-11) suspected of harboring the estimated ~1.5kbp insert after transformation. Clones 3 and 4 (lanes 5 and 6) appear to be migrating slower than the undigested pBS plasmid control (lane 2) and were chosen for further analysis. Lane 1: DNA molecular weight marker (HyperLadder I, BIOLINE)

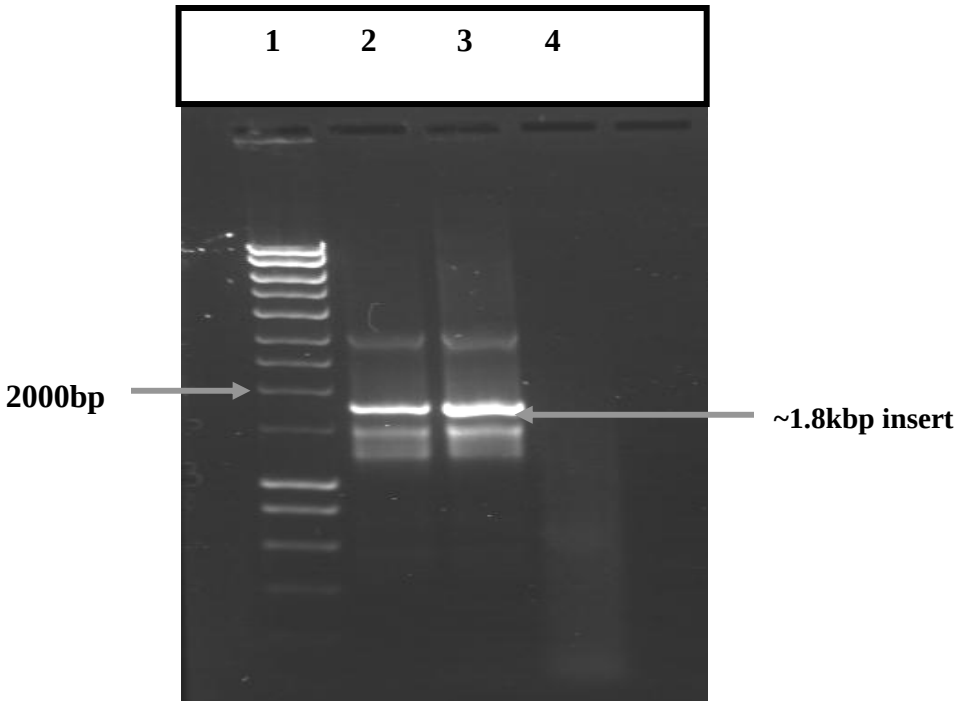


Figure 3.8 0.8% agarose gel of PCR amplification products of clones suspected of harboring the estimated ~1.8kbp insert. The insert was amplified from clone1 (lane 2) and clone 3 (lane 3), as indicated by the presence of the expected size fragments. Lane1: DNA molecular weight marker (HyperLadderI, BIOLINE). Lane4: Negative control.

3.5 Cloning of Putative Satellite DNAs

The plasmid vector was successfully extracted using the High Pure Plasmid Isolation kit (Roche). The vector was dephosphorylated and subsequently purified. The concentration was determined to be 40ng/µl by running on a 0.8% agarose gel alongside a concentration marker (HyperLadder I, BIOLINE) (results not shown).

The ligation and transformation were successful at ligation ratio 10:1 (insert:vector) and numerous white colonies were produced. The positive control produced a lawn of bacteria. No colonies were observed on the negative control.

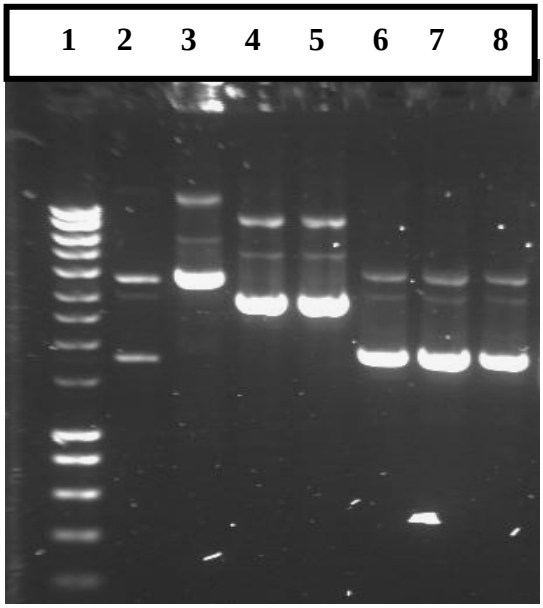


Figure 3.6 0.8% agarose gel of clones suspected of harboring the estimated ~1.8kbp insert after transformation. Clones 1, 2 and 3 (lanes 3-5) appear to be migrating slower than the undigested pBS plasmid control (lane 2). Lane 1: DNA molecular weight marker (HyperLadder I, BIOLINE)

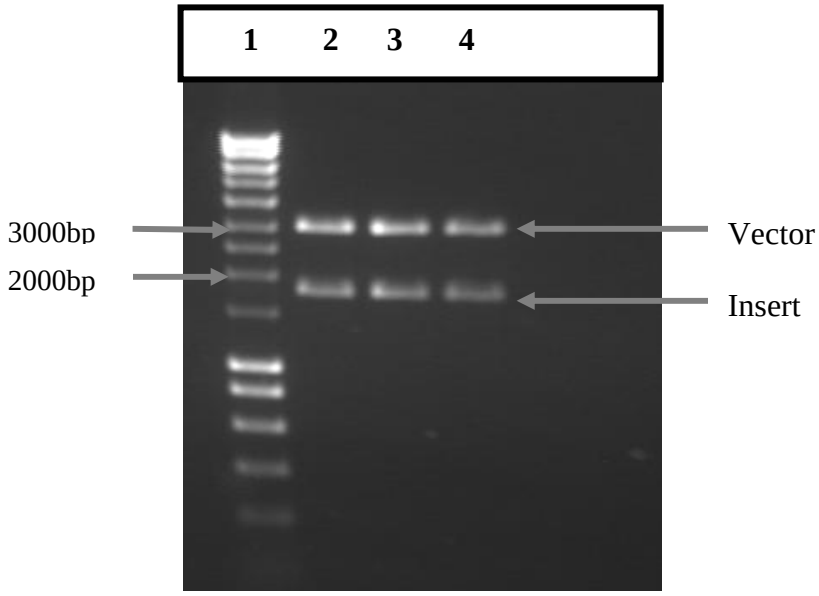


Figure 3.7 0.8% agarose gel of clones 1-3, suspected of harboring the estimated ~1.8kbp insert. The clones were double digested with *Eco*RI and *Bam*HI to try and cut the insert out. The insert was successfully cut out using these restriction enzymes from all three clones. Lane 1: HyperLadder I, Lane 2: Clone 1, Lane 3: Clone 2, Lane 3: Clone 3.

3.4 Cloning of Two/Third DNA-A fragment

The plasmid vector was successfully extracted using the High Pure Plasmid Isolation kit (Roche). Following dephosphorylation, the vector was purified and the concentration determined to be 20ng/µl by running on a 0.8% agarose gel alongside a concentration marker (HyperLadder I, BIOLINE) (results not shown).

The ligation and transformation were successful at ligation ratio 10:1 (insert:vector) and numerous white colonies were produced. The positive control produced a lawn of bacteria. No colonies were observed on the negative control

3.4.1 Screening of Recombinant Clones

Following transformation, 6 white colonies were selected for plasmid extraction. Three clones (lanes 3-5), figure 3.6, appeared to be harboring inserts as they were migrating slower than the pBluescript (KS) vector (lane 2), figure 3.6. These clones were selected for further screening by restriction digestion and PCR amplification. The clones were double digested with *Eco*RI and *Bam*HI, and the insert was successfully cut out from the cloning vector (figure 3.7) from all the chosen clones. The clones were also screened by PCR amplification using the primers AC515 and AV1707. The insert, estimated ~1.8kbp was amplified from the clones 1 and 3 (figure 3.10), using these primers.

3.3 Southern Blot Analysis

Southern blot analysis confirmed the presence of *Begomovirus* CCP in the total DNA extracted from ToCSV [01/2521]-infected tomato plants. *Begomovirus* CCP (~550bp) was detected from ToCSV [01/2521]-infected plant material via hybridization against DIG-labeled ToCSV [99/0631] CCP (Fig3.5, lane 1). The levels of ToCSV [01/2521] DNA (Fig3.5, lane 1) detected by southern blot were very low compared to ToCSV [99/0631] DNA levels (Fig3.5, lane 2). Hybridization of the probe to the positive control (ToCSV isolate 99/0631 CCP) produced a smear (Fig3.5, lane).

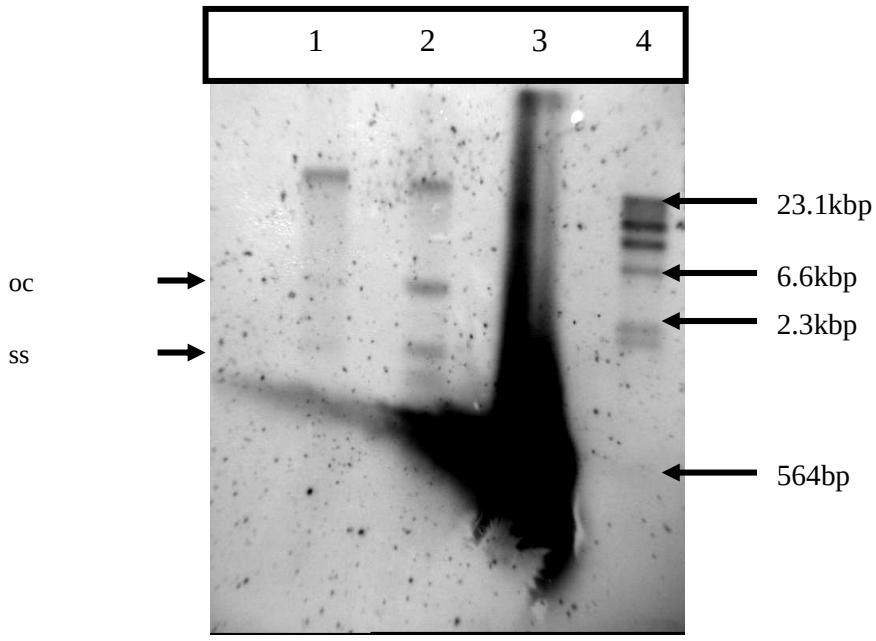


Figure 3.5. Southern blot analysis confirming the presence of *Begomovirus* CCP in total DNA extracted from ToCSV [01/2521]-infected plants. Equal amounts of total DNA were gel separated and blotted. *Begomovirus* CCP was detected via hybridization against DIG-labeled ToCSV [99/0631] CCP. Lane1: DNA from ToCSV [01/2521]-infected plants. Lane 2: DNA from ToCSV [99/0631]-infected plant. Lane 3: ToCSV [99/0631] CCP, positive control. Lane4: DIG-Labeled DNA molecular marker II. Left: Indication of viral DNA forms (oc= open circular, ss= single-stranded).

PCR master mix. PCR amplification of DNA stabbed from the ~1.5kbp band, yielded a fragment of similar size, lane 3, (figure 3.4A). PCR amplification of DNA stabbed from bands ~0.9kbp, ~2.7kbp and ~3.5kbp repeatedly gave products of ~0.9kbp (lanes 2, 4 and5), figure 3.4A, respectively. Both full-lenghth abutting primers FL-V and FL-C, and near-full length pimers AL1c2745 and PAR1v32 failed to re-amplify the estimated full-length DNA-A fragments of ~2.7kbp from both isolates using the band stab technique.

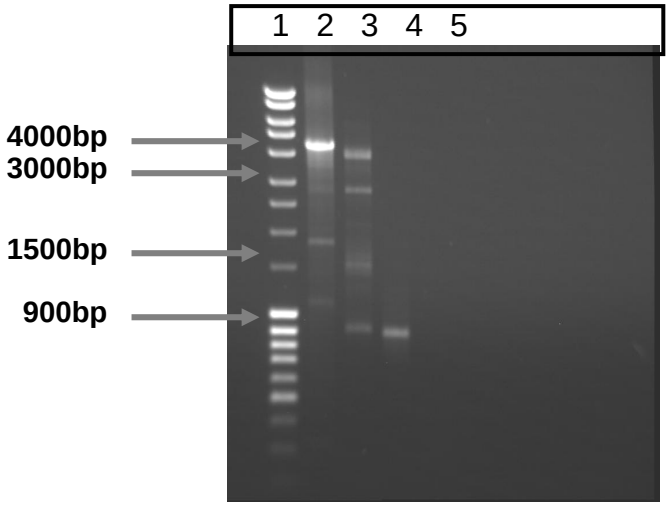


Figure 3.4 0.8% agarose gel of PCR amplification product with primers AL1c2745 and PAR1v32. Lane 1: DNA molecular weight marker (Fermentas), Lane 2: SACMC DNA-A in pBS, Lane 3: ToCSV [01/2521], Lane 4: ToCSV [99/0631], Lane 5: Negative control.

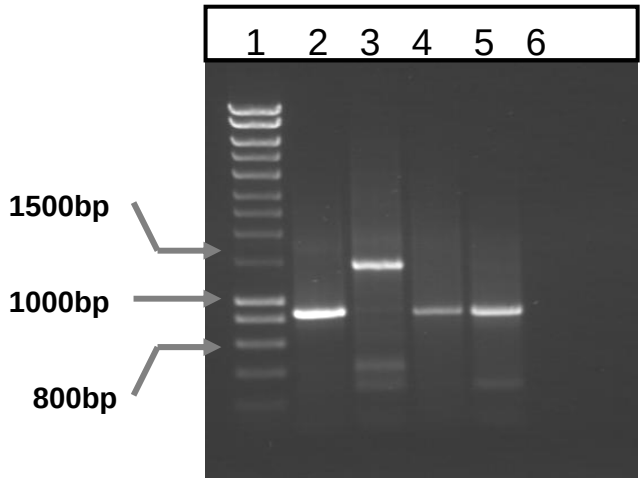


Figure 3.4A 0.8 % agarose gel of band-stab PCR re-amplification products from figure 3.3 using primers AL1c2745 and PAR1v32. Lane 1: DNA molecular weight marker, HyperLadder I (BIOLINE), Lane 2: amplified from ~0.9kbp fragment, Lane 3: amplified from ~1.5kbp fragment, Lane 4: amplified from ~2-7kbp fragment, Lane5: amplified from ~3.5kbp fragment. Lane 6: negative control.

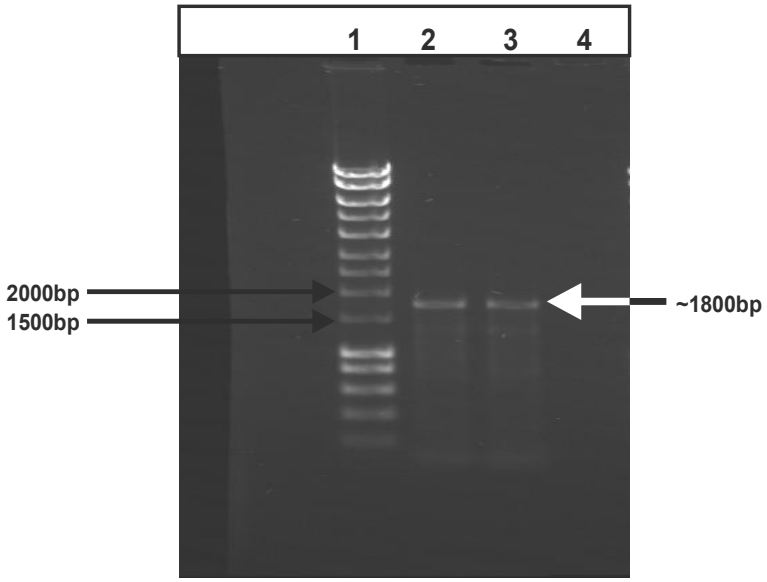


Figure 3.3 0.8% agarose gel of PCR amplification products obtained with two/third primers. Lane 1: DNA molecular weight marker, HyperLadder I (BIOLINE), Lane 2: Isolate 99/0631 (positive control), Lane 3: Isolate 01/2521, Lane 4: Negative control. Lanes 2 and 3 show amplification of a ~1800bp band, the expected DNA fragment size

3.2.4 Abutting Primers and Near-full length Primers

Primers AL1c2745 and PAR1v32 were designed to amplify almost the whole sequence of ToCSV [99/0631] DNA-A, except for a few residues of the intergenic region, to give ~2.7kbp product. When DNA from tomato plants infected with ToCSV [99/0631] was PCR amplified with these primers, products of ~2.7kbp and ~0.9kbp were amplified, although the ~2.7kbp band was too weak to reproduce photographically (Figure 3.4). The estimated ~0.9kbp fragment was unexpected. PCR amplification of DNA from tomato plants infected with ToCSV [01/2521], yielded four different size fragments (Figure 3.4), ~0.9kbp, ~1.5kbp, ~2.7kbp and ~3.5kbp. Only the estimated ~2.7kbp fragment was expected. The four fragments amplified from ToCSV [01/2521]-infected plants were subsequently individually PCR amplified utilizing a band-stab technique, where a small pipette tip is stabbed into the desired band and then into a tube containing

JSP001/JSP003 and JSP001/JSP003 designed to amplify the full CP region of ACMV and EACMV, respectively, did not yield any DNA product when analyzed on a 0.8% agarose gel (Results not shown). The expect DNA fragment size is ~750bp.

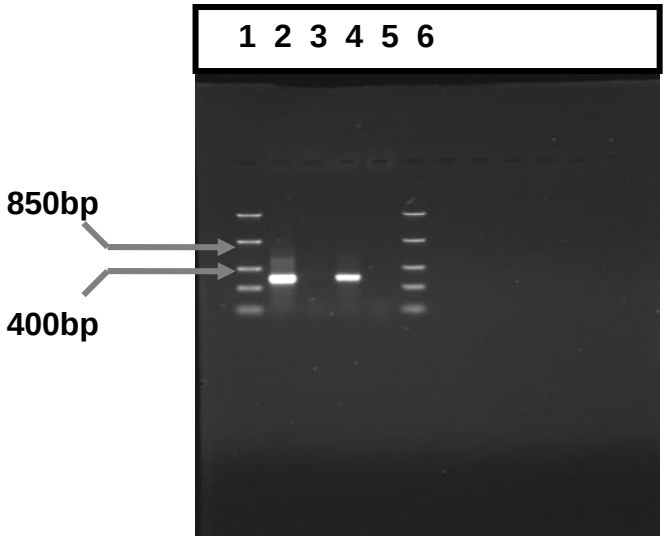


Figure 3.2 0.8% agarose gel of PCR amplification products obtained with CCP primers. Lanes 1 and 6: Middle Range DNA molecular weight marker (Fermentas), Lane 2: SACMV DNA-A in pBS (positive control), Lane 3: ToCSV [01/2521] Lane 4: ToCSV [99/0631], Lane 5: Negative control. Lanes 2 and 4 show amplification of ~570bp DNA fragment. Lanes 5 and 6 show no amplification.

3.2.3 Two/Third DNA-A Primers

TNA samples extracted from tomato plants infected with ToCSV isolates 99/0631 and 1252/10 respectively, were PCR amplified with virus specific primers AC515 and AV1707 designed to amplify 2/3 DNA-A of ToCSV [99/0631]. When analyzed on a 0.8% agarose gel (Figure 3.3), amplification from TNA samples infected with ToCSV [99/063] and ToCSV [01/2521] resulted in DNA fragments estimated to be ~1.8kbp.

expected PCR product size (~550bp) was amplified (figure 3.1). TNA samples extracted from young, symptomatic tomato leaves, infected with ToCSV [01/2521] were screened for the presence of begomovirus DNA by PCR with degenerate primers, AV1c1048 and AV1v514. No amplified product was detected (figure 3.2). The degenerate primer pair AV1c1048/AV1v514 was also used to screen a number of symptomatic tomato leaves, collected from Mozambique, for the presence of begomovirus DNA by PCR. Bands of expected PCR product size were not amplified (Results not shown)

Table 3.1. Characterization of begomovirus infections by PCR

Viruses	CCP Primers	CP Primers	Universal Abutting Primers	Two/Third DNA-A Primers	Near-full Length Primers
ToCSV (01/2521)	-	-	-	+ ~1800bp	+ ~2700bp
					+~3500bp
					+ ~1500bp
					+ ~900bp
ToCSV (99/0631)	+ ~550bp	-	-	+ ~1800bp	+ ~900bp
					+ ~2700bp

PCR results were scored as positive (+) where samples and positive control gave positive reactions, and negative (-) where the samples gave a negative reaction while the positive control gave a positive reactions. Positive (+) results are followed by the size fragments obtained.

3.2.2 Full Coat Protein (CP) Primer PCR

PCR amplification on TNA samples extracted from tomato plants infected with ToCSV, isolates 99/0631 and 01/2521, using virus specific primer pairs

CHAPTER THREE

Results

3.1 Total Nucleic Acid Extractions

TNA was successfully extracted from young leaves of ToCSV-infected tomato plants. The TNA concentrations were determined by fluorometry to be 20ng/µl.

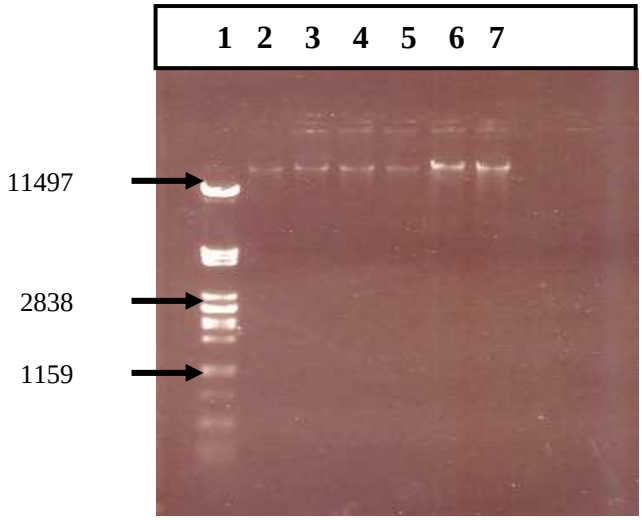


Figure 3.1 0.8% agarose gel of TNA extracted from infected tomato leaves.
Lane1: Molecular weight marker (Pst1). Lanes 2 & 3: TNA extracted from tomato leaves infected with ToCSV [99/0631]. Lanes 4 & 5: TNA extracted from tomato leaves infected with ToCSV [01/2521]. Lanes 6 & 7: TNA extracted from tomato leaves infected with ToCSV [Moz1].

3.2 Begomovirus Detection in the Symptomatic Plant Material

PCR amplification results with the various primers are summarized in table 3.1

3.2.1 PCR Amplification with Core Coat Protein (CCP) Primers

TNA samples extracted from young symptomatic tomato leaves, infected with ToCSV [99/0631] were screened for the presence of begomovirus DNA by PCR with degenerate primers AV1c1048 and AV1v514. A band corresponding to the

for bombardment. Fifty microlitres of the resuspended microparticles was used to bombard a single plant. The microparticles were accelerated by helium pressure at 1300psi. The meristem of the following three week old test plants were bombarded: pepper, pumpkin, cowpea, eggplant, okra, cotton, malva, *Nicotiana tabacum*, bean and tomato (positive control). Experimental control plants were inoculated with tungsten and sterile distilled water only. Plants were examined for symptom development 7, 14, 21, 28 and 35 days post-inoculation. Positive virus inoculation was confirmed by PCR using core CP primers to amplify the diagnostic ~550bp of the CP.

2.9 Host Range Study

2.9.1 Preparation of DNA for Biolistic inoculation

A total of nine test plants, representing four different plant families, *Solanaceae*, *Malvacea*, *Leguminosae* and *Cucurbitaceae*, were tested for their susceptibility to ToCSV [99/0631]. Numerous seeds were sown per plastic pot and later thinned to one seedling per pot. At least four seedlings of each test plant were biolistically inoculated. Total DNA was chosen as viral source because full-length ToCSV [99/0631] DNA-A clones were not available. TNA was extracted from young tomato leaves, infected with ToCSV [99/0631] as described in 2.2. Samples were analyzed on a 1% agarose gel and the TNA concentration determined by fluorometry.

2.9.2 Biolistic Inoculation of Indicator Plants

Experimental host range study was conducted by biolistic bombardment of test plants to determine the host range of ToCSV [99/0631]. Biolistic inoculation of indicator plants was carried out in a vacuum chamber, constructed by ARC, Roodeplaat. The mechanism of particle bombardment involved the acceleration of tungsten particles through a partial vacuum under the pressure of helium gas. Two micrograms of RNaseA-treated TNA from infected tomato plants was used per plant. Four plants were used per experiment using a single reaction. The particle bombardment procedure used 5mg tungsten particles to which 250µl 100% ethanol was added and vortexed prior to incubation on ice for 5 minutes. The suspension was subsequently vortexed and centrifuged briefly to collect the particles. The ethanol was aspirated and the tungsten particles washed by resuspending in 500µl of sterile distilled water, vortexing and centrifuging as above. The wash step was repeated once. Fifty microlitres of sterile distilled water was then added to the pelleted tungsten particles and TNA, 50µl 2.0M $CaCl_2$ and 45µl spermidine were added sequentially to the tungsten particle while vortexing the particle during and between each addition. After incubation on ice for 10 minutes, particles were collected by centrifugation. The supernatant was aspirated and the pellet containing the microparticles resuspended in 100µl 100% ethanol

AJ457819) and Y25 (TYLCCNVβ-Y25, AJ421619), BYVMV from India (BYVMVβ, AJ308425), ToLCNDV (ToLCNDVβ, AY438562), okra leaf curl disease (OLCDβ, AJ316029), ToLCV satellite from Australia (ToLCV-sat; NC_002743.

The GeneBank database accession numbers of the presumed defective DNA β molecules used for comparison were: Ageratum yellow vein disease associated defective DNA β molecule (AYVDβΔ01-Ind, AJ316042), Okra yellow vein disease associated defective DNA β molecule (OYVDβΔ01-Egy) and Okra leaf curl disease associated defective DNA β molecule (OLCDβΔ01-Egy).

The database accession numbers of begomovirus DNA-A sequences used for comparison are as follows: *Tomato curly stunt virus* (ToCSV; AF261885), *African cassava mosaic virus* (ACMV; J02057), *South African cassava mosaic virus* (SACMV; AJ575560), *East African cassava mosaic virus* (EACMV; AF126807), *Ageratum yellow vein virus* (AYVV, X74516), *Tomato leaf curl virus* (ToLCV; S53251), *Tomato leaf curl Bangalore virus* (ToLCBV; Z48182). *Tomato yellow leaf curl virus* from Egypt (TYLCV, AY594174).

The database accession numbers of begomovirus DNA-B sequences used for comparison are as follows: *South African cassava mosaic virus* (SACMV; AF155807), *African cassava mosaic virus* (ACMV; J02058); *East African cassava mosaic virus* (AF126807), *Tomato golden mosaic virus* (TGMV; X04485) and *Tomato mottle Taino virus* (ToMV; AF012301).

Database accession numbers of defective molecules derived from begomovirus DNA-A used for comparison are as follows: those derived from AYVV (Y14167; Y14168), *Tobacco leaf curl Zimbabwe virus*, isolate HG (AF368275), *Tobacco leaf curl Zimbabwe virus*, Mild isolate (AF368274). Database accession number of defective molecule derived from begomovirus DNA-B is as follows: *Tomato golden mosaic virus* subgenomic DNA derived from DNA-B (X04485).

2.7 Sequence Determination

Transformed clones, confirmed by PCR amplification or PCR amplification and restriction digestion for the presence of putative satellite DNA molecules associated with ToCSV [01/2521] and ToCSV [99/0631] respectively, were sent to Inqaba Biotechnical, in Pretoria, South Africa for sequencing. All clones were sequenced from both orientations.

2.8 Sequence Comparison

Sequence data were assembled and analyzed with the aid of DNAMAN Version 4.0 software (Lynnon Biosoft, Quebec, Canada). Because the full-length ToCSV [01/2521] DNA was not amplified, a few begomoviruses whose nucleotide sequences are available in GenBank, were chosen to compare with the small DNA molecules amplified from ToCSV-infected leaf material. The B components of some bipartite begomovirses were chosen because at this stage it was not yet known whether the so called ToCSV isolate [01/2521], was indeed a different ToCSV isolated, and therefore a monopartite begomovirus. Because of the size of these unexpected DNA molecules, it was suspected that they could possibly be DNA β satellite molecules, defective DNA β molecules or subgenomic molecules derived from DNA-A or DNA-B, or they could also be defective interfering (DI) DNA molecules. Therefore DIs, subgenomic molecules derived from DNA-A and those derived from DNA-B and full-length and defective DNA β molecules were chosen from the GenBank database to compare with the small DNA molecules from ToCSV-infected leaf material.

The GenBank database accession numbers of the DNA β molecules used for comparison were: DNA β of AYVV (AYVVβ, AJ252072); BYVMV (BYVMVβ, AJ3008425); CLCuMV from Pakistan (CLCuMVβ-01, AJ292769; CLCuMVβ-02, AJ298703); tobacco leaf curl disease (TobLCDβ01-Pak, AJ316033; TobLCDβ02-Pak, AJ316034); tomato leaf curl disease (TomLCDβ01-Pak, AJ316035; TomLCDβ02-Pak, AJ316036); TYLCCNV isolates Y8 (TYLCCNVβ-Y8, AJ421622), Y10 (TYLCCNVβ-Y10, AJ421621), Y88 (TYLCCNVβ-Y88,

Ligation Buffer (Rapid DNA Ligation kit, Roche) and 1µl T4 DNA Ligase (Rapid DNA Ligation kit, Roche). Ligations were carried out in a waterbath at 18°C for 5 minutes.

E. coli DH5α cells were transformed with 5µl of the ligation reaction according to the Transformation and Storage Buffer (TSB) method of Chung and Miller (1988). Transformed cells were plated onto LB plates containing 100µl/ml ampicillin, 0.9 mg X-gal and 0.8 mg IPTG. Undigested pBluescript (KS) was transformed into cells and plated as a positive control. Linearised vector was re-ligated and transformed into *E. coli* cells as a ligation control, and linearized and dephosphorylated vector was re-ligated and transformed into *E. coli* cells as confirmation of total dephosphorylation of the vector. Water was used in the transformation as a negative control. Plates were incubated at 37°C for 17 hours and then at 4°C for at least 24 hours to eliminate false positives and to distinguish the blue and white selection colours.

2.6.4 Screening for Transformants

White colonies were picked off the plates and inoculated into 4ml LB broth containing ampicillin (100µg/ml) and incubated at 37°C with shaking at 200 rpm overnight. The plasmid DNA was extracted using an alkaline lysis based plasmid extraction method by Birnboim and Doly (1979) as described in 2.5.4. A sample of each clone was run on a 0.8% agarose gel alongside pBluescript (KS) vector to identify slower-migrating plasmids. Two methods were employed to screen for slower-migrating plasmids: Restriction analysis and PCR amplification. Transformants were screened by performing a double digest with *EcoR*I and *BamH*I in Buffer E (Promega) at 37°C for 3 hours to cut out the insert. Transformants were also screened by PCR amplification using the primers AC515 and AV1707 as described in 2.3.2.

A sample of each clone was run on a 0.8% agarose gel alongside pBluescript (KS) vector to identify slower-migrating plasmids. Two methods were employed to screen for slower-migrating plasmids: Restriction analysis and PCR amplification. Transformants were screened by double digestion with restriction enzymes *Eco*RI and *Bam*HI in Buffer E (Promega) at 37°C for 3 hours to cut out the insert. Transformants were also screened by PCR amplification using the primers AL1c2745 and PAR1v32 as described in 2.3.3.

2.6 Cloning of two/third DNA-A

2.6.1 Preparation of Insert DNA for Cloning

During PCR-mediated amplification of ToCSV [01/2521]-infected plants with primers AC515 and AV1707, a DNA fragment estimated to be ~1.8kbp was obtained. This fragment was prepared for cloning as described in 2.5.1.

2.6.2 Preparation of Vector for Cloning

The vector used in this cloning procedure is pBluescript (KS). The vector was prepared for cloning as described in 2.5.2.

2.6.3 Cloning of two/thirds DNA-A into pBluescript (KS)

Ligation reactions were based on a pmol ligation ratio of 5:1, 7:1 and 10:1 (insert:vector). The amount of vector and insert needed for these ratios was calculated using the formula:

$$\frac{\text{Vector concentration (ng) x insert length (kb) x 5 (or 10)}}{\text{Vector length (kb)}}$$

The 20µl blunt-end ligation reactions contained 10ng of vector, the appropriate concentration of insert as determined by the relevant ligation ratio, 10µl T4 DNA

E. coli XL1 Blue cells were transformed with 10µl of the ligation reaction mix according to the Transformation and Storage Buffer (TSB) method of Chung and Miller (1988). Transformed cells were plated onto LB plates containing 100µl/ml ampicillin, 0.9 mg X-gal and 0.8 mg IPTG. Undigested pBluescript (KS) was transformed into cells and plated as a positive control. Linearised vector was re-ligated and transformed into *E. coli* cells as a ligation control, and linearized and dephosphorylated vector was re-ligated and transformed into *E. coli* cells as confirmation of total dephosphorylation of the vector. Water was used in the transformation as a negative control. Plates were incubated at 37°C for 17 hours and then at 4°C for at least 24 hours to eliminate false positives and to distinguish the blue and white selection colours.

2.5.4 Screening of Transformants

White colonies were picked off the agar plates and inoculated into 4ml LB broth containing ampicillin (100µg/µl) and incubated at 37°C with shaking at 200 rpm overnight. The plasmid DNA was extracted using an alkaline lysis based plasmid extraction method by Birnboim and Doly (1979) with minor modifications. A 1.5ml aliquot of the overnight culture was centrifuged at maximum speed (14000 rpm) at 4°C for 3 minutes. The supernatant was poured off and the pellet resuspended in 100µl of ice-cold lysis buffer (50mM glucose, 50mM Tris pH 8.0 and 10mM EDTA) and incubated at room temperature for 5 minutes. Two hundred microlitres of freshly prepared detergent buffer (0.2N NaOH and 1% SDS) was added, mixed by inversion and incubated on ice for 10 minutes. Following incubation, 150µl of potassium-acetate containing buffer (5M potassium-acetate and 11.5% glacial acetic acid) was added, mixed by inversion and incubated for 10 minutes on ice. The tubes were centrifuged at maximum speed for 10 minutes and the supernatant transferred to a new tube. DNA was precipitated by adding 0.5 volumes of sodium acetate and 3 volumes of ice-cold 100% ethanol and incubation at -70°C for 1 hour. Following the incubation, DNA was pelleted by centrifuging at 4°C for 20 minutes. The pellet was vacuum dried and resuspended in 14µl TE buffer (10mM Tris, pH 8.0, 1mM EDTA) containing 20µg.ml RNaseA.

resuspended in 10µl sterile distilled water. A sample (1µl) was run on agarose gel with a concentration marker.

The linearized vector (250ng) was dephosphorylated using 2U Calf Intestinal Phosphotase (CIP) enzyme in a 10x CIP Dephosphorylation Buffer (Roche) at 37°C for 60 minutes. The reaction was stopped by incubating at 37°C for 10 minutes. Dephosphorylation of the vector prevents vector ends from being religated by T4 DNA Ligase during Ligation reactions. The vector was purified using phenol-chloroform and precipitated with 0.1 volume 3M sodium acetate and 3 volumes 100% ice cold ethanol. The pellet was resuspended in 5µl 1x dilution buffer (Rapid DNA Ligation kit, Roche). Samples were analyzed on a 0.8% agarose gel.

2.5.3 Cloning of Putative Satellite DNAs into pBluescript (KS)

Ligation reactions were based on a pmol ligation ratio of 5:1 and 10:1 (insert:vector). The amount of vector and insert needed for these ratios was calculated using the formula:

$$\frac{\text{Vector concentration (ng) x insert length (kb) x 5 (or 10)}}{\text{Vector length (kb)}}$$

The 20µl blunt-end ligation reactions contained 10ng of vector, the appropriate concentration of insert as determined by the relevant ligation ratio, 10µl T4 DNA Ligation Buffer (Rapid DNA Ligation kit, Roche) and 1µl T4 DNA Ligase (Rapid DNA Ligation kit, Roche). Ligations were carried out in a waterbath at 18°C for 30 minutes.

associated with genomic DNA, even though their origin and biological function was unknown at this stage.

2.5.1 Preparation of Insert DNA for Cloning

Insert DNA (1.5kbp and 0.9kbp) was amplified by PCR using primer pair AL1c2745/PAR1v32. DNA was isolated from a low melting temperature agarose gel (LMT) using the QIAquick Gel Extraction kit (QIAGEN) as per manufacturer's instructions. Inserts were phosphorylated using 2U T4 Polynucleotide Kinase (PNK) (Fermentas) and 10x PNK Buffer A (Fermentas) at 37°C for I hour. The reaction was stopped by incubating at 70°C for 10 minutes. Inserts were purified with the High Pure PCR Purification kit (Roche), precipitated (0.1 volume 3M acetate and 3 volumes ice-cold 100% ethanol) and resuspended in 5µl 1x dilution buffer (Rapid DNA ligation kit, Roche).

2.5.2 Preparation of Vector for Cloning

The vector used in this cloning procedure is pBluescipt (KS). This vector contains an ampicillin antibiotic resistant gene, a *Lac Z* gene, gene for blue/white colour selection and a multiple cloning site (MCS) which includes a *Sma*I restriction enzyme site. The vector pBluescript (KS) was isolated from an overnight culture of *Escherichia coli* XLI Blue (Stratagene) cells using the High Pure Plasmid Isolation kit (Roche). This kit employs an alkaline lysis method (Birnboim and Doly, 1979). Alkaline lysis releases plasmid DNA from bacteria. In the presence of the chaotropic salt guanidine-HCL, plasmid DNA binds selectively to special glass fibers pre-packed in the High Pure filter tubes that come with the kit. After a series of rapid wash-and-spin steps, to remove contaminating bacterial components, low salt elution releases bound plasmid DNA from the glass fiber. The extracted plasmid DNA was analyzed by agarose gel electrophoresis. The vector was linearized with 10U of *Sma*I in 1x Buffer J (Promega) at 30°C for 3 hours. The linearized plasmid DNA was purified on a low-melting temperature agarose gel as described by Favre (1992). Pellets were vacuum dried and

T_{opt} = Tm – 20 to 25 ^{o}C [T_{opt} = hybridization temperature]

2.4.5 Post-hybridization Stringency Washes

First, the membrane was washed for two consecutive 5 minutes in 100 ml 2x SSC, 0.1% SDS at room temperature with shaking, and then washed for two consecutive 15 minutes in 100ml 0.5x SSC, 0.1% SDS (pre-warmed) at 65°C under constant agitation.

2.4.6 Immunological detection

After post-hybridization and stringency washes, the membrane was rinsed in 50ml washing buffer (0.1M maleic acid, 0.15M NaCl, 0.3 % (v/v) tween 20) for 5 minutes. The membrane was incubated for 30 minutes in 100ml blocking solution (Roche) and 20ml antibody solution (Roche), respectively followed by two consecutive 15 minute washes in 100ml washing buffer. The membrane was equilibrated for 5 minutes in 20ml detection buffer (0.1M Tris-HCL, 0.1M NaCl, pH 9.5). The membrane was incubated in 10ml color substrate solution (Roche) in a small container covered with foil until desired band intensities were achieved. The reaction was stopped by washing the membrane for 5 minutes with 50ml TE-buffer.

2.5 Cloning of Putative Satellite DNAs

During an attempt to amplify near-full length (~2.7kbp) DNA from ToCSV [01/2521]- and ToCSV [99/0631]- infected plants, with primers AL1c2745 and PAR1v32, amplicons of unexpected size were repeatedly amplified. Two were found in association with ToCSV [01/2521] (~900bp and ~1.5kbp) and one was found in association with ToCSV [99/0631] (~900bp). These small DNA molecules, were isolated from 1% low melting temperature agarose gel. We proceeded to clone these fragments. At this point we decided to temporarily call these small DNA molecule putative satellite DNA molecules because they are

DNA was capillary transferred to a nylon membrane (Amersham Hybond-N) according to a method adapted from Sambrook *et al*. (1989). Samples were run on a 0.8% agarose gel, free of ethidium bromide for 2 hours. DNA samples were denatured by incubating the gel in 0.25M HCL for 15 minutes followed by two consecutive 30 minutes incubations in denaturation buffer (1.5M NaCl, 0.5 N NaOH) at room temperature with shaking.. The gel was rinsed in sterile distilled water and subsequently incubated in neutralization buffer (1.5M NaCl, 0.5N NaOH) for two consecutive 30 minutes at room temperature with shaking. The DNA was allowed to transfer from the gel onto the nylon membrane overnight (18 hours) in the presence of 20x SSC blotting buffer (3M NaCl, 0.3M trisodium citrate, pH 7.0).

2.4.3 DNA Fixation

The membrane was placed on a Whatman 3MM paper, pre-soaked in 10x SSC, DNA side down and UV-crosslinked for 5 minutes without prior washing. After the UV-crosslinking, the membrane was rinsed briefly in double distilled water and allowed to air-dry for 1 hour.

2.4.4 Hybridization

Hybridization solution (DIG Easy Hyb, Roche) was pre-heated to hybridization temperature (47°C). The nylon membrane, with fixed DNA was pre-hybridized at 47°C for 30 minutes with gentle agitation in a small container. DIG-labeled DNA probe was denatured by boiling for 5 minutes followed by rapid cooling on ice. Denatured probe was then added to the pre-heated hybridization solution and mixed well while trying to avoid foaming (bubbles lead to background). Pre-hybridization solution was poured off and the probe/hybridization mixture was added to the membrane and incubated in a hybridization chamber at 47°C with gentle agitation, overnight. The hybridization temperature was calculated according to GC content and percent homology of probe to target according to the following equation:

$T_m = 49.82 + 0.41(\%G + C) – (600/L)$ [L = length of hybrid in base pairs]

sequence which stretches between nucleotides 2760 and 2 within the genome of ToCSV [99/0631] (GenBank accession no AF261885). Total nucleic acid preparations (100ng) of ToCSV isolates were added to each tube containing PCR master mix and a total volume of 50µl. The master mix contained 10mM dNTPs, 3.75U Expand Long Template Enzyme mix (Roche), 5U 10x Expand Long template buffer with 17mM MgCl2 (Roche) and 20pmol of each primer. Experimental controls included a complete PCR master mix to which was added 100ng SACMV DNA-A in pBS as a positive control and a PCR master mix minus DNA as a negative control. PCR was carried out in a thermal cycler (BIO-RAD). PCR reaction consisted of an initial cycle of 2 min at 94°C followed by 10 cycles of 10 sec at 94°C, 30 sec at 52°C and 2 min at 68°C; 20 cycles of 15 sec at 94°C, 30 sec at 52°C, 3 min at 68°C and a final extension at 68°C for 10 min. PCR amplified products were electrophoresed at 50mA through a 0.8% (w/v) agarose gel in 1X TAE electrophoresis buffer and visualized under UV after staining with in µg/ml ethidium bromide.

2.4. Southern Blot Analysis

When a ~550bp DNA fragment repeatedly failed to PCR amplify from ToCSV [01/2521]-infected plants, using degenerate CCP primers AV1c1048 and AV1v514, southern blot analysis was employed to ascertain whether these plants were indeed infected with a begomovirus. TNA samples, containing ToCSV [01/2521] DNA, were analyzed by southern blotting with the probe constructed in 2.4.1 according to a method adapted from Sambrook *et al*. (1989).

2.4.1 Probe Construction

A probe to detect *Begomovirus* DNA-A was constructed. The core coat protein obtained by PCR-mediated amplification of ToCSV [99/0631] DNA, was randomly prime labeled using a non-radioactive Digoxigenin-dUTP DNA labeling and detection kit (Roche) according to the manufacturer's instructions.

2.4.2 DNA Transfer

Figure3: Begomovirus DNA-A. The diagram illustrates the binding sites of primers AC515 and AV1707. The dotted lines between the primers illustrate the area of ToCSV genome targeted and amplified during PCR amplification using the primer set AC515/AV1707.

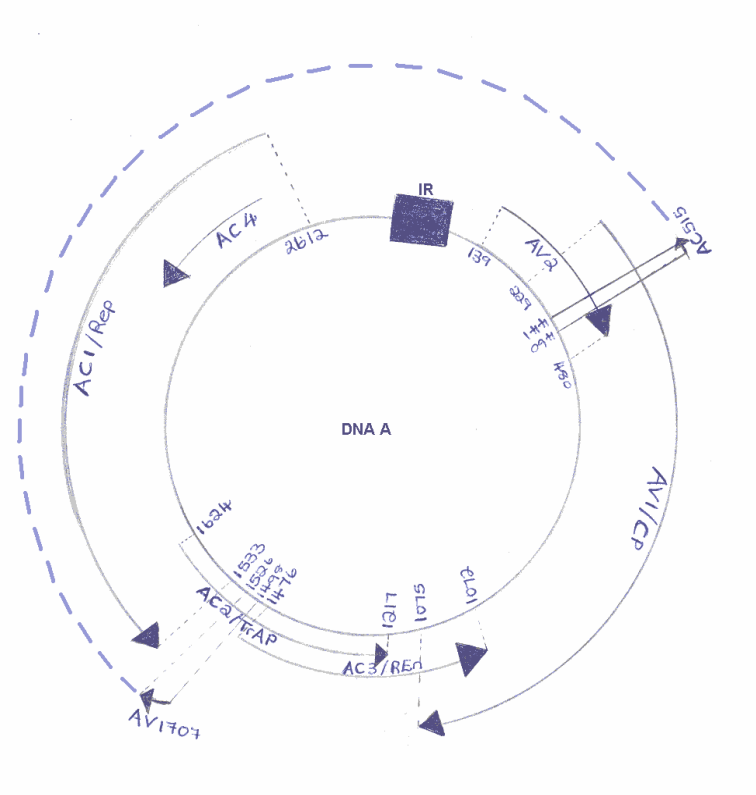
2.3.3 Abutting Primers

Non-overlapping, abutting primers, FL-C and FL-V designed to the coding region of the coat protein gene of the ACMV clone, pJS092 to allow amplification of full-length linear DNA (Briddon *et al*., 1993) were use to try and amplify the full-length DNA-A of ToCSV [01/2521] and ToCSV[99/0631]. The highly conserved nature of the coat protein gene of whitefly-transmitted viruses makes this the ideal priming site for this sub-group of viruses. Total nucleic acid preparations (100ng) of ToCSV isolates were added to each tube containing PCR master mix and a total volume of 50µl. The master mix contained 10mM dNTPs, 3.75U Expand Long Template Enzyme mix (Roche), 5U 10x Expand Long template buffer with 17mM MgCl2 (Roche) and 20pmol of each primer. Experimental controls included a complete PCR master mix to which was added 100ng SACMV DNA A in pBS as a positive control and a PCR master mix minus DNA as a negative control. PCR was carried out in a thermal cycler (BIO-RAD). PCR reaction consisted of an initial cycle of 2 min at 94°C followed by 10 cycles of 10 sec at 94°C, 30 sec at 58°C and 2 min at 68°C; 20 cycles of 15 sec at 94°C, 30 sec at 58°C, 3 min at 68°C and a final extension at 68°C for 10 min. PCR amplified products were electrophoresed at 50mA through a 0.8% (w/v) agarose gel in 1X TAE electrophoresis buffer and visualized under UV after staining with in µg/ml ethidium bromide.

2.3.4 Near-full length DNA-A Primers

Primers AL1c2745 and PAR1v32 were designed using the internet program Primer3, to amplify almost the full length genome sequence of ToCSV [99/0631], except for about 60 residues of the intergenic region, to give a ~2.7kbp product. The forward primer binds between nucleotides 32 and 51 and the reverse primer binds between nucleotides 2726 and 2745, thus missing the nonanucleotide

plants could be used to design virus specific primers to amplify more informative genomic regions and possibly the full length DNA-A of this isolate. The annealing sites for the reverse primer is between nucleotides 441 and 462 (Fig. 3) and the annealing site for the forward primer is between nucleotides 1498 and 1526 (Fig. 3). The area amplified by these primers is depicted by the dotted lines (Fig. 3). TNA samples extracted from tomato plants infected with ToCSV isolates 99/0631 and, were tested using these primers. Total nucleic acid preparations (50ng) were added to each tube containing PCR master mix and a total volume of 50µl. Each PCR master mix consisted of 5µl 10x AccuBuffer (BIOLINE), 1µl 50mM $MgCl_2$ solution (BIOLINE), 10mM dNTPs, 1µl ACCUZYME DNA Polymerase (BIOLINE) and 20pmol of each primer. Experimental controls were as described in 2.3.1. PCR amplification was carried out in a thermal cycler (BIO-RAD) and consisted of an initial cycle of 2 minutes at 94°C, followed by 30 cycles of 15 seconds at 94°C, 30 seconds at 52°C and 2 minutes at 72°C. PCR amplified products were electrophoresed at 50mA through a 0.8% (w/v) agarose gel in 1X TAE electrophoresis buffer and visualized under UV after staining with in 1µg/ml ethidium bromide.



To screen for the presence of begomovirus DNA in the symptomatic leaf DNA samples, PCR amplification using begomovirus-specific primers AV1c1048 and AV1v514 (Wyatt and Brown, 1996) was performed. The degenerate primer pair was designed specifically to target the "core" region of the coat protein gene of whitefly-transmitted geminiviruses. These primers are designed to anneal to begomovirus universally conserved sequences that flank the core region of the coat protein (CP) gene.

The primer pairs JSP001/JSP003 and JSP001/JSP002, designed to amplify the full CP genes (~ 700bp) of EACMV and ACMV respectively, and available in our laboratory, were tested in an attempt to amplify the full CP gene from the DNA of ToCSV isolates 01/2521 and 99/0631. PCRs were performed in 50µl reaction mixes containing 50ng of template DNA, 10mM dNTPs, 0.05% gelatin, 5% tween, 2U *Taq* polymerase (Roche), 10x PCR reaction buffer (Roche) and 20pmol of each forward and reverse primers. Experimental controls included, a complete PCR master mix to which was added 50ng TNA extracted from SACMV infected leaves, as a positive control. As a negative control, double-distilled water, substituted for viral DNA, was added to a complete PCR master mix. PCR amplification was carried out in a thermal cycler (BIO-RAD) with an initial cycle of 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1min at 45°C and 1min at 72°C and a final extension at 72°C for 10min. PCR amplified products were electrophoresed at 50mA through 0.8% (w/v) agarose gels in 1x TAE electrophoresis buffer, and visualized under UV after staining in 1µg/ml ethidium bromide.

2.3.2 Two/Thirds DNA-A Primers

Virus specific primers AC515 and AV1707, designed by A.M. Idris, University of Arizona, to amplify 2/3 of ToCSV [99/0631] DNA-A were used to try and amplify a fragment of similar size from ToCSV [01/2521] DNA. The ToCSV [99/0631] genome region amplified by these primers included the IR, AC1 and AC4 ORFs, and parts of AC2, AV2 and AV1 ORFs. We tested these primers hoping that the fragment obtained from amplifying ToCSV [01/2521]-infected

Table2.1 Sequences and ToCSV DNA-A target region of PCR primers used

Primer designation	**Sequence (5'→3')****	**Target region**
AV1c1048	GGRTTDGARGCATGHGTACATG	Core CP gene
AV1v514	GCCCWTGTAGAGRAAGCCMAG	Core CP gene
JSP001	ATGTCGAAGCGACCAGGAGAT	CP gene
JSP002	TGTTTATTAATTGCCAATACT	CP gene
JSP003	CCTTTATTAATTTGTCAGTGC	CP gene
FL-C	GGGTCACATCACTAATCACC	Full-length DNA
FL-V	GGGGGCCTGGGCTGACACAC	Full-length DNA
AC515	CT(TG)GGCTT(TC)CT(AG)TA(CT)AT(AG)GGCC	2/3 DNA-A
AV1707	GGTAGTATGAGGATCCACAGTCTAGGTCT	2/3 DNA-A
AL1c2745	TATTAATCGGATGGCCGCTT	Near-full length DNA-A
PAR1v32	TGCCCCCAAAAAAAAGTGGT	Near-full length DNA-A

****R = (A,G); D = (A,G,T); H = (A,G,T); H = (A,C,T); M = (A,C); W = (A,T).**

2.3.1 Core Coat Protein Primers and Full Coat Protein Primers

70% ethanol and spun at maximum speed at 4°C for 10 minutes. The pellet was vacuum dried and resuspended in 50µl TE buffer (10mM Tris, pH 8.0, 1mM EDTA) containing 20 µg/ml RNAase A. A sample of the TNA product was analyzed by electrophoresis on a 0.8% agarose gel and the concentration was determined by fluorometry.

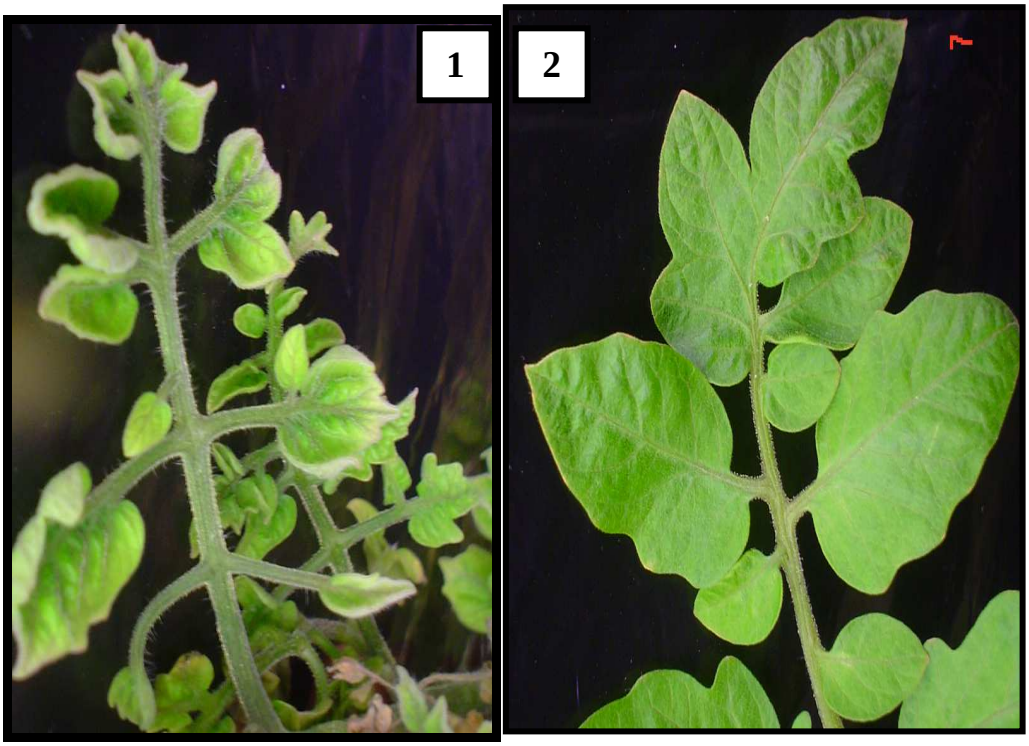


Figure 1: Tomato plant infected with ToCSV [99/0631]. Note: curling up of leaf margins, yellowing of leaves and reduced leaf size.
Figure 2: Tomato plant infected with ToCSV [01/2521]. Note slight downward curling and mild yellowing of leaves.

2.3 Polymerase Chain Reaction Amplification

Total nucleic acids (TNA), containing geminivirus DNA, were extracted from tomato leaves infected with ToCSV isolates 01/2521, 99/0631 and Moz1 respectively, as described in 2.2 and used as template DNA for PCR amplification of viral DNA using a range of different primer sets, details of which are given in table 2.1.

CHAPTER TWO
Materials and Methods

2.1 Virus source

Symptomatic tomato plants, infected with the original isolate, ToCSV [99/0631] (Fig. 1) were collected from the Onderberg region of Mpumalanga, South Africa. Tomato plants exhibiting begomoviral symptoms (leaf curling and leaf yellowing) and infected with what is thought to be a second isolate, ToCSV [01/2521] (Fig. 2), were collected from a tunnel, near Pietermaritzburg, KwaZulu-Natal, South Africa. The ToCSV isolates were maintained through grafting on cv. Red Kaki tomato. These were grown in temperature-controlled growth rooms at 25°C (day/night) under banks of fluorescent tubes (Osram L36W/77) with a 16 hour photoperiod. Symptomatic tomato plants were also collected from Mozambique and infected leaves were frozen and kept at -70°C.

2.2 Total Nucleic Acid Extraction

Total nucleic acid (TNA) was extracted from young leaves of ToCSV infected cv. Red Kaki tomato plants using the cetyl trimethyl ammonium bromide (CTAB) extraction method developed by Doyle and Doyle (1987). A 50mg sample of young leaf tissue was snap frozen with liquid nitrogen and ground into a powder using mortar and pestle and immediately suspended in warm (65°C) 500µl extraction buffer (2% CTAB, 20mM EDTA, 1.4M NaCl 100mM Tris, pH 8.0) and 1µl β-mercaptoethanol (final concentration of 0.1% v/v). The tubes were incubated at 65°C for 60 minutes. TNA was extracted from the aqueous layer, twice, by adding 500µl chloroform:isoamyl alcohol (24:1), inverting and centrifuging at maximum (14000 rpm) at 4°C for 10 minutes. Nucleic acids were precipitated with an equal volume (500µl) of isopropanol, and centrifuged at maximum speed at 4°C for 10 minutes. The pellet was washed with 500µl ice-cold

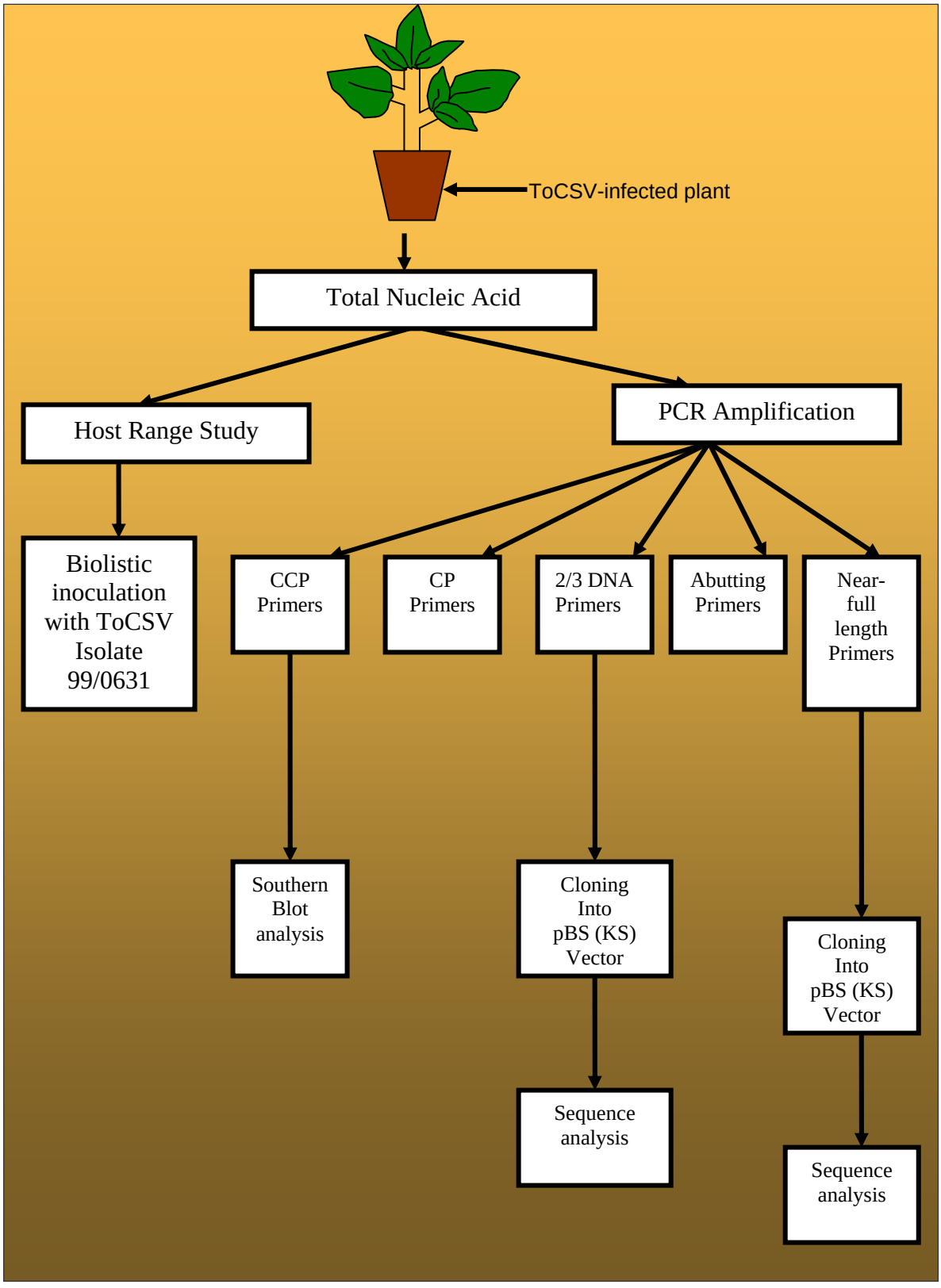


Figure 1.2 A summary of the procedure followed in this study.

1.12 Specific Aims

1) To characterize ToCSV isolate 01/2521, using molecular techniques.
    a) Amplify the core coat region, coat protein region and full-length DNA of 01/2521.
    b) Clone PCR fragments into pBluescript KS (+) vector.
    c) Sequence PCR amplified regions of isolate 01/2521 and compare with the original isolate (99/0631) and other begomoviruses.
2) Screen for the presence of satellites in ToCSV-infected tomato plants.
3) Conduct a host range study
    a) Biolistic inoculation of indicator host plants and test for infectivity by symptoms and PCR.

implying that they are encapsidated (Czosnek *et al*., 1989). Circular subgenomic DNA molecules of smaller size have also been observed, and these have also been shown to be transmissible by whiteflies (Stanley *et al*., 1992). The transmission of defective molecules by whiteflies was also observed by Stanley *et al.* (1997) for AYVV and Liu *et al*. (1998) for CLCuV-PK, thus further supporting the suggestion made by Stenger *et al*. (1992), that molecules half the size of the standard viral genome may predominate as a result of stringent size selection for encapsidation.

1.11 Background and Objective

A new disease of tomatoes (*Lycopersicon esculentum*) emerged during 1997 and 1998 in South Africa (Pietersen *et al*., 2000). Initially the disease was found to occur at incidences up to 50% and confined only to the Strydomblok Distrik close to the Mozambique border (Pietersen *et al*., 2000). The disease quickly spread to other tomato producing areas of the country such as Onderberg in Mpumalanga, Pongola and Nkwalini in KwaZulu-Natal, and Trichardtsdal in Limpopo where incidences of 100% were noted (Pietersen and Smith, 2002). Symptoms of the disease include reduced leaf size, yellowing of the upper leaves, upward curling of the margins and stunted internodes. The causative virus of this devastating disease, *Tomato curly stunt virus*, referred to in this study as ToCSV [99/0631], has been characterized and it is reported to be closely related to SACMV, EACMV and TYLCV-Is. Tomato plants showing similar but milder symptoms (slight leaf yellowing and downward curling of leaf margins) were collected in 2001, from a tunnel in KwaZulu-Natal near Pietermaritzburg. Initial attempts by Dr G. Pietersen to amplify total DNA extracted from these plants with ToCSV [99/0631]-specific primers were unsuccessful. These result together with the observation of milder symptoms led to the belief that these tomato plants could be infected with a different ToCSV isolate, referred to in this study as ToCSV [01/2521]. The main objective of this study was to characterise ToCSV [01/2521].

1.10.2 Defective Interfering DNA

Begomoviruses are reported to have the ability to acquire extra DNA components. This stems from the fact that cultures of several begomoviruses have been shown to contain circular DNA molecules, about half the size of the genomic components(Harrison and Robinson, 1999).. The first type of these circular DNA molecules to be discovered in ACMV (Stanley and Townsend, 1985) and other bipartite begomoviruses such as TGMV (MacDowell *et al*., 1986) were shown to be derived exclusively from the DNA-B component by a single large deletion that removes the *BV1* gene and part of the *BC1* gene without affecting the IR sequence (Harrison and Robinson, 1999). The defective DNA molecules are maintained in cultures, are produced in significantly large amounts. Because they cause a decrease in the accumulation of genomic DNA and attenuate symptoms (Stanley *et al*.,1997), they are considered to be defective interfering (DI) nucleic acids (Stenger *et al.,* 1992).

Circular molecules that act as defective interfering DNAs have also been found in association with monopartite begomoviruses such as AYVV from Singapore (Stanley *et al*., 1997) and CLCuV from Pakistan (Liu *et al*., 1998). Small circular recombinant components, each containing AYVV origin of replication together with sequences of unknown origin were isolated from infected ageratum. These recombinants were shown to behave as defective interfering DNAs because when co-inoculated with AYVV into *Nicotiana benthamiana,* they ameliorated the symptoms of AYVV and reduced its accumulation in the host plant. In contrast to the DIs found associated with bipartite begomoviruses, the DIs associated with monopartite begomoviruses are derived from DNA-A, their only genomic component. They are circular, about half the size of genomic DNA, include the IR and 5’ part of the Rep gene, are produced in substantial amounts and also cause a decrease in virus genome accumulation and attenuate symptom expression (Stanley *et al*., 1997; Liu *et al*., 1998).

Defective molecules associated with both monopartite and bipartite begomoviruses are transmitted by whiteflies along with the genomic DNA, thus

conservation between distinct DNA β molecules. The A-rich region, assumed to be a "stuffed" region in order to comply with size constraints for encapsidation and transmission (Saunders *et al*., 2001), is typically between 160 and 280 bases in length and is reported to have between 57 and 65% A content, with repeated blocks of up to 11 consecutive A residues. The overall A content of DNA β molecules is between 28 and 38% (Briddon *et al*., 2003).

Various studies have been conducted to determine the role of the conserved βC1 ORF, Qian and Zhou (2005) have recently demonstrated that a C1 deletion DNA β (ΔC1β) associated with TYLCCNV, can be trans-replicated by TYLCCNV and stably exist in *Nicotiana benthamiana* and *N. glutinosa*, an indication that the C1 gene is dispensable for the replication of both DNA β and the helper virus. They also showed that the presence of this gene can indeed increase both TYLCCNV and DNA β accumulation in plants. Zhou and associates (2003) provided evidence of the involvement of the βC1 ORF in modulation of symptom expression in host plants by demonstrating that an in-frame mutation of the βC1 initiation codon of a few DNA β species associated with tomato- and tobacco- infecting begomoviruses, resulted in loss of symptom severity in *N. benthamiana*. Saunders and associates (2004) subsequently demonstrated that disruption of the βC1 ORF prevented infection of the AYVV-DNA β complex in ageratum and altered their phenotype in *N. benthamiana* to that produced by AYVV alone, thus supporting the findings by Zhou *et al*. (2003). To further test the role of βC1 ORF as a pathogenicity determinant, Saeed and associates (2005) constructed two βC1 mutants, one with a stop codon at amino acid position 41 and another carrying two stop codons at positions 9 and 41. They found that both resulted in loss of pathogenicity in tobacco when co-inoculated with *Tobacco leaf curl virus*. These results, together with the findings by Zhou and associates (2003) and by Saunders and associates (2004), confirm that βC1 is responsible for pathological symptoms associated with DNA β infections and that the strong phenotype exhibited by βC1 protein expression seems to be a common feature of DNA β satellites.

replication nor symptoms caused by ToLCV, thus making DNA β the first reported symptom modulating DNA satellite (Saunders *et al*., 2000). DNA β, unlike ToLCV-sat, does affect the replication of the helper virus. However, just like ToLCV-sat, DNA β molecules have no significant sequence homology to their helper virus, and they require the helper virus for replication and movement in plants and insect transmission ((Saunders *et al*., 2000, Saunders *et al*., 2001).

1.10.1 DNA β Satellite

DNA β molecules are defined as symptom-modulating single-stranded DNA satellites associated with monopartite begomoviruses (Briddon *et al*., 2003). Recently more monopartite begomovirus species have been found to be associated with DNA β molecules, including *Bhendi yellow vein mosaic virus* (Jose and Usha, 2003) and *Eupatorium yellow vein virus* (Saunders *et al*., 2003). In Yunnan, China, Zhou *et al*. (2003) identified 18 DNA β species found to be associated with begomoviruses isolated from tobacco, tomato and weed species. Cui and associates (2004) later reported 25 isolates of *Tomato yellow leaf curl China virus* (TYLCCNV) collected from tobacco, tomato or *Siegesbeckia orientalis* plants in different regions of Yunnan province to be associated with DNA β molecules.

Comparison of nucleotide sequences of 28 DNA β species by Briddon *et al*. (2003), identified three absolutely conserved features: (1) a predicted stem loop structure with the loop sequence TAA/GTATTAC (the nonanucleotide sequence), (2) a region of high sequence similarity, first identified by Briddon et al. (2001) and referred to as the satellite conserved region (SCR) and (3) an adenine (A)-rich region approximately 370 to 420nts upstream of the SCR. The presumed full-length DNA β molecules did however differ in the total number of open reading frames (ORF) identified, but they all contained a single ORF, βC1, previously identified as ORF C1 by Saunders *et al*. (2000) for AYVD DNA β and ORF C4 for CLCuD DNA β (Briddon *et al*., 2001). The βC1 ORF is universally conserved in size and position. The SCR of DNA β molecules is reported to be greater than 65% conserved compared to a typical level of 50% overall nucleotide sequence

components but have no biological role in the disease process and are dispensable for both infectivity to and symptom induction in the host plant. These satellite-like molecules do however encode a single product that is similar to the replication-associated protein of nanoviruses. Because of this, DNA1 molecules are capable of autonomous replication in the host cell, but require helper begomovirus for spread in plants and insect transmission (Mansoor *et al*., 1999; Saunders and Stanley, 1999; Stanley, 2004). DNA1 molecules are larger than the typical 1000-1100 nucleotide length of nanoviruses. This is attributed to the presence of an A-rich "stuffed" region within the intergenic region. The inclusion of the A-rich region is assumed to represent size adaptation of these subviral molecules in order to allow their functional interaction with begomovirus proteins (Stanley, 2004). It has been established that begomovirus components are subjected to strict size constraints to enable their systemic movement (Rojas *et al*., 2000).

During an attempt to resolve the aetiology of AYVD, an additional subgenomic DNA component, termed DNA β, was isolated from ageratum exhibiting AYVD (Saunders *et al*., 2000). Agroinoculation of ageratum with AYVV DNA-A resulted in an asymptomatic infection and viral DNA levels less than 5 % to that found in naturally infected plants. However, co-agroinoculation of AYVV with DNA β produced characteristic symptom phenotypes and viral DNA levels accumulated to levels observed in naturally infected symptomatic tissues. Subsequently, DNA β was also shown to be associated with *Cotton leaf curl Multan virus* from Pakistan (Briddon *et al*., 2001). Because the DNA β molecules isolated from ageratum and cotton respectively, share no extensive sequence homology with AYVV DNA-A and CLCuMV DNA-A, yet they are essential components of the respective diseases, they are considered to be begomovirus satellite DNAs (Saunders *et al*., 2000; Briddon *et al*., 2001; Saunders *et al*., 2001).

The first reported DNA satellite is a 682-nt single-stranded DNA satellite molecule shown to be associated with *Tomato leaf curl virus* (ToLCV) from northern Australia (Dry *et al*., 1997). The ToLCV satellite (ToLCV-sat), previously abbreviated as TLCV-sat, had no recognizable effects on either viral

1.10 Geminivirus Subgenomic DNAs

The systemic spread of geminiviruses is reported to be size-dependent (Stanley *et al*., 1990). The size of geminivirus genomic DNA varies between 2.5 and 3.0 kbp in length (Harrison and Robinson, 1999; Stanley, 2004). Small DNA molecules, approximately half this size and associated with geminivirus infection, have been identified. These small DNA molecules are either virus-derived (Stenger *et a*l., 1992; reviewed by Frischmuth and Stanley, 1993) or have very little to no sequence homology with the helper virus as in the case of satellite DNAs (Dry *et al*., 1997; Stanley *et al*., 1997, Saunders *et al*., 2000). Some of these molecules have similarities to the genome component of nanoviruses, another ssDNA plant virus family (Mansoor *et al*., 1999; Saunders and Stanley, 1999), while some are reported as DNA β because of their involvement in symptom production comparible to the DNA-B function of begomoviruses (Saunders *et al*., 2000). All of the above mentioned half-size DNA molecules are dependent on the co-infecting geminivirus for either replication or systemic spread or both.

Small subviral recombinant DNAs (recDNAs), each approximately half the size of the begomovirus component, have been isolated from ageratum plants that are infected with AYVV. The recDNAs are believed to play a biological role in modulating disease development, because when co-agroinoculated with AYVV DNA-A, they caused a delay in the accumulation of the viral DNA and resulted in the amelioration of the disease symptoms in *Nicotiana benthamiana* (Stanley *et al*., 1997).

Another subviral DNA component, termed DNA1 and associated with cotton leaf curl disease (CLCuD) from Pakistan, was isolated from diseases cotton plants (Mansoor *et al*., 1999). Its homologues, associated with ageratum yellow vein disease (AYVD) originating from Singapore, and okra leaf curl disease (OLCD) from Pakistan, were subsequently isolated from diseased okra and ageratum plants respectively (Saunders and Stanley, 1999; Mansoor *et al*., 2001). DNA1 homologues are approximately half the size of their co-infecting begomovirus

Table 1.1. Begomoviruses Infecting Tomato

Virus	Acronym	No. isolates	Distribution
Chino del tomate virus	CdTV	5	Mexico
Pepper huasteco yellow vein virus	PHYVV	1	Mexico, USA (Texas
Potato yellow mosaic virus	PYMV	2	Guadeloupe, Venezuela
Potato yellow mosaic Panama virus	PYMPV		Panama
Potato yellow mosaic Trinidad virus	PYMTV		Trinidad & Tobago
Pepper golden mosaic virus	PepGMV	1	Mexico, USA (Arizona, Texas)
Tomato chlorotic mottle virus	ToCMoV	2	Brazil
Tomato curly stunt virus	ToCSV	1	South Africa
Tomato dwarf leaf curl virus	TDLCV	1	Jamaica
Tomato golden mosaic virus	TGMV	2	Brazil, Costa Rica, Venezuela
Tomato golden mottle virus	ToGMoV	1	Guatemala
Tomato leaf curl Bangalore virus	ToLCBV	4	India
Tomato leaf curl Gujarat virus	ToLCGV	3	India
Tomato leaf curl India virus	ToLCIV	1	India
Tomato leaf curl Indonesia virus	ToLCIDV	1	Indonesia
Tomato leaf curl Karnataka virus	ToLCKV	1	India
Tomato leaf curl Vietnam virus	ToLCVV	1	Vietnam
Tomato leaf curl Laos virus	ToLCLV	1	Vietnam
Tomato leaf curl Malaysia virus	ToLCMV	1	Malaysia
Tomato leaf curl New Dehli virus	ToLCNDV	5	India
Tomato leaf curl Nicaragua virus	ToLCNV	1	Nicaragua
Tomato leaf curl Phillipines virus	ToLCPV	1	Phillipines
Tomato leaf curl Senegal virus	ToLCSV	1	Senegal
Tomato leaf curl Sinalao virus	ToLCSinV	1	Sinalao
Tomato leaf curl Sri Lanka virus	ToLCSLV	1	Sri Lanka
Tomato leaf curl Taiwan virus	ToLCTWC	1	Taiwan,Japan
Tomato leaf curl virus	ToLCV	3	Australia
Tomato mosaic Barbados virus	ToMBV	1	Barbados
Tomato mosaic Havana virus	ToMHV	1	Cuba
Tomato mottle Taino virus	ToMoTV	1	Cuba
Tomato mottle virus	ToMoV	1	Puerto Rico, USA
Tomato rugose mosaic virus	ToRMV	2	Brazil
Tomato severe leaf curl virus	ToSLCV	4	Guatemala
Tomato severe rugose virus	ToSRV	1	Brazil
Tomato Uberlandia virus	ToUV		Uberlandia
Tomato yellow dwarf virus	ToYDV	1	Taiwan,Japan
Tomato yellow leaf curl China virus	TYLCCnV	9	China
Tomato yellow leaf curl Gezira virus	TYLCGV	3	Sudan
Tomato yellow leaf curl Kuwait virus	TYLCKWV		Kuwait
Tomato yellow leaf curl Malaga virus	TYLCMalV	1	Spain
Tomato yellow leaf curl Nigeria virus	TYLCNV	1	Nigeria
Tomato yellow leaf curl Sardinia virus	TYLCSV	4	Sardina, Italy France
Tomato yellow leaf curl Saudi Arabia virus	TYLCSAV	1	Saudi Arabia
Tomato yellow leaf curl Tanzania virus	TYLCTZV	1	Tanzania
Tomato yellow leaf curl Thailand virus	TYLCTHV	4	Thailand, Myanmar
Tomato yellow leaf curl virus	TYLCV	12	Israel , Caribbean basin, Cuba, USA (Florida)
Tomato yellow leaf curl Yemen virus	TYLCYV	1	Yemen
Tomato yellow mosaic virus	ToYMV	2	Brazi, Venezuela
Tomato yellow mottle virus	ToYMoV	1	Costa Rica
Tomato yellow vein streak virus	ToYVSV	1	Brazil

Source of virus names and acronyms: Varma and Malathi, (2003). Italicised names refer to species, non-itelicised names refer to tentative species

and Sicily have been identified as *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (Varma and Malathi, 2003).

Tomato leaf curl disease (ToLCD) has been occurring in India for many years, resulting in yield reduction and economic losses to the growers. Viruses causing TYLCD are reported to be closely related to each other, whereas those causing ToLCD are more diverse (Varma and Malathi, 2003). In India, five different begomoviruses are reported to cause ToLCD (Table 1.1). Three of these viruses, *Tomato leaf curl Gujarat virus* (ToLCGV), *Tomato leaf curl India virus* (ToLCIV), and *Tomato leaf curl New Delhi* virus (ToLCNDV) have bipartite genomes, and the other two, *Tomato leaf curl Bangalore virus* (ToLCBV) and *Tomato leaf curl Karnataka virus* (ToLCKV), occur in southern India and have monopartite genomes (Muniyappa *et al.,* 2000; Padidam *et al*., 1995). Tomato leaf curl is continuously emerging in new areas and has also been reported to occur in Bangladesh, Bhutan, Nepal, Pakistan, and Sri Lanka (Varma and Malathi, 2003). Apart from the Indian subcontinent, ToLCV is reported to occur in geographically widely separately areas including Australia and Taiwan (Dry *et al*., 1993)

In Africa, TYLCD was first described in the Sudan (Yassin and Nour, 1965). By the late 1970s a high incidence of the disease was observed throughout Nigeria. The disease has also been reported in Burkina Faso (Konate *et al*., 1995), Cape Verde, the Ivory Coast, Mali (d'Hondi and Russo, 1985), Egypt (Czosnek *et al*., 1990) and Cameroon (Czosnek and Laterrot, 1997). A new tomato-infecting begomovirus, designated *Tomato curly stunt virus* (ToCSV), is currently a major cause of concern in South Africa. It was first reported in 2000 (Pietersen *et al*., 2000). So far this virus is reported to be confined to certain tomato producing regions of the country.

Israeli isolate of TYLCV (Cohen and Nitzany, 1966). Within a very short space of time, TYLCV went from causing sporadic outbreaks to becoming a major economic problem with reported yield losses in the Jordan Valley of up to 100% in the early seventies. By the end of the seventies the virus had spread to other tomato production areas in the Middle East.

TYLCV has since spread to other parts of the world. In Morocco, where tomato is the second most important crop for export, the occurrence of TYLCD was first reported from the area south of Casablanca in 1996-1997, and by 1998 it spread to all the tomato growing areas (Varma and Malathi, 2003). TYLCV was identified for the first time in the New World when the virus was isolated from infected tomato plants in the Caribbean Islands (Dominican Republic, Cuba and Jamaica) (Czosnek and Laterrol, 1997). TYLCV from Israel is believed to have been unintentionally introduced to the Western Hemisphere, arriving in the Dominican Republic probably through importation of infected transplants from Israel (Polston and Anderson, 1997). From there, the virus spread to Haiti, the Bahamas, Cuba, Jamaica, Mexico and the United States (Ascencio-Ibanez *et al*., 1999; Pappu *et al*., 2000; Polston *et al*., 1999; Ramos *et al*., 1996). Tomato-infecting begomoviruses emerged as a problem in the USA agriculture in 1989 when *Tomato mottle virus* (ToMoV) was first observed in Naplesh Florida in the spring tomato field of 1989. By 1991, annual economic losses due to ToMoV were estimated to be around US $125 million as a result of reduced fruit yields and the cost of pesticide application to control whiteflies (Polston *et al*.,1996; Polston and Anderson, 1997). ToMoV have since spread to other tomato producing parts of the USA. TYLCV also emerged in Florida a couple of years ago. It was first observed in July 1997 and by April 1998 incidences of up to 100% were recorded in tomato fields.

TYLCD has also been reported in Europe. It was first reported in Sardinia in 1988 and subsequently in Sicily and southern Italy, Spain, Portugal and France (Pico *et al*. 1996; Louro *et al*., 2001, Varma and Malathi, 2003). All the Portuguese isolates and some recent Spanish isolates have been identified as the Israeli strain of TYLCV whereas some isolates from Spain and various isolates from Sardinia

1.9 Tomato-infecting Begomoviruses

Tomato (*Lycopersicon esculentum* Mill.) seems to be an ideal host for begomoviruses. There are 34 recognized and 18 tentative species of begomoviruses that have been reported to naturally infect tomato (Table 1.1). The majority of these viruses have been identified during the last 10 to 15 years and originate mostly from the Americas, where 17 distinct begomoviruses have been isolated from tomato (Polston and Anderson, 1997). It is believed that many more await detection considering the limited tomato growing areas of the world that have been examined for these viruses so far (Varma and Malathi, 2003).

The most devastating and most frequent tomato-infecting begomoviruses are those with generic names “tomato leaf curl virus” and “tomato yellow leaf curl virus”. The majority of tomato diseases caused by begomoviruses are collectively described as either “leaf curl” or yellow leaf curl” based on their biological properties and subtle differences in symptoms. They induce symptoms characteristic of leaf curl diseases including severe reduction in leaf size, downward curling, crinkling of interveinal areas, interveinal and marginal chlorosis, occasional development of enations, purple discolouration of the abaxial surface of leaves, shortening of internodes, development of small branches and reduced fruiting, some even have bright yellow spots on leaves (Varma and Malathi, 2003). It is therefore not possible to distinguish these viruses from each other by symptoms or other biological properties such as host range, and transmission alone. Reactions with panels of monoclonal antibodies can be used to a limited extent, but they are best distinguished by DNA hybridization, PCR and nucleotide sequence analysis (Rojas *et al*., 2000; Varma and Malathi, 2003).

So far, 18 begomoviruses associated with Tomato leaf curl disease (ToLCD) and 11 associated with Tomato yellow leaf curl disease (TYLCD) have been identified (Table 1.1). These viruses are widely distributed in Africa, the Americas, Asia, Australia and parts of Europe. TYLCD was first observed in Israel in 1939-1940 and the appearance of the disease paralleled an increase in the whitefly *Bemisia tabaci* population. Twenty years later the causal begomovirus was identified as the

(reviewed by Harrison and Robinson, 1999). Geminiviruses have exploited recombination as a means of acquiring distinct characteristics that might have selective advantage, and possibly resulting in the emergence of new geminiviral diseases (Padidam *et al*., 1999). Recombination has also been involved in the evolution of begomovirus genomes and has occurred between strains and between species. This is supported by the identification of chimeric sequences, i.e. contiguous regions of the nucleotide sequence that are of apparently different origins. The most devastating genomic recombination event amongst the begomoviruses has been between EACMV and ACMV giving rise to the virulent EACMV-Uganda variant (UGV2), which caused a pandemic in East Africa (reviewed by Harrison and Robinson, 1999). This was the first example of naturally occurring recombination between two different, identified begomovirus species. Recombination among viruses from different genera within the family *Geminiviridae*, have also been reported. BCTV, a curtovirus, is reported to combine properties of species in the genera *Begomovirus* and *Mastrevirus* (Stanley *et al*., 1986). Recombinants are also evident in the CLCuD complex. CLCuMV in Pakistan is reported to have more that 50% of its genomic DNA-A identical to that of BYVMV (Zhou *et al*., 1998). CLCuMV and AYVV DNA-A components are also reported to develop recombinants with DNA β and nanovirus-like components (Bridddon *et al*., 2001; Saunders *et al*., 2001). Three factors contribute significantly to geminiviruses having such high propensity for recombination (Padidam *et al*., 1999; Power, 2000).

- Mixed infections, a pre-requisite for recombination, are common in geminivirus diseases.
- High levels of replication; geminiviruses replicate via a double-stranded replicative form and achieve high copy numbers.
- Increased host range; the host range of geminiviruses has expanded greatly, this is owing to the emergence of the B biotype whitefly that can feed on hundreds of host species, transmitting begomoviruses among host plants that did not previously share insect vectors.

multiplication proceeds by RCR (reviewed by Hanley-Bowdoin *et al*., 1999). Both the complementary-sense and virion-sense strands of the intermediate dsDNA encode viral genes and transcription of the viral genome is bidirectional with independently controlled transcripts initiating within IR (Rybicki *et al*., 2000). The RCR model explains how viral ssDNA is produced from the double-stranded template at the final stage of the multiplication cycle. However, since geminiviruses transcribe bidirectionally, and thus increasing the risk of collisions between replication and transcription complexes, Jeske *et al*. (2001), wondered whether RCR was indeed the only model for geminivirus replication. Following numerous studies, they reported, based on two-dimensional gel electrophoresis and electron microscopy results, that geminiviruses might multiply by two mechanisms, RCR and recombination-dependent replication (RDR) (Jeske *et al*., 2001). Subsequent studies involving distantly related geminiviruses, mainly from the genera *Begomovirus* and *Curtovirus* have revealed replicative intermediates consistent with both RCR and RDR (Preiss and Jeske, 2003; Alberter *et al*., 2005). In contrast to RCR, where open circular DNA is the template for the synthesis of ssDNA, template for RDR is covalently closed circular DNA (Alberter *et al*., 2005)

The third stage in geminivirus replication is the production and encapsidation of mature genomic circular ssDNA into viral particles. In order to propagate infection in the host, the virion needs to move from cell to cell, and therefore must be able to exit the nucleus and be transported through the lattice-like structure of the cytoplasm and through plasmodesmata, which are wall spanning co-axial membranous organelles that bridge the cytoplasm of contiguous cells (Gafni and Epel, 2002). For bipartite begomoviruses, this movement and subsequent systemic infection, is made possible by the DNA-B encoded NSP and MP.

1.8 Recombination

Diversity or variability among viruses is common and it is usually generated through mutations, *de novo* gene acquisition, re-assortment and recombination

1.7 Replication of Geminiviruses

In order for a plant virus to survive in nature, it needs to replicate its genome, assemble genomic nucleic acid into nucleoprotein particles within infected plant cells, spread within the infected plant cells and must be transmitted from one plant to another. The overall strategy used by geminiviruses to replicate their ssDNA genome is similar to that of prokaryotic ssDNA phages and plasmids (Gutierrez, 2000). The geminivirus replication cycle relies entirely on DNA intermediates and occurs within the nucleus of infected cells.

Before infection and replication in a host cell can take place, geminiviruses must cross the cell wall plasma-membrane interface with the ultimate aim of localizing its genome within the host nucleus. When a geminivirus first enters its host cell, for example through injection by insect vector, there are no viral proteins present except for the coat protein (CP). The virus's movement towards the nucleus is thus entirely dependent on the CP and exploitation of host transport mechanisms (Gafni and Epel, 2002). Upon entry into the nucleus, amplification of the viral genome, which involves a DNA replication process, is divided in the following three stages, as described by Gutierrez, (2000): During the first stage, the genomic circular single stranded DNA is converted into the transcriptionally active intermediate supercoiled, covalently closed circular double stranded DNA (dsDNA). This replicative form then associates with cellular histones to form viral minichromosomes. The second stage is the amplification of the dsDNA intermediates through a rolling-circle mechanism in which only one virally encoded protein, the initiator protein Rep, encoded by the *C1* gene, is absolutely required. Until recently, rolling circle replication (RCR), was believed to be the only mechanism of geminivirus DNA replication (Alberter *et al*., 2005). Initiation of rolling circle DNA replication, which has been mapped within an invariant nonanucleotide sequence, TAATATT/AC, depends on the specific interaction of the Rep protein with its cognate binding site(s) and involves a directly repeated Rep-binding motif (iteron) within the intergenic region (Arguella-astorga *et al*., 1994; Stanley, 1995). The virion-sense strand is nicked by Rep downstream of the motif within the universally conserved nonanucleotide sequence and virus genome

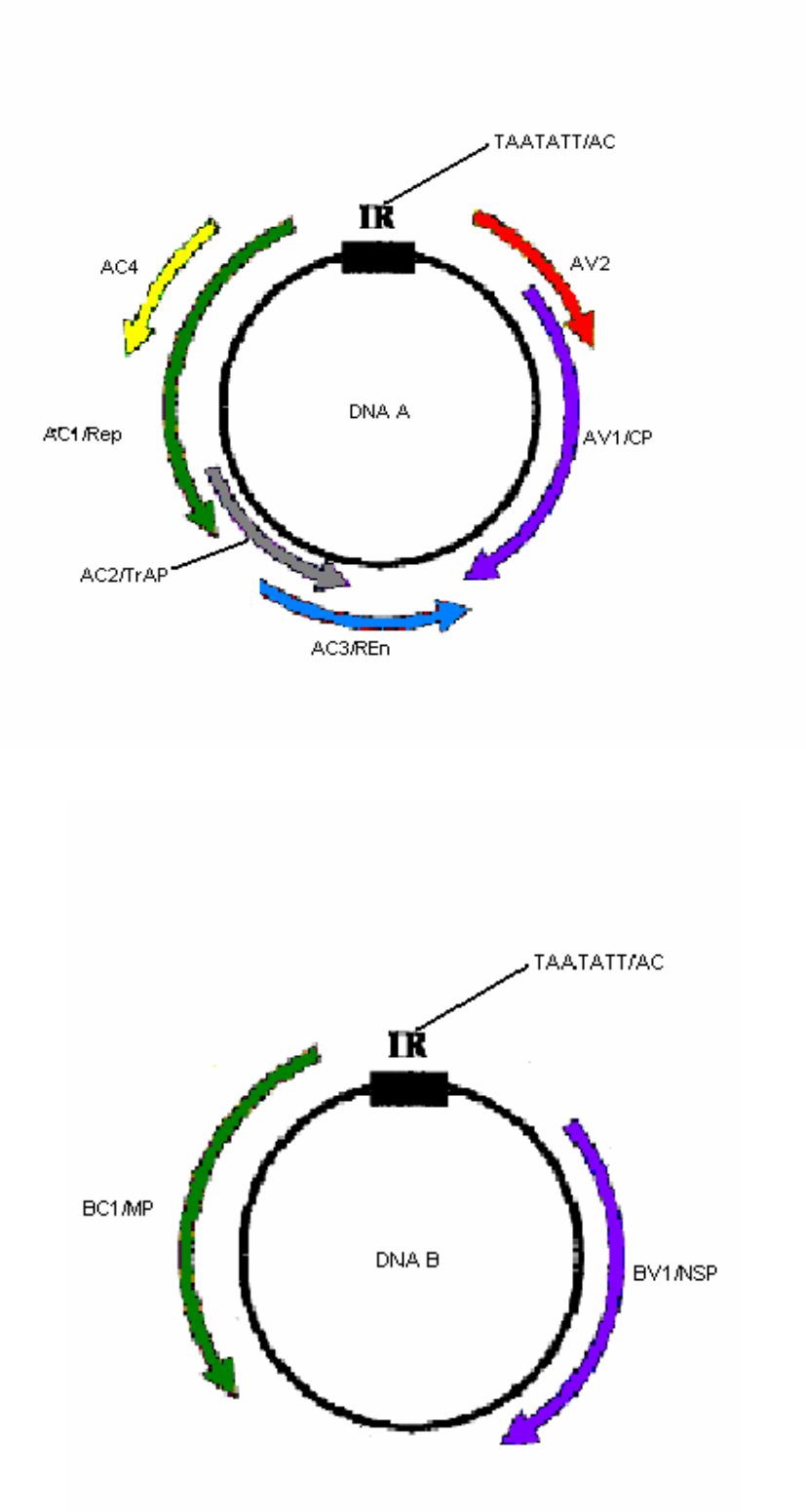


Figure 1.1 Genome Organization of Begomoviruses. DNA A contains six open reading frames (ORFs) and DNA B contains two ORFs. ORFs are indicated with arrows.

geminivirus replication (Settlage *et al*., 2001). The function of the protein product encoded by *AC4* of bipartite begomoviruses was previously not clearly determined, but Van Wezel *et al*. (2002) have recently shown that the *AC4* ORF of *African cassava mosaic virus* triggers systemic necrosis in the hypersensitive response-like reaction initiated by Rep and Vanitharani *et al*. (2004) implicated the protein product of *AC4* in the suppression of RNA silencing. The gene AV2 (Pre-CP), which is only found in begomoviruses from the Old World (Gafni and Epel, 2002) encodes a protein that is involved in symptom expression and virus movement (Padidam *et al*., 1996)

The viral B component of bipartite begomoviruses encode two non-structural, functionally distinct movement proteins (MPs), (*BC1* and *BV1*), that are not required for viral replication and encapsidation (Sanderfoot *et al*., 1996), but act in a coordinated manner to facilitate movement of viral genome (Ingham *et al*., 1995). *BV1* encodes a nuclear shuttle protein (NSP) which is involved in intracellular trafficking of replicated viral ssDNA genome between nucleus and cytoplasm, while the protein product of *BC1* functions at the cell plasma membrane and wall to facilitate the movement of ssDNA to adjacent phloem cells and into the sieve elements for long distance movement, to systemically infect the host plant (Jeffrey *et al*., 1996; Sanderfoot *et al*., 1996; Hehnle *et al*., 2004)

The protein product of *BC1* is also involved in the production of disease symptoms and in viral pathogenicity (Ingham *et al*., 1995; Jeffrey *et al*., 1996). In *Squash leaf curl virus*, *BV1* and *BC1* have been implicated as host range determinants (Ingham *et al*., 1995).

agricultural practices and global trade has in recent years resulted in the accidental introduction of the monopartite begomovirus, *Tomato yellow leaf curl virus*, which originated in the Middle East (Navot *et al*., 1991) to the Carribean and the Americas (Polston and Anderson, 1997; Polston *et al*., 1999).

1.6 Begomovirus Genome Organization

The majority of begomoviruses have bipartite genomes consisting of a DNA-A and DNA-B component, each between 2.5 – 3.1kbp in length (Reviewed by Briddon and Stanley, 2006). Genes of the DNA-A component encode proteins required for viral replication and encapsidation, while genes on the DNA-B component encode proteins required for viral movement in plants. DNA-A and DNA-B of bipartite begomoviruses share a large noncoding region, ~200 nt long, termed the "common region" (CR), or "intergenic region" (IR) in monopartite begomoviruses, which is very similar or identical in the two DNA components of the same virus (Padidam *et al*., 1995). The CR/IR contains modular *cis*-acting sequences that are involved in transcriptional regulation of certain viral genes and the sequence elements essential for virus replication (Idris and Brown, 1998).

Protein encoding sequences are present on both the viral and complementary strands of begomoviral genomes. Six partially overlapping open reading frames (ORFs) are organized bidirectionally in two transcriptional units that are separated by the CR/IR. DNA-A contains one gene (*AV1*) on the viral strand and three genes (*AC1*, *AC2* and *AC3*) on the complementary strand. DNA-B contains one gene (*BV1*) on the viral strand and one gene (*BC1*) on the complementary strand (figure 1.1). The gene *AC1* encodes the replication-associated protein (Rep) which is essential for viral replication (Fontes *et al*.,1994; Gutierrez, 2000), while *AC2* encodes a transcriptional activator protein (TrAP). TrAP functions as a transcriptional activator of virion-sense gene expression and has also been implicated in the suppression of RNA silencing (Vanitharani *et al*., 2004) and other host defence mechanisms. (Hao *et al*., 2003). The gene *AC3* encodes the replication enhancer protein (REn) which interacts with Rep and enhances

1.5 Begomoviruses

There are currently 133 officially recognized geminivirus species of which 117 belong to the genus Begomovirus (Stanley *et al*., 2005). Begomoviruses cause numerous diseases of cultivated and non-cultivated dicotyledonous plants in the tropics and subtropics, where the whitefly vector, *Bemisia tabaci* (Gennadius) species occurs (Idris *et al*., 2005). Begomoviruses have a wide host range and cause significant yield losses to many crops such as tomato, cassava and cotton in many countries (Jiang and Zhou, 2005). Some of these diseases are among the world's most economically important plant virus diseases. For example, cassava mosaic disease (CMD) of cassava in sub-Saharan Africa is reported to cause annual yield losses exceeding $2 billion in value of staple food of millions of poor people (Harrison and Robinson, 1999). Tomato leaf curl disease and Tomato yellow leaf curl disease cause devastating damage to crops in many countries (Varma and Malathi, 2003). Cotton leaf curl disease is reported to affect over two million acres in Pakistan, where cotton normally makes up to 60% of the country's exports with serious effects on yield and the country's economy (Briddon *et al*., 2000; Briddon *et al*., 2001).

The majority of begomoviral species are bipartite, they each have two DNA components, referred to as DNA-A and DNA-B, that are transcribed bidirectionally (Harrison and Robinson, 1999; Saunders *et al*., 2002). However, a small number of begomoviruses such as *Tomato yellow leaf curl virus* (TYLCV) and *Tomato leaf curl virus* (TLCV) isolated from plants growing in Israel (Navot *et al*., 1991) and Australia (Dry *et al*., 1993) respectively, are monopartite and posses a single DNA component that resembles the DNA-A component of bipartite begomoviruses. These DNA components induced symptomatic infections in their original host (tomato) and *Nicotiana spp* when cloned copies were introduced into the plant by *Agrobacterium*-mediated inoculation, an indication that these viruses have overcome the need for a DNA-B component (Kheyr-Pour *et al*., 1991; Navot *et al*., 1991). Bipartite begomoviruses are reported to occur in both the Eastern and Western Hemisphere, while monopartite begomoviruses are found only in the Eastern Hemisphere (Rybicki *et al*., 2000). However,

Curtovirus, *Topocovirus* and *Begomovirus*, based on the transmitting insect vector, host range and genome organization. The leafhopper-transmitted members of *Mastrevirus*, type species *Maize streak virus* (MSV), have monopartite genomes containing two virion-sense and two complementary-sense open reading frames (ORFs) with one long and one short intergenic region. They primarily infect monocotyledonous plants, the only two exceptions being *Tobacco yellow dwarf virus* (TYDV) and *Bean yellow dwarf virus* (BYDV), which infect dicotyledonous plants (Gutierrez, 2000). The leafhopper-transmitted curtoviruses, type species *Beet curly top virus* (BCTV), infect dicotyledonous plants. Their monopartite genomes have three ORFs in the virion-sense with one intergenic region. Members of the genus *Curtovirus* are believed to be intermediates between mastreviruses and begomoviruses in evolution (Rybicki, 1994). This is supported by the fact that while the capsid protein gene of BCTV exhibit a high degree of homology with capsid protein genes of mastreviruses, the genes encoded on BCTV complementary sense DNA strand however, are homologous to those of the DNA-A component of begomoviruses. (Briddon *et al*., 1990; Briddon *et al*., 1998). The genus *Topocovirus*, recently recognized by the ICTV has one member, *Tomato pseudo-curly top virus*, which has a monopartite genome with two ORFs in the virion-sense and two in the complementary-sense and is transmitted by a treehopper vector to dicotyledonous plants. More than 80% of known geminiviruses are transmitted by whiteflies in a persistent, circulative, non-propagative manner (Rybicki *et al*., 2000) to dicotyledonous plants, and belong to the genus *Begomovirus*, type species *Bean golden yellow mosaic virus* (BGYMV) (Varma and Malathi, 2003). The majority of begomoviruses have bipartite genomes with two DNA components designated DNA-A and DNA-B, but a few species like *Tomato yellow leaf curl virus* from Israel, have been identified with a single genome component equivalent to the DNA-A component of bipartite begomoviruses (Gafni and Epel, 2002).

subtropical regions, is the mot prominent of the tomato-infecting geminiviruses (Reviewed by Pico *et al*, 1996 ; Varma and Malathi, 2003).

1.4 Geminiviruses

Geminiviruses are important plant pathogens and a major constraint to agricultural productivity in all tropical and subtropical regions of the world (Mansoor *et al*., 2003; Varma and Malathi, 2003). Even though symptoms, now known to be associated with geminiviruses, have been observed in plants grown in tropical and subtropical regions of the world for over 100 years, it wasn't until the 1970s that a distinct group of single-stranded DNA viruses was shown to be associated with these symptoms (Goodman, 1977; Harrison *et al*., 1977). Over the last couple of years, new geminiviruses have emerged and some known geminiviruses have re-emerged as economically serious constraints to various agricultural systems around the world. Several factors such as evolution of new variants of the viruses, appearance of efficient vectors, weather events, changing cropping systems, movement of infected planting material and introduction of susceptible plant varieties have singly, or in combination, contributed to the emergence of geminivirus problems around the world (Varma and Malathi, 2003). Geminiviruses are also versatile, reported to infect everything from monocots such as maize to dicots such as cassava and tomato. Infections can result in leaf mottling that interferes with photosynthesis, decreased yields of starchy foods such as cassava, as well as disruption of flower and fruit formation in crops such as tomato, pepper and cotton (Moffat, 1999; Varma and Malathi, 2003)).

Geminiviruses form the second largest family of plant viruses, *Geminiviridae*, (Varma and Malathi, 2003). They have circular, single-stranded DNA genomes of 2.5 – 3.1kbp in length (Reviewed by Briddon and Stanley, 2006), although double-stranded DNA (dsDNA) forms appear as intermediates. They have characteristic twinned (geminate) quasi-isometric particles encapsidating either a monopartite or bipartite genome (Zhang *et al*., 2001). According to the International Committee on Taxonomy of Viruses (ICTV) (Fauquet *et al*., 2003), the family *Geminiviridae* has been grouped into four genera: *Mastrevirus*,

them. A good intake of chromium, a mineral of which tomatoes are a good source, has been shown to help diabetic patients keep their blood sugar levels under control (Rao and Rao, 2003; Leonardi *et al*., 2000). Tomatoes are also a very good source of vitamin K. One cup of raw tomato provides one with 13.5% of the daily value for vitamin K which is important for maintaining bone health. Vitamin K1 activates osteocalcin, the major non-collagen protein in bone. Osteocalcin anchors calcium molecules inside of the bone. Therefore, without enough vitamin K1 osteocalcin levels are inadequate, and bone mineralization is impaired (Rao and Rao, 2003).

1.3 Diseases of Tomato

Tomato is a horticultural commodity of great economic importance, with reported total annual production above 70million t and over 3 million ha in cultivation worldwide (Rosello *et al*., 1996). The tomato crop is susceptible to more than 200 diseases (Kalloo, 1991), pests include bacteria, fungi and many viruses. The most common diseases of tomato can be classified as either systemic or foliar. Foliar diseases are most common, they affect primarily the leaves but some may also affect the fruit. In contrast, systemic diseases affect the entire plant. A large number of bacteria infect tomato. Bacterial canker, caused by *Clavibacter michiganensis*, bacterial speck of tomato, caused by *Pseudomonas syringae* and bacterial spot of tomato, caused by *Xanthomonas campestri* and *Xanthomonas vesicatoria* are among the most economically important bacterial diseases in many tomato growing regions of the world (Boudyach *et al*., 2001; Ji *et al*., 2006). Tomatoes also suffer from fungal diseases, the two most harmful being fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici* Sacc. and verticillium wilt caused by *Verticillium albo-atrum* and *V. dahliae* (Kalloo, 1991). Viral diseases can cause great economic losses to the tomato crop (Pico *et al*., 1996; Rosello *et al*., 1996). Some of the most devastating diseases of cultivated tomato (*Lycopersicon esculentum* Mill.) are attributed to viruses belonging to the plant family *Geminiviridae*. *Tomato yellow leaf curl virus* (TYLCV), responsible for economic losses of up to 100% in the tomato crop in many tropical and

components as they are good sources of carotenoids (in particular, lycopene), ascorbic acid (vitamin C), vitamin E, folate, flavonoids and potassium (Beecher 1998; Leonardi *et al.*, 2000). Lycopene, the carotenoid found in tomatoes (and everything made from them) has been extensively studied for its antioxidant and cancer-preventing properties. Unlike many other food nutrients whose effect have only been studied in animals, Lycopene from tomatoes has actually been studied in humans and found to be protective against a growing list of cancers. The antioxidant function of lycopene, which is its ability to help protect body cells from oxygen damage has been linked in human research to prevention of heart disease (Agarwal and Rao, 2000)

In addition to the center-stage lycopene, tomatoes are also high in traditional nutrients. They are an excellent source of vitamin C and vitamin A, the latter mostly through its concentration of carotenoids including beta-carotene. Vitamin A and C, are antioxidants that travel through the body neutralizing free radicals that could otherwise damage cells and cell membranes, subsequently escalating inflammation and the progression or severity of atherosclerosis, diabetic complications and asthma. Tomatoes are also a very good source of fiber, which has been shown to lower high cholesterol levels, preventing blood sugar levels from getting too high and help prevent colon cancer (Leonardi *et al*., 2000).

Tomatoes are also reported to be a very good source of potassium and a good source of niacin, vitamin B6 and folate. Niacin has been used for many years as a safe way to lower high cholesterol levels. Diets rich in potassium have been shown to lower high blood pressure and reduce the risk of heart diseases. Vitamin B6 and folate are needed by the human body to convert a potentially dangerous chemical called homocysteine into other benign molecules. This is very important because high levels of homocysteine which have been reported to directly damage blood vessel walls are associated with an increased risk of heart attack and stroke. In addition, folate in tomatoes is reported to help reduce the risk of colon cancer. Tomatoes are also a good source of riboflavin. Studies have shown that riboflavin helps reduce the frequency of migraine attacks in those people who suffer from

Nightshade family, which includes poisonous henbane, mandrate and the deadly nightshade (belladona). The French call it *pomme d' amour*, meaning "love apple", a name stemming from the belief that the fruit had aphrodisiacal qualities, while the Italians call it *pomodoro* or "golden apple" owing to the fact that the first known species with which they were familiar may have been yellow in colour. Regardless of its name, tomato has become one of the most popular vegetables of the world. While most tomatoes produced worldwide are used in the production of tomato paste, an ingredient in different processed tomato products such as ketchup, sauces and soups a significant number of tomatoes are consumed fresh as a raw vegetable in sandwiches and salads (Sanchez *et al*., 2003).

In South Africa, thousands of tons of fresh tomatoes are produced yearly, mainly by subsistence and resource-poor farmers. The Tomato Producers Organization of South Africa reported on a yearly fresh tomato production of over 345000 tons in 2003 alone. In South Africa, the crop is widely used as a fresh vegetable and in the form of an onion-tomato-amaranth stew to supplement the local diet of maize meal. Tomatoes are also grown commercially, creating direct employment opportunity to thousands of South Africans. The Department of Agriculture reported that in 1998 about 5465 ha were planted to tomatoes in South Africa, subsequently creating direct employment opportunity for 16295 people. The tomato is also one of the main vegetables used for hawking by small-scale entrepreneurs in the informal sector (Department of Agriculture, 2003).

1.2 Health and Antioxidant Properties of Tomatoes

Tomatoes are a great vegetable crop loaded with a variety of vital nutrients. They also make a wonderful addition to a heart-healthy and cancer-preventing diet. For the past five years, a lot of attention has focused on the antioxidant content of tomatoes. This is because numerous epidemiological studies suggested that regular consumption of fruits and vegetables, including tomatoes, can play an important role in preventing cancer and cardiovascular diseases (Rao and Agarwal, 2000). Tomatoes and tomato products are rich in health-related food

CHAPTER ONE

Review of Literature

1.1Tomato, the crop

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important solanaceous vegetable crops grown worldwide, both under indoor and outdoor conditions (Kalloo, 1991). The mountainous regions of the Andes, in South America, is believed to be the origin of wild tomato. However, domestication and cultivation of tomato first took place in Mexico (Taylor, 1986; Kalloo, 1991), infact the name tomato is suspected to have been derived from "tomatl" in the Nahua tongue of Mexico (Kalloo, 1991). The original botanical name of tomato was *Solanum lycopersicum* until Miller in 1788 suggested the name *Lycopersicon esculentum* for the cultivated forms and *L. pimpinellifolium* for the wild form of tomato. The name *Lycopersicon lycopersicum* was also suggested in the early 1900s, however, Broome *et al*. (1983) proposed to retain *Lycopersicon esculentum* since it had already been used popularly for a long time.

Tomato is universally treated as a vegetable, and is extensively grown as an annual plant worldwide. Tomato plants are herbaceous, annual to perennial, sexually, but occasionally asexually propagated (Kalloo, 1991). Growth habits of the plants are either determinate or indeterminate with a sympodial branching pattern. Determinate types grow to a certain height and stop in order to bear fruit, while the indeterminate types grow and branch until the growing tips are cut off.

Originally, tomato fruits were considered poisonous because of the association of the species with the solanaceous (Nightshade) family. They carried this stigma even into the twentieth century in some parts of the world. The name given to the tomato fruit in various languages reflects some of the history and mystery surrounding it. *Lycopersicon* means "wolf peach" in Latin and refers to the former belief that, like a wolf, this fruit was dangerous, hence its inclusion in the

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In memory of my cousin

Vuyani Phillip

1968-2004

ABSTRACT

Tomato (*Lycopersicon esculentum*) is a horticultural commodity of great economic importance in many parts of the world, including South Africa. A previous study identified a new begomovirus, *Tomato curly stunt virus* (ToCSV), as the causative virus of a new and potentially devastating disease of tomatoes in South Africa. In this study, symptomatic plants, suspected of infection with an uncharacterized ToCSV isolate (01/2521) were collected for screening from Pietermaritzburg, South Africa. A host range study was conducted with the original ToCSV isolate (99/0631). Two small DNA molecules (1449 nts and 755 nts) were found associated with ToCSV [01/2521] using near-full length primers AL1c2745 and PAR1v32 specific for ToCSV. A single small DNA molecule (842 nts) was also found in association with the original ToCSV isolate. Nucleotide sequence analysis revealed that the two small DNA molecules (1449bp and 755bp) have no significant nucleotide sequence identity (less than 20%) with any known begomovirus. The 842bp molecule has the most significant nucleotide sequence identity (48%) to that of ToCSV (AF261885), while less than 20% nucleotide sequence identities were found when compared with other begomoviruses. Nucleotide sequence alignment of the 842bp DNA molecule to the ToCSV sequence, showed that this small DNA molecule is a chimeric molecule that could have arisen through recombination, partly from the coding regions of the ToCSV genome, but the rest of the molecule is of unknown origin. All three small DNA molecules identified in this study were compared to some known begomovirus associated subgenomic molecules and satellite molecules, and sequence identities of less than 20% were found. To our knowledge, this is the first report of a small DNA molecule found associated with the ToCSV genome. The complete genome sequence of ToCSV [01/2521] was not determined. Based on the results we obtained from the host range study, all the chosen test plants are not susceptible to ToCSV infection. The infectivity of all the small molecules identified in this study, is currently being investigated.

DECLARATION

I declare that this research report is my own, unaided work. It is being submitted for the degree of Masters of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____
(Signature of Candidate)

_____ day of _____ 2006

AN INVESTIGATION OF TOMATO CURLY STUNT VIRUS IN SOUTH AFRICA

Azola Kuhle Fali



A research report submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree of Masters of Science

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